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Research Section

PHENOLIC ANTIOXIDANTS AND THE INHIBITION OF HEPATOTOXICITY FROM *N*-DIMETHYLNITROSAMINE FORMED *IN SITU* IN THE RAT STOMACH

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(*Received 22 November 1976*)

Abstract—The effect of the widely used edible stabilizers propyl gallate (PG), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *tert*-butylhydroquinone (TBHQ) on intragastric *N*-nitrosamine formation was studied. Rats were given by gastric intubation, sodium nitrite (125 mg/kg) and dimethylamine (1000 mg/kg) followed immediately by the test compound in doses of 25, 75 or 225 mg/kg. Ascorbate (200 mg/kg) was used as a positive control. Indices of *N*-nitrosamine formation 48 hr after dosing were the activities of serum glutamic–oxalacetic transaminase (GOT) glutamic–pyruvic transaminase (GPT), and ornithine–carbamoyl transferase (OCT), and the extent of hepatic necrosis. The nitrosamine-forming mixture alone produced extensive hepatic necrosis and 24-, 19- and 4-fold increases in serum GOT, GPT and OCT activities respectively. Enzyme induction was completely suppressed by ascorbate. PG completely protected against hepatic necrosis and enzyme induction at 225 mg/kg, and to a lesser extent at 75 mg/kg. TBHQ gave 60% protection against necrosis and appreciably suppressed enzyme activity increases at 225 mg/kg. BHA and BHT at all dose levels, PG at 25 mg/kg and TBHQ at 25 and 75 mg/kg neither demonstrated protective activity nor induced any lethality. Hence at approximately equimolar levels of nitrite and antioxidant, PG and TBHQ exerted an inhibitory effect on nitrosamine formation, but at lower levels all four antioxidants tested had no observable effect on the nitrosamine-forming system.

INTRODUCTION

N-Nitrosamines (NOAs) are of considerable interest because of their powerful carcinogenicity (Shank, 1975). There is ample evidence that they can be formed intragastrically following intake of nitrite together with various secondary amines (Wogan & Tannenbaum, 1975). Thus nitrite and morpholine or piperazine, administered to mice simultaneously, induced lung adenomas similar to those induced by the corresponding NOAs (Greenblatt, Mirvish & So, 1971). Nitrite and dimethylamine, given simultaneously, produced a rapid onset of hepatic necrosis in mice similar to that produced by the corresponding NOA (Asahina, Friedman, Arnold, Millar, Mishkin, Bishop & Epstein, 1971). NOA formation can be mediated by a variety of substances both *in vivo* and *in vitro*. Thiocyanate, iodide and bromide catalyse NOA formation (Boyland, Nice & Williams, 1971), and ascorbate, tannins, urea and ammonium sulphamate inhibit formation (Mirvish, 1975), presumably by respectively activating or competing for the nitrosating species. Hepatic necrosis and tumour induction as a result of dosing with an NOA-forming system are suppressed by inhibitors of NOA formation given immediately after dosing (Kamm, Dashmann, Conney & Burns, 1973; Mirvish, Cardesa, Wallcove & Shubik, 1975).

Phenols are usually readily *C*-nitrosated and should be competitive inhibitors of NOA formation (Challis, 1973). Thus hydroquinone, α-tocopherol and the phenolic antioxidants *tert*-butylhydroquinone (TBHQ), propyl gallate (PG) and butylated hydroxytoluene (BHT), inhibit *N*-nitrosopyrrolidine formation in simulated food-processing systems, particularly in the presence of emulsifiers (Gray & Dugan, 1975). PG also inhibits *N*-nitrosopyrrolidine formation during the cooking of nitrite-cured bacon (Sen, Donaldson, Seaman, Iyengar & Miles, 1976). Gallic acid is effective in blocking the *in vitro* nitrosation of morpholine (Mirvish, 1975) and when given in the food inhibits the lung adenoma induced in mice by nitrite and morpholine (Mirvish *et al.* 1975). However, chlorogenic acid, a major phenolic constituent of coffee, and 4-methylcatechol effectively promote *N*-nitrosopiperidine formation in aqueous solution (Challis & Bartlett, 1975). Gallic acid inhibits the *in vitro* formation of *N*-nitrosodiethylamine at pH 2 but promotes it at pH 4 (Walker, Pignatelli & Castegnaro, 1975).

The widespread use of phenolic antioxidants as edible stabilizers makes it desirable to investigate their effect on NOA formation. Their behaviour in simulated food systems, reported above, suggests that they may inhibit NOA formation in food, but there is no information on their effect on intragastric NOA formation. This paper reports on the *in vivo* action of these substances on an NOA-forming system of nitrite and dimethylamine in the rat stomach. The acute hepatotoxicity of the system (Cardesa, Mirvish, Haven & Shubik, 1974) was used as an index of NOA formation and the effects of the antioxidants were compared with the effect of ascorbate, a known inhibitor of NOA formation.

EXPERIMENTAL

Materials. The test compounds, BHT, Tenox® TBHQ, Tenox® BHA and Tenox® PG, were provided by Eastman Chemical Products Inc., Kingsport, Tenn.

Animals, diet, dosages. Male caesarean-derived albino rats (Sprague–Dawley strain, Charles River Laboratory, Wilmington, Mass.), weighing 240–296 g, were housed individually and given Purina Laboratory Rat chow and drinking-water *ad-lib.* They were divided into groups of ten to give similar inter-group weight ranges and mean weights.

Doses were administered singly by gastric intubation after a 12-hr fast period. Dimethylamine (DMA)(1000 mg/kg) was given in 20% (w/v) aqueous solution, sodium nitrite (125 mg/kg) in 2·5% (w/v) aqueous solution, and sodium ascorbate (200 mg/kg) in 4·0% (w/v) aqueous solution. All three solutions were adjusted to pH 7·0 with N-NaOH. Test compounds were dissolved in warm corn oil and the cooled solutions were given in doses of 25, 75 and 225 mg/kg of test compound. Rats were dosed in groups as follows: corn-oil vehicle or 225 mg/kg of the test antioxidant as controls; NOA-forming system (DMA followed rapidly by sodium nitrite) followed immediately either by the corn-oil vehicle providing a hepatotoxicity control or by ascorbate as inhibition control; NOA-forming system followed at once by the test compound.

Indices of toxicity. Animals were anaesthetized with sodium pentobarbitone 48 hr after dosing, when a mid-ventral incision was made and blood samples were collected by puncture of the dorsal aorta. Animals were then killed by exsanguination. Livers were removed, inspected and classified as normal, slightly abnormal (showing colour changes and granular appearance, slight enlargement or slightly rounded margins and small yellow foci), showing focal necrosis (a combination of three or more of the preceding observations on one lobe) and liver necrosis (a combination of the preceding observation on two or more lobes). Livers were preserved in 10% buffered formalin. Blood samples were allowed to clot and serum was tested for glutamic–pyruvic transaminase activity by the Trans-AC procedure (Warner-Lambert Co., Morris Plains, NJ), glutamic–oxalacetic transaminase activity (Sigma Chemical Co., St. Louis, Mo.) and ornithine–carbamoyl transferase activity (Stanjord & Clayson, 1966). Two lobes of each liver were sectioned, stained with haematoxylin and examined microscopically for areas of necrosis. These were distinguished, in increasing order of severity, as absent, slight centrilobular, moderate centrilobular, centrilobular and mid-zonal, and centrilobular, mid-zonal and interlobular (Cardesa *et al.* 1974).

RESULTS

Serum-enzyme activities

The effects on serum-enzyme activities of the corn-oil vehicle, the NOA-forming system alone and the NOA-forming system followed by ascorbate are presented in Table 1. In the absence of the NOA-forming system, activities were essentially unaltered. The NOA-forming system produced dramatic increases in the activities of GOT, GPT and OCT by factors of approximately 24, 19 and 4, respectively. As expected (Cardesa *et al.* 1974) these increases were all repressed by ascorbate to normal or near normal values. The effects of the test compounds on NOA-produced enzyme increases are given in Table 2 as ratios of the mean activities for test groups to the mean for the corn-oil control. Comparing ratios indicates that the highest dose levels of TBHQ and PG produced marked inhibition of the enzyme induction due to the NOA-forming system.

The variability of enzyme activity within groups responding to the NOA-forming system was usually appreciable. Thus values for ten rats receiving the NOA-forming system followed by 25 mg TBHQ/kg were GOT, 3784 ± 2552 units, GPT, 816 ± 480

Table 1. *Rat serum enzyme activities: control values**

Compound(s)	Dose (mg/kg)	Serum enzyme activities†		
		GOT	GPT	OCT
Corn oil vehicle		149 ± 33	43 ± 4	0·127 ± 0·014
NOA-forming system‡		3628 ± 1077	830 ± 288	0·495 ± 0·028
NOA-forming system‡ + ascorbate	200	133 ± 18	38 ± 5	0·190 ± 0·150
TBHQ	225	133 ± 20	41 ± 7	0·138 ± 0·015
BHT	225	157 ± 20	49 ± 7	0·130 ± 0·013
BHA	225	132 ± 21	44 ± 5	0·138 ± 0·014
PG	225	151 ± 27	42 ± 3	0·135 ± 0·013

*Determined on sera collected 48 hours after dosing under pentobarbitone anaesthesia. Values are means ± SD for each group of ten rats.

†Units are GOT, Karmen; GPT, Reitman Frankel; OCT, μmoles.

‡In all cases the NOA-forming system comprised 1000 mg DMA/kg and 125 mg $NaNO_2$/kg.

Table 2. *Effect of phenolic antioxidants on the elevation of serum enzyme activity produced by the NOA-forming system*

Compound(s)	Dose (mg/kg)	Serum enzyme activity ratio†		
		GOT	GPT	OCT
NOA-forming system‡		24·4	19·2	3·9
NOA-forming system‡ + TBHQ	25	25·4	19·0	3·7
	75	17·6	14·9	3·3
	225	6·7*	5·7	2·1*
NOA-forming system‡ + BHT	25	20·7	18·1	3·8
	75	27·3	22·5	3·7
	225	18·0	14·2	3·8
NOA-forming system‡ + BHA	25	26·6	22·3	3·8
	75	19·6	14·0	3·4
	225	23·8	16·6	3·6
NOA-forming system‡ + PG	25	17·5	15·0	3·2
	75	6·8*	8·5	2·6*
	225	1·0*	1·0*	1·1*

†Enzyme activity units and control values are in Table 1; the ratio is of the mean value for test compound treatment to the mean value for corn oil control.
‡In all cases the NOA-forming system comprised 1000 mg DMA/kg and 125 mg $NaNO_2$/kg.
Values marked with asterisks differ significantly (*$P < 0.05$) from the value produced by the NOA-forming system alone.

units and OCT, 0·475 ± 0·124 units. Group mean enzyme levels for the test compounds were compared with those for the NOA-forming system alone by the *F* test (analysis of variance) the Student's *t* test (Snedecor & Cochran, 1967). Statistically significant ($P < 0.05$) inhibitions were found for PG at the 225 mg/kg and 75-mg/kg dose levels, and for TBHQ at the 225-mg/kg level. In the latter case, individual values within the test group showed complete inhibition of enzyme induction in five rats, no inhibition in three and partial elevations in two rats, good correlation being obtained between all three enzyme activities. The partial inhibition obtained with 75 mg PG/kg was mainly due to intermediate values of enzyme activity.

Gross and microscopic liver observations

Liver observations are summarized in Tables 3 (controls) and 4 (test antioxidants). Gross changes were absent from the animals receiving only the corn oil. The gross liver damage seen in all rats receiving the NOA-forming system alone was absent from those receiving ascorbate. The test compounds administered alone produced slight liver enlargement in a few animals, characteristic of the stress-response to high doses of these materials (Gilbert & Golberg, 1965). PG and TBHQ, respectively, provided 100 and 60% protection against NOA-induced gross liver damage at 225 mg/kg, and less protection at lower dose levels. BHA and BHT provided no significant protection at any dose level tested.

Extensive necrosis was seen, as expected, in rats receiving only the NOA-forming system; this also was suppressed by ascorbate but BHA and BHT provided virtually no protection against necrosis, while TBHQ provided limited protection. PG afforded complete protection against necrosis at 225 mg/kg, but gave almost no protection at lower dose levels. Statistical analysis of the incidence of microscopic liver necrosis by the chi-square method (Snedecor & Cochran,

Table 3. *Control gross and microscopic liver observations*

Compound(s)	Dose (mg/kg)	No. of animals affected								
		Gross observations*				Microscopic observations†				
		N	SN	FN	LN	A	SC	MC	CM	CI
Corn oil vehicle		10				10				
NOA-forming system‡					10				2	8
NOA-forming system‡ + ascorbate	200	10				9	1			
TBHQ	225	5	5			10				
BHT	225	7	3			10				
BHA	225	5	5			10				
PG	225	9		1		9		1		

*Definitions are N, normal colour, size, surface appearance, sharp margins; SN, colour change, granular, slightly enlarged, slightly rounded margins, small yellow foci; FN (focal necrosis) three or more of the preceding on one lobe; LN (liver necrosis), a combination of the preceding on two or more lobes.
†Definitions are A, necrosis absent; SC, slight centrilobular necrosis; MC, moderate centrilobular necrosis; CM, centrilobular and mid-zonal necrosis; CI, centrilobular, mid-zonal and interlobular necrosis.
‡In all cases the NOA-forming system comprised 1000 mg DMA/kg and 125 mg $NaNO_2$/kg.

Table 4. *Effect of phenolic antioxidants on gross and microscopic NOA-induced liver effects*

Compound	Dose (mg/kg)	No. of rats affected: Gross observations† N	SN	FN	LN	% Protection from gross effects‡	No. of rats affected: Microscopic observations† A	SC	MC	CM	CI
NOA-forming system§	25	1			9	10			1		9
+ TBHQ	75	1	1	3	5	20	2			1	7
	225	4	2	1	3	60	3	1	2	1	3
NOA-forming system§	25	1			9	10			1	2	7
+ BHT	75			1	9	0			1		9
	225				10	0			1	3	6
NOA-forming system§	25				10	0				1	9
+ BHA	75			1	9	0			1	1	8
	225		1	1	8	10	1		1		8
NOA-forming system§	25	2		1	7	20	1		1	1	7
+ PG	75	2		4	4	20	1			3	6
	225	10*				100	10*				

†Definitions in Table 3.
‡No. of normal (N) or slightly abnormal (SN) rats × 100/10.
§In all cases the NOA-forming system comprised 1000 mg DMA/kg and 125 mg $NaNO_2$/kg.
Values marked with asterisks differ significantly (*$P < 0.05$) in the incidence of hepatic necroses from those of the NOA-forming system alone.

1967), and comparison of the incidence in the antioxidant-treated groups with that in the group given the NOA-forming system revealed that only PG at 225 mg/kg reduced hepatic necrosis significantly ($P < 0.05$).

DISCUSSION

The results in this study apply to the acute formation of dimethylnitrosamine in the acid milieu of the rat stomach, using dose levels of 1000 mg DMA/kg, 125 mg $NaNO_2$/kg and 25–225 mg phenolic antioxidant/kg. The approximate concentrations in the rat stomach immediately after dosing, assuming a stomach volume of 5 ml, were 1 M-DMA, 0·09 M-$NaNO_2$ and 0·005–0·05 M for the test compound. Concentrations which inhibited *in vivo* NOA formation cannot be ascertained directly because of variable stomach emptying times, the heterogeneous dosing mixture, and the possibility of nitrite absorption. However there is an approximate equivalence of nitrite and PG or TBHQ concentrations for inhibition of the NOA-forming system, since 2 mol nitrite are required to produce 1 mol N_2O_3, the nitrosating species (Mirvish, 1975). Inhibition by PG or TBHQ is dose-related, and in this respect these compounds are similar to ascorbate, with which for example the extent of inhibition of *N*-nitrosomorpholine formation is proportional to the ascorbate/nitrite concentration ratio (Archer, Tannenbaum, Fan & Weisman, 1975). A PG or TBHQ/nitrite concentration ratio of less than one would thus presumably imply the absence of significant inhibition.

It is estimated that in the USA daily intake of phenolic antioxidants per head does not exceed 10 mg (Food and Drug Administration, 1972) and the daily intake of nitrite into the stomach is about 12·8 mg, of which almost 70% is salivary in origin (White, 1975). For a stomach contents volume of about 500 ml, the resulting concentrations are 0·34 mM for $NaNO_2$ and 0·09 mM for the test compound. The PG or TBHQ/nitrite ratios are thus lower than those presumably required for inhibition of NOA formation, but ratios for all four test compounds are close to those where no effects on the NOA-forming system were encountered.

Precursors of NOAs occur in the human diet, originating from natural and man-made sources (Wogan & Tannenbaum, 1975). Mediation of NOA formation by phenolic antioxidants, if it occurred, would probably take place during food processing or in the stomach. The studies of Gray & Dugan (1975) indicate that PG, TBHQ and BHT tend to depress NOA formation in simulated food systems at pH 3·5, which would lead one to expect no enhancement of NOA formation from natural precursors by phenolic antioxidants during food precessing and storage. The studies reported in this paper indicate also that under normal conditions of use, phenolic antioxidants would be unlikely to promote NOA formation in the stomach, and that under conditions of low or diminished nitrite intake PG and TBHQ might well completely inhibit any NOA formation.

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REGENERATION OF RAT LIVER IN THE PRESENCE OF ESSENTIAL OILS AND THEIR COMPONENTS

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Abstract—Liver regeneration was followed over a 10-day period in partially hepatectomized rats given sc injections of high levels of essential oils, terpenes or aromatic compounds daily for the first 7 days or fed *ad lib.* for 10 days on diets supplemented with oils, spices and botanicals. The liver increment (the amount of tissue regenerated) was increased significantly by sc injection of oils of anise, fennel, tarragon, parsley seed, celery seed and oleoresin, nutmeg, mace, cumin and sassafras and of the aromatic principles, 4-allylanisole, 4-propenylanisole, *p*-isopropylbenzaldehyde, safrole and isosafrole. Most of the essential oils were ineffective in total doses up to 3000 mg/kg because they contained a high percentage of terpenes, which proved inert. Many of the agents effective by the sc route were also active when added to the diet, although a possible exception was tarragon oil (0·50%). Azulenes (e.g. *Matricaria chamomilla* L. oils and guaiazulene) stimulated regeneration when given by injection but Haitian vetivert oil, the alcohol mixture vetiverol and guaiene, in massive doses, did not. However, these last three agents, as well as the blue chamomile oils and ground *Matricaria* flowers, stimulated liver regeneration when given in the diet. Several of the test agents also increased the wet and dry liver weight expressed relative to body weight, in intact rats, whether given by the sc or oral route. To throw further light on the tests with the active principles, certain aromatic or phenolic compounds, including monosubstituted derivatives, were screened in operated animals by the sc route.

INTRODUCTION

Essential oils and flavourings are widely used and must figure prominently in nutritional surveys as possible factors in cancer and some other diseases. Safrole (4-allyl-1,2-methylenedioxybenzene), an important component of a number of oils, has been shown to be a hepatic carcinogen (Borchert, Miller, Miller & Shires, 1973a; Epstein, Fujii, Andrea & Mantel, 1970; Hagan, Jenner, Jones, Fitzhugh, Long, Brouwer & Webb, 1965; Homburger, Kelley, Baker & Russfield, 1962; Long, Nelson, Fitzhugh & Hansen, 1963) with 1′-hydroxysafrole constituting a strongly hepatocarcinogenic metabolite (Borchert *et al.* 1973a; Borchert, Wislocki, Miller & Miller, 1973b; Wislocki, Borchert, Miller & Miller, 1976). In a short-term high-dosage toxicological study, gross pathological changes were reported as extensive with safrole, isosafrole, dihydrosafrole and aliphatic allyl compounds, moderate to minimal with the 4-allyl-, 4-propenyl- and 4-*n*-propylanisoles and essentially negative with benzene, allylbenzene and anisole (Taylor, Jenner & Jones, 1964). Reports are also available on safrole and other principles in relation to accidental poisoning (Craig, 1953), to hypothermic activity (Caujolle & Meynier, 1960) and to differences in gastro-intestinal absorption (Fritsch, de Saint Blanquat & Derache, 1975). The effect of nutmeg or myristica powder, as well as of its principle, myristicin (4-allyl-6-methoxy-1,2-methylenedioxybenzene), has been determined on the nervous system (Weiss, 1960; Wesley-Hadzija & Bohinc, 1956) and an explanation of the psychotropic activity has been attempted on the basis of a suggested relationship with the structure of sympathomimetic amines (Seto & Keup, 1969; Shulgin, 1966; Shulgin & Sargent, 1967). Yet another principle of several oils, namely asarone or 1,2,4-trimethoxy-5-propenylbenzene, has been studied from the standpoint of physiological and psychotropic properties (Dandiya & Sharma, 1962; Seto & Keup, 1969; Sharma & Dandiya, 1962). Recent biological data on safrole have been reviewed by Opdyke (1974), and for more general information on essential oils and flavourings, the reader is referred to the latter work as well as to several treatises, especially that of Guenther (1948–1952).

The general problem of enzyme induction has been investigated in relation to safrole but the findings have varied with the criteria employed. Liver microsomal-enzyme activity has been reported as decreased (Friedman, Arnold, Bishop & Epstein, 1971; Fujii, Jaffe, Bishop, Arnold, Mackintosh & Epstein, 1970; Seto & Keup, 1969), as increased (Gray, Parke, Grasso & Crampton, 1972; Lake & Parke, 1972; Lotlikar & Wasserman, 1972; Parke & Rahman, 1970) and as essentially unchanged (Borchert *et al.*, 1973b; Lotlikar & Wasserman, 1972). The formation of a safrole metabolite-cytochrome *P*-450 complex which can be cleaved by safrole and other agents has been advanced recently (Elcombe, Bridges, Gray, Nimmo-Smith & Netter, 1975).

The present investigation was undertaken with a view to screening the effect of a number of essential oils, flavourings, spices and plant materials on the extent of liver regeneration in partially hepatectomized rats. Where an oil displayed a definite activity, a correlation was attempted on the basis of the action of terpenic and aromatic components tested in a similar manner. In this connexion, previously published findings with a variety of aromatic compounds engen-

dering stimulatory effects on the regenerating rat liver were applied to the current experiments (Gershbein, 1958, 1966, 1973 & 1975). For several agents, changes in liver-to-body weight ratios were followed in intact rats.

EXPERIMENTAL

Test materials. Of the essential oils, bay, eucalyptus, peppermint and spearmint originated from Gentry Corp., Fairlawn, NJ, cadinene, caryophyllene, myrcene, rhodinol, cajaput, carrot-seed, opoponax natural G, origanum Spanish, parsley-seed, petit-grain and black pepper from Givaudan Corp., New York, cade rectified, cananga, cassia USP Chinese, cedarwood, celery-seed, citronella, copaiba NF IX, geranium Bourbon, lemon, patchouly and ylang ylang from J. Manheimer Inc., Long Island City, NY, citral, geraniol, isosafrole, safrole and piperonal from Matheson Coleman & Bell Co., Cincinnati, Ohio, vetivert (Bourbon and Haiti) and vetiverol from Florosynth Inc., Bronx, NY, oleoresins from Stange Co., Chicago, Ill., except for oleoresin celery, which was from Fritzsche Dodge & Olcott Inc., New York, carvone, cineole, *p*-isopropylbenzaldehyde, L-menthone and isoeugenol from Eastman Kodak Co., Rochester, NY, 4-allylanisole (estragole or methylchavicol), 4-allyl-2,6-dimethoxyphenol, 3-allylguaiacol, 2-allylphenol, allyl phenyl ether, anisole, 4-cumylphenol, dihydrosafrole, farnesol, guaiacol, guaiazulene, guaiene, 2-hydroxy-4-methoxybenzaldehyde, 2-hydroxy-5-methoxybenzaldehyde, 4-methoxybenzyl alcohol, 3-*p*-methoxyphenylpropanol, *β*-methylstyrene, piperine, piperonyl alcohol, 4-propenylanisole (anethole), 4-*n*-propylanisole, allylbenzene and *n*-propylbenzene from Aldrich Chemical Co., Milwaukee, Wisc., and camphene, dipentene, *trans*-*p*-menthane, *α*-pinene, *α*- and *γ*-terpinenes, terpinolene and wood turpentine from Hercules Inc., Wilmington, Del. The remaining oils and principles were furnished by Fritzsche Dodge & Olcott Inc. S. B. Penick & Co., New York, supplied Spanish whole safron, sarsaparilla ethanol extract and yarrow flowers. The garlic and onion instant powders, ground nutmeg, decorticated cardamom and Jamaican allspice were commercial products (McCormick & Co., Inc., Baltimore, Md), as were the herbal teas (Alvita Products Co., Huntington Beach, Cal.) and the tea from *Matricaria chamomilla* L. (Seelect Dietary Products, Inc., Huntington Park, Cal.). Of the chamomile oils, J. Manheimer Inc. supplied four that were essentially free of blue colour (Egypt, Romaine and two English batches) and two very viscous and intensely blue-coloured oils (H-4 and H-5). A sample (H-1) similar to the latter originated from S. B. Penick & Co. The herbs and other plant materials were ground in a Waring blender and sieved prior to use.

Animals and treatment. The test materials were administered to rats either in the diet or by sc injection. The diets were prepared by admixture of the agents with Rockland rat meal, all percentages being calculated on a weight basis. For sc injection of the oils and principles, solutions were prepared in peanut oil and the corresponding controls received the oil alone, in daily volumes ranging up to 0·50 ml.

Prior to treatment, Charles River rats of specified weight and sex were partially hepatectomized under ether anaesthesia, two-thirds of the organ being extirpated (Higgins & Anderson, 1931) and dried to constant weight at 100°C. The rats were housed in individual cages and given diet and water *ad lib.* Supplemented diets were fed for 10 days following surgery and the rats were then killed. In the sc series, injections were carried out daily for the first 7 days after surgery and the rats were killed with ether on day 10, at which time the entire livers were removed and dried as described above. Small sections of the organs were retained for microscopic examination. The liver increment, or the amount of tissue regenerated, was calculated from the dry liver weight by taking the product of the weight at surgery and a factor of 0·46 and subtracting this from the weight at autopsy (Gershbein & Labow, 1953). Rats losing more than about 12–15% of their initial body weight were excluded from the calculations, since starvation or inanition can cause extensive decreases in the liver increment.

In another series, intact rats were subjected to several of the treatments over a period of 10 days.

Table 1. *Body weight and liver increment findings for partially hepatectomized rats injected sc with essential oils and compounds*

Oil or other test material and dose† (mg/rat/day; overall)	No. of rats	Body weight (g)		Liver increment (g)	*t*
		Initial‡	Terminal‡		
Series 2E					
Control	15	260 ± 6·9	289 ± 7·5	2·244 ± 0·094	
Nutmeg (100; 2535)	12	261 ± 8·1	291 ± 9·5	2·665 ± 0·093	3·14**
Series 3E					
Control	11	292 ± 9·0	301 ± 9·5	2·034 ± 0·092	
Celery-seed (100; 2375)	9	290 ± 7·7	300 ± 11·2	2·472 ± 0·175	2·34*
Series 5E					
Control	12	269 ± 8·7	283 ± 8·8	2·024 ± 0·103	
Mace (100; 2500)	11	279 ± 10·0	280 ± 9·0	2·421 ± 0·102	2·74*
Fennel (100; 2415)	11	282 ± 11·6	299 ± 10·9	2·629 ± 0·157	3·29**
Ylang ylang (150; 3670)	11	274 ± 11·1	298 ± 7·5	2·240 ± 0·152	1·19
Series 6E					
Control	9	247 ± 10·3	254 ± 12·0	1·879 ± 0·121	
Cardamom seed (100; 2705)	10	249 ± 11·3	269 ± 12·8	2·184 ± 0·131	1·69
Coriander seed (100; 2780)	8	243 ± 12·1	260 ± 13·1	2·136 ± 0·076	1·74
Carrot-seed (100; 2725)	8	250 ± 9·8	264 ± 12·8	2·220 ± 0·211	1·44

Table 1.—*continued*

Oil or other test material and dose† (mg/rat/day; overall)	No. of rats	Body weight (g)		Liver increment (g)	*t*
		Initial‡	Terminal‡		
Series 6E—*continued*					
Parsley-seed (100; 2830)	5	242 ± 19·0	251 ± 17·6	2·746 ± 0·243	3·60**
Oleoresin celery (100; 2940)	9	225 ± 9·8	251 ± 11·3	2·402 ± 0·107	3·23**
Series 7E					
Control	12	244 ± 3·9	265 ± 5·3	2·171 ± 0·093	
Anise (100; 2735)	11	256 ± 4·3	276 ± 6·0	2·593 ± 0·112	2·91**
Cumin (100; 2825)	10	242 ± 4·5	253 ± 6·6	2·490 ± 0·096	2·38*
Eugenol (50; 1365)	11	249 ± 4·5	262 ± 5·5	1·997 ± 0·066	1·50
4-Allylanisole (50; 1335)	12	252 ± 4·1	271 ± 6·4	2·592 ± 0·143	2·46*
Series 8E					
Control	12	289 ± 15·6	296 ± 15·2	2·267 ± 0·126	
Tarragon (100; 2400)	5	288 ± 13·3	296 ± 7·7	2·938 ± 0·241	2·71*
Guaiac wood (100; 2475)	12	288 ± 14·4	277 ± 14·4	2·268 ± 0·114	0·01
Series 9E					
Control	15	344 ± 7·2	339 ± 7·7	2·148 ± 0·097	
Nutmeg (100; 2065)	13	332 ± 7·0	346 ± 5·7	2·623 ± 0·097	3·42**
Series 10E—females					
Control	10	203 ± 2·6	213 ± 4·3	1·607 ± 0·073	
Tarragon (100; 3450)	7	202 ± 2·9	205 ± 8·3	2·097 ± 0·158	3·10**
4-Allylanisole (50; 1675)	12	203 ± 2·4	215 ± 5·0	1·914 ± 0·094	2·50*
Carrot-seed (75; 2480)	10	208 ± 2·5	221 ± 2·7	1·833 ± 0·144	1·40
Parsley-seed (50; 1640)	8	209 ± 2·4	216 ± 3·0	2·130 ± 0·103	4·25**
Series 11E					
Control	12	302 ± 12·3	299 ± 13·7	1·773 ± 0·115	
Sassafras (50; 1130)	6	316 ± 23·4	313 ± 26·1	3·095 ± 0·164	6·61**
Camphor sassafrassy (50; 1240)	6	282 ± 26·6	283 ± 30·3	2·187 ± 0·159	2·09
Series 12E					
Control	10	264 ± 10·8	292 ± 7·4	2·127 ± 0·095	
4-Propenylanisole (100; 2450)	12	269 ± 8·9	295 ± 7·6	2·613 ± 0·088	3·77**
Isoeugenol (100; 2640)	8	252 ± 7·3	276 ± 7·1	2·248 ± 0·120	0·80
Isosafrole (15; 375)	11	272 ± 10·3	290 ± 9·7	2·423 ± 0·087	2·31*
Series 13E					
Control	13	213 ± 2·5	264 ± 3·9	2·206 ± 0·115	
Safrole (10; 295)	13	210 ± 3·4	266 ± 6·6	2·565 ± 0·078	2·58*
Series 14E					
Control	13	283 ± 8·8	292 ± 9·6	1·891 ± 0·108	
Dihydrosafrole (15; 360)	12	282 ± 7·1	299 ± 11·7	2·396 ± 0·155	2·70*
Series 15E					
Control	12	285 ± 6·4	313 ± 6·6	2·168 ± 0·105	
4-Allylanisole (50; 1160)	15	285 ± 5·8	311 ± 5·3	2·585 ± 0·088	3·07**
n-Propylanisole (150; 3440)	13	280 ± 6·1	323 ± 6·5	2·555 ± 0·140	2·19*
n-Propylbenzene (200; 4670)	11	288 ± 5·7	310 ± 5·1	2·481 ± 0·088	2·27*
Tarragon (50; 1160)	12	287 ± 5·0	314 ± 6·0	2·543 ± 0·112	2·45*
Series 16E					
Control	11	249 ± 4·5	271 ± 8·8	1·985 ± 0·098	
Allyl phenyl ether (50; 1320)	12	256 ± 4·3	275 ± 4·6	2·487 ± 0·106	3·46**
Piperine (25; 660)	10	250 ± 4·4	277 ± 9·1	2·481 ± 0·071	4·03**
Series 17E					
Control	11	301 ± 6·4	355 ± 9·0	2·503 ± 0·091	
Allylbenzene (100; 2330)	12	289 ± 9·8	319 ± 8·1	2·893 ± 0·087	3·22**
β-Methylstyrene (100; 2330)	14	291 ± 5·6	320 ± 6·9	2·665 ± 0·126	1·05
Allyl phenyl ether (100; 2260)	15	302 ± 8·0	321 ± 6·5	3·273 ± 0·118	5·17**
Series 18E					
Control	16	282 ± 7·2	291 ± 8·9	1·892 ± 0·088	
p-Isopropylbenzaldehyde (50; 1250)	15	282 ± 7·9	279 ± 6·6	2·278 ± 0·074	3·36**
3-*p*-Methoxyphenylpropanol (150; 3750)	11	282 ± 8·5	278 ± 8·2	2·076 ± 0·082	1·46
Series 19E					
Control	11	324 ± 8·3	335 ± 7·1	2·256 ± 0·115	
Guaiene (100: 2135)	13	325 ± 7·7	331 ± 7·5	2·402 ± 0·130	1·40
Guaiazulene (50; 1065)	12	322 ± 7·3	336 ± 8·0	2·697 ± 0·062	3·45**
Vetivert, Haiti (150; 3330)	13	320 ± 7·1	310 ± 8·6	2·383 ± 0·121	0·75
Vetiverol (100; 2170)	12	318 ± 7·6	327 ± 6·8	2·587 ± 0·112	2·06
Chamomile, H-1 (50; 1100)	12	321 ± 8·8	313 ± 7·8	2·548 ± 0·118	1·77

†Injected on days 1–7 after surgery in a daily volume of peanut oil ranging up to 0·50 ml, the controls receiving peanut oil alone. Except for series 10E, males were used throughout.

‡Initial weights were recorded prior to surgery and terminal weights at autopsy 10 days later.

Results are means ± SEM for the numbers of rats stated. Significant *t* values are marked with asterisks: *$P < 0.05$; **$P < 0.01$.

They were killed as described above and the percentages of wet and dry liver were determined.

RESULTS

Subcutaneous injection of agents in partially hepatectomized rats

Positive findings in the liver following sc injection of very high doses of oils, flavourings or principles on the first 7 days after surgery appear in Table 1. Of the oils, nutmeg (series 2E and 9E), mace, fennel, celery-seed, anise, cumin, parsley-seed and tarragon (series 3E, 5E, 6E, 7E and 8E) stimulated liver regeneration in male rats. The principles 4-allylanisole, 4-propenylanisole and *p*-isopropylbenzaldehyde, screened at overall dosages of 1335, 2450 and 1250 mg/kg, respectively, increased the extent of liver regeneration, as did safrole, isosafrole and dihydrosafrole injected daily in a dose of 10–15 mg/rat (overall dosage 295–375 mg/kg), piperine (660 mg/kg), sassafras oil (1130 mg/kg) and a high safrole-containing fraction of camphor oil (1240 mg/kg). The incremental increase with the last agent lacked significance at the 5% level of probability.

The activity of tarragon oil was compared with that of several aromatic compounds (series 15E). 4-Allylanisole was very effective at 1160 mg/kg ($P < 0.01$) but the increases in increment with tarragon oil at a comparable level, *n*-propylanisole (3440 mg/kg) and *n*-propylbenzene (4670 mg/kg) were significant at the 5% level of probability. Allylbenzene was a more marked stimulant than *n*-propylbenzene, whereas β-methylstyrene (2330 mg/kg; series 17E), and comparable levels of eugenol, isoeugenol, 3-allylguaiacol and 2-allylphenol were inactive. Allyl phenyl ether proved an effective compound at an overall dosage of 2260 mg/kg. When tested in adult female rats (series 10E), 4-allylanisole (1675 mg/kg), tarragon oil (3450 mg/kg) and parsley-seed oil (1640 mg/kg) elicited significant rises in liver increment and, again in line with observations in males, carrot-seed oil (2480 mg/kg) was without significant action.

Of the azulene-containing products (Table 1; series 19E), the increases in liver increment with guaiene (2135 mg/kg), Haitian vetivert oil (3330 mg/kg), vetiverol (2170 mg/kg) and one blue chamomile oil sample (H-1; 1100 mg/kg) lacked significance. However, in several preliminary runs, two other chamomile oils (H-4 and H-5) and guaiazulene, at similar levels, stimulated the regenerative process.

Other compounds eliciting little change from control values in the extent of liver regeneration, when given in an overall dosage of 2450–2950 mg/kg except where indicated otherwise, were the terpenes cadinene, camphene, carvone, caryophyllene, cineole, citral, citronellol, dipentene, farnesol, geraniol, hydroxycitronellol, linalool, *trans-p*-menthane, L-menthone, myrcene, α-phellandrene, α-pinene, rhodinol, α-terpinene, γ-terpinene, α-terpineol, terpinolene and wood turpentine, as well as 4-allyl-2,6-dimethoxyphenol, allyl isothiocyanate (80 and 120 mg/kg, the latter being rather poorly tolerated), anisole, 4-cumylphenol, guaiacol, guaiene, 2-hydroxy-4 and 5-methoxybenzaldehydes (1200 mg/kg), 4-methoxybenzyl alcohol, 3-*p*-methoxyphenylpropanol (3750 mg/kg), phenetole and piperonyl alcohol. The following oils were similarly without significant effect when injected sc in an overall dosage of 2400–2950 mg/kg or as specified: *Abies sibirica*, bay, bergamot, cade, cajaput, cananga, cardamom seed, cassia (1100 mg/kg), cedarwood, citronella, chenopodium (25 and 125 mg/kg, the latter being rather poorly tolerated), clove, copaiba, coriander (oleoresin), cubeb, davana, dill, eucalyptus, geranium, ginger, guaiac wood, juniper, lemon, marjoram, neroli, opoponax, origanum, paprika (oleoresin), patchouly, pennyroyal (1300 mg/kg), pepper (black), peppermint, perilla, petitgrain, pimento essence berries, pimento leaf, rosemary, sage, sandalwood, spearmint, thuja (1440 mg/kg), thyme, wormwood (1200 mg/kg) and ylang ylang.

Body-weight losses were fairly high in animals injected with parsley-seed, tarragon, sassafras and the camphor sassafrassy oils at the levels indicated, 30–50% of the rats being excluded from consideration. Several of the terpenes, oils and phenols were responsible for extensive skin necrosis, which necessitated the shifting of the site of injection from day to day.

Administration of diets supplemented with oils, principles and plant materials to partially hepatectomized rats

The feeding of diets containing chamomile and sassafras-bark teas at both 1·5 and 7·0%, blue Hungarian chamomile oils, H-1 (0·20%), H-4 (0·35%) and H-5 (0·20%), guaiazulene (0·20%), *p*-isopropylbenzaldehyde (0·35%), celery-seed and parsley-seed oils, each at 0·60%, and ground nutmeg (6·0%) to males caused increases in the liver increments over a period of 10 days (Table 2). The Egyptian, Romaine and English chamomile oils (each at 0·35%), ground cumin seed

Table 2. *Action of diets supplemented with essential oils and principles and chamomile and sassafras teas on rat-liver regeneration*

Test material and dietary level† (%)	No. of rats	Body weight (g)		Liver increment (g)	*t*
		Initial‡	Terminal‡		
Series 20E					
Control	15	227 ± 5·3	266 ± 7·2	2·194 ± 0·060	
Chamomile tea (1·5)	13	232 ± 5·5	271 ± 4·4	2·602 ± 0·112	3·40**
Sassafras bark tea (7·0)	10	230 ± 6·1	250 ± 6·7	2·743 ± 0·115	4·61**
Series 21E					
Control	17	280 ± 6·6	303 ± 8·0	2·126 ± 0·084	
Sassafras bark tea (1·5)	13	275 ± 6·4	302 ± 7·1	2·460 ± 0·113	2·62*
Series 22E					
Control	15	243 ± 8·4	277 ± 8·3	2·026 ± 0·111	
Chamomile tea (7·0)	11	247 ± 9·7	262 ± 11·7	2·624 ± 0·090	3·96**

Table 2.—*continued*

Test material and dietary level† (%)	No. of rats	Body weight (g)		Liver increment (g)	*t*
		Initial‡	Terminal‡		
Series 24E					
Control	12	273 ± 7·5	304 ± 6·8	2·276 ± 0·063	
Safrole (0·25)	12	272 ± 9·3	302 ± 11·1	3·303 ± 0·161	7·35**
Series 25E					
Control	14	332 ± 7·8	349 ± 7·4	2·540 ± 0·115	
Guaiene (0·40)	11	320 ± 11·1	332 ± 11·2	2·958 ± 0·113	2·53*
Series 27E					
Control	14	239 ± 7·5	278 ± 7·8	2·034 ± 0·076	
Chamomile oil, English (0·35)	12	235 ± 7·5	263 ± 9·9	2·234 ± 0·112	1·52
Chamomile oil, H-4 (0·35)	11	235 ± 7·4	276 ± 8·3	2·431 ± 0·112	3·03**
Chamomile oil, H-5 (0·20)	13	238 ± 6·7	272 ± 6·8	2·775 ± 0·188	3·76**
Vetivert oil, Haiti (0·35)	10	243 ± 8·9	282 ± 10·5	2·367 ± 0·135	2·30*
Isosafrole (0·25)	9	254 ± 10·7	256 ± 12·0	2·636 ± 0·119	4·49**
Series 28E					
Control	17	230 ± 11·3	259 ± 10·8	2·135 ± 0·066	
Vetivert oil, Bourbon (0·35)	14	243 ± 14·2	257 ± 13·4	2·166 ± 0·073	0·32
Series 29E					
Control	16	250 ± 3·1	287 ± 4·5	2·178 ± 0·070	
Vetivert oil, Haiti (0·50)	12	258 ± 3·8	297 ± 4·7	2·715 ± 0·077	5·11**
Vetiverol (0·50)	13	256 ± 5·8	291 ± 10·1	2·775 ± 0·126	4·59**
Guaiene (0·50)	13	254 ± 3·9	291 ± 6·1	2·713 ± 0·109	3·30**
p-Isopropylbenzaldehyde (0·35)	12	253 ± 3·6	288 ± 5·8	2·528 ± 0·079	3·30**
Series 30E					
Control	12	245 ± 5·4	264 ± 5·4	2·005 ± 0·118	
Guaiazulene (0·20)	10	243 ± 7·4	251 ± 9·1	2·814 ± 0·186	3·80**
Series 32E—females					
Control	16	182 ± 2·9	192 ± 3·8	1·630 ± 0·071	
Chamomile tea (1·5)	12	182 ± 3·3	186 ± 5·0	1·911 ± 0·102	2·32*
Safrole (0·25)	9	172 ± 1·0	183 ± 4·9	2·218 ± 0·068	5·44**
Series 32E—females					
Control	15	191 ± 2·3	213 ± 3·0	2·003 ± 0·063	
Chamomile, H-1 (0·20)	13	193 ± 3·8	214 ± 2·9	2·363 ± 0·091	3·36**
Series 35E—females					
Control	16	245 ± 2·4	260 ± 3·1	1·897 ± 0·062	
Nutmeg oil (0·60)	11	243 ± 2·9	260 ± 5·5	2·438 ± 0·140	3·92**
Series 36E					
Control	12	242 ± 7·5	255 ± 9·8	2·092 ± 0·081	
Cassia oil (0·60)	8	247 ± 9·4	265 ± 11·9	2·170 ± 0·224	0·38
Celery-seed oil (0·60)	8	245 ± 11·9	260 ± 9·2	2·420 ± 0·093	2·62*
Oleoresin tumeric (0·60)	8	245 ± 10·1	258 ± 12·0	2·083 ± 0·143	0·06
Series 38E					
Control	16	325 ± 9·7	328 ± 9·8	2·236 ± 0·076	
Cardamom-seed oil (0·60)	13	330 ± 10·6	339 ± 9·0	2·382 ± 0·084	1·28
Parsley-seed oil (0·60)	11	335 ± 14·9	307 ± 15·6	3·212 ± 0·166	5·92**
Series 39E					
Control	15	272 ± 5·0	298 ± 6·5	2·294 ± 0·056	
Tarragon oil (0·50)	12	271 ± 5·5	282 ± 7·4	2·240 ± 0·114	0·36
Series 40E					
Control	11	264 ± 7·6	270 ± 6·6	1·783 ± 0·076	
Nutmeg, decorticated and ground (6·0)	13	274 ± 8·6	279 ± 7·0	3·555 ± 0·167	9·09**
Series 41E					
Control	13	275 ± 9·9	281 ± 8·2	2·083 ± 0·118	
Jamaica allspice, ground (6·0)	13	275 ± 13·8	273 ± 12·2	2·037 ± 0·117	0·28
Series 44E					
Control	14	276 ± 11·8	284 ± 12·4	1·910 ± 0·087	
Cardamom, ground (5·0)	10	279 ± 14·4	289 ± 12·6	2·035 ± 0·22	0·86
Cumin seed, ground (6·0)	9	290 ± 15·5	283 ± 12·6	2·011 ± 0·110	0·51
Series 46E—females					
Control	13	223 ± 9·0	235 ± 7·9	1·998 ± 0·090	
Isosafrole (0·25)	15	218 ± 8·0	213 ± 8·1	2·345 ± 0·093	2·67*
Dihydrosafrole (0·25)	13	222 ± 9·3	233 ± 9·9	2·655 ± 0·123	4·29**

†Test diets were fed *ad lib.* for 10 days following surgery. Males were used in each series except where indicated otherwise (series 32E, 33E, 35E and 46E).

‡Initial weights were recorded prior to surgery and terminal weights at autopsy on day 10.

Results are means ± SEM for the numbers of rats stated. Significant *t* values are marked with asterisks: *$P < 0.05$; **$P < 0.01$.

(6·0%) and Bourbon vetivert oil (0·35%) were inactive as was tarragon oil (0·50%). However, Haitian vetivert oil (0·35 and 0·50%), vetiverol (0·50%) and guaiene (0·40 and 0·50%) increased the extent of regeneration (Table 2). Other agents having no significant effect when fed at the dietary levels (%) indicated in parentheses were allspice, Jamaica (6·0), alfalfa seed and leaf teas (7·0 each), cardamom, decorticated (6·0), cardamom-seed oil (0·60), cassia oil (0·60), chia-seed tea (7·0), chicory root, green and roasted (7·0 each), cinnamon (12·0), cloves (5·0), fennel tea (7·0), fenugreek-seed tea (7·0), garlic, instant powder (4·5), mustard seed (3·5), onion, instant powder (4·5), poppy seed, Dutch (7·0), sesame seed (9·0), saffron, Spanish whole (1·5), sarsaparilla, ethanol extract (2·7), tea, black (7·0), turmeric, oleoresin (0·60), and yarrow flowers, whole (6.0).

Diets shown to be active in the male were also checked in the female rats. Thus, chamomile oil, H-1 (0·20%), nutmeg oil (0·60%) and safrole, isosafrole and dihydrosafrole (each at 0·25%) stimulated the regenerating liver (series 32E, 33E, 35E and 46E; Table 2).

Effect of agents on the intact liver of rats of either sex

The sc injection of parsley-seed and tarragon oils, 4-allylanisole and *p*-isopropylbenzaldehyde at overall dosages of 1300, 2550, 1280 and 1215 mg/kg, respectively, to intact male rats caused marked increases in the ratios of both wet and dry liver weights to body weight (series 48E and 49E; Table 3). Liver enlargement was also noted in females injected with oils of nutmeg (3200 mg/kg), cumin (3100 mg/kg) and parsley seed (1400 mg/kg) and with the blue chamomile oil H-1 (3100 mg/kg), but even in similarly massive doses, eugenol was ineffective in rats of either sex. Table 3 also presents data for immature males fed chamomile tea (1·5%) and safrole (0·25%). Only the latter diet caused rises in the liver weights.

Microscopic examination of liver sections removed from treated groups of operated and intact rats revealed few notable differences from the corresponding controls.

DISCUSSION

Most of the essential oils, flavourings and plant materials included in the present study were conspicuous for their lack of any significant effect on the regenerating rat liver. These findings are understandable when it is considered that the main matrix comprises terpenes and that such agents, when tested individually in massive repeated doses, were found to have little influence on the regenerative process. Invariably, when a stimulatory response was evident, the behaviour was due to an aromatic principle such as 4-allylanisole, 4-propenylanisole, safrole and isosafrole, among others, although components such as eugenol and isoeugenol had no such effect.

Of the oils producing an effect on the regenerating liver, tarragon oil contains 4-allylanisole as the main aromatic principle, and significant amounts of this compound occur also in fennel-seed and anise oils. 4-Propenylanisole is present in fennel-seed oil at a level of 50–60% and at even higher levels (80–90%) in oil of anise (Bouchardat & Tardy, 1896). Oils of nutmeg and mace are very similar in make-up and

Table 3. *Body- and liver-weight findings for intact rats given daily sc injections of essential oils for 7 days or fed diets supplemented with chamomile tea or safrole for 10 days*

Oil or other test material and dose (mg/rat/day:mg/kg overall) or dietary level (%)	No. of rats	Body weight (g)		Liver weight (g/100 g body weight)			
		Initial†	Terminal†	Wet	*t*	Dry	*t*
Series 48E (sc)—males							
Control	12	245 ± 6·4	295 ± 5·8	4·343 ± 0·103		1·248 ± 0·026	
Parsley-seed (50; 1300)	12	245 ± 4·5	286 ± 6·5	4·754 ± 0·072	3·29**	1·391 ± 0·029	3·67**
4-Allylanisole (50; 1280)	12	254 ± 5·9	296 ± 6·9	4·694 ± 0·082	2·68*	1·394 ± 0·034	3·40**
Eugenol (100; 2610)	11	252 ± 5·9	284 ± 11·2	4·461 ± 0·085	0·87	1·324 ± 0·029	1·95
Tarragon (100; 2550)	11	255 ± 5·7	291 ± 6·1	4·931 ± 0·088	4·29**	1·442 ± 0·018	6·06**
Series 49E (sc)—males							
Control	12	264 ± 8·1	297 ± 11·2	3·931 ± 0·058		1·138 ± 0·020	
p-Isopropylbenzaldehyde (50; 1215)	12	271 ± 7·8	307 ± 10·1	5·278 ± 0·103	11·42**	1·538 ± 0·024	12·66**
Series 49E (sc)—females							
Control	12	206 ± 3·1	234 ± 3·8	4·207 ± 0·096		1·216 ± 0·032	
Cumin (100; 3100)	12	214 ± 2·8	231 ± 3·5	5·371 ± 0·078	9·46**	1·552 ± 0·030	7·64**
Eugenol (100; 3200)	12	212 ± 2·9	226 ± 2·7	4·325 ± 0·069	1·00	1·221 ± 0·026	0·12
Nutmeg (100; 3200)	12	208 ± 2·7	223 ± 2·7	4·752 ± 0·096	4·04**	1·314 ± 0·033	2·13*
Chamomile H-1 (100; 3100)	12	210 ± 3·2	239 ± 2·0	4·527 ± 0·073	2·74*		
Series 50E (sc)—females							
Control	8	240 ± 5·9	252 ± 6·0	3·844 ± 0·094		1·195 ± 0·032	
Parsley-seed (50; 1400)	8	240 ± 2·8	261 ± 3·4	4·371 ± 0·060	4·71**	1·319 ± 0·012	3·21**
Series 51E (diet)—males							
Control	12	97 ± 1·8	157 ± 3·8	4·500 ± 0·056		1·208 ± 0·020	
Chamomile tea (1·5%)	12	98 ± 1·3	145 ± 4·0	4·660 ± 0·122	1·19	1·296 ± 0·048	1·69
Safrole (0·25%)	12	100 ± 1·5	147 ± 3·7	5·222 ± 0·109	5·92**	1·479 ± 0·048	5·21**

†Initial weights were recorded prior to surgery and terminal weights at autopsy on day 10.
Results are means ± SEM for the numbers of rats stated. Significant *t* values are marked with asterisks: $*P < 0·05$; $**P < 0·01$.

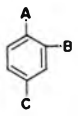
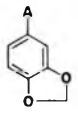
contain small amounts of safrole in addition to high myristicin contents, but their compositions are subject to wide variation. Among the compounds shown to be present in oil of parsley are apiole or 1-allyl-2,5-dimethoxy-3,5-methylenedioxybenzene (Thoms, 1903), 1-allyl-2,3,4,5-tetramethoxybenzene (Thoms, 1908) and myristicin (Ciamician & Silber, 1890). These agents may contribute to the efficacy of the oil in stimulating liver regeneration, and a study of their individual action would be timely. Cumin-seed oil contains up to about 60% *p*-isopropylbenzaldehyde or cuminaldehyde, an agent shown, in the present study, to heighten the regenerative process. The stimulatory action of safrole, a principle widely distributed in nature, is reflected in the findings with sassafras bark tea or the oil, among others. The propenyl derivative, isosafrole, simulated the action of safrole on the liver and presumably occurred at a relatively low level in the ylang ylang oil sample tested (3670 mg/kg; Table 1).

The observed activity of celery-seed oil and the oleoresin (Series 3E and 6E, Table 1) on the regenerating liver is of interest. These mixtures are high in the terpenes sedanolide and sedanonic anhydride, which are responsible for the characteristic odour, and also contain small amounts of aromatic components. Although terpenes tested at high sc levels were invariably without effect on liver regeneration, it might be useful to fractionate the above agents and aromatic moieties with the aim of pin-pointing the one or more active principles.

Representative agents were also administered by the dietary route with results which, with a few exceptions, followed the sc findings. In contrast to nutmeg, ground cumin seed at 6·0% was ineffective (Table 2), presumably because of its low level of *p*-isopropylbenzaldehyde. As tarragon oil added to the diet at 0·50% was inactive, it appears that effects may vary with the route as well as with dosage, since a positive effect followed sc injection of 2400 mg/kg (Table 1).

The configuration of phenols and other aromatic agents and their efficacy in stimulating liver regeneration when given by the sc route are summarized in Table 4. In marked contrast to *n*-propylanisole, the corresponding alcohol, 3-*p*-methoxyphenylpropanol, did not alter the increment from the range seen in controls. Inactive phenolic agents included guaiacol, the two phenolic aldehydes, 4-allyl- and 4-propenyl-2-methoxyphenols (eugenol and isoeugenol, respect-

Table 4. *Summary of liver findings in partially hepatectomized rats given seven daily sc injections of various aromatic compounds*

Compound*	Group A	Group B	Group C	Effect
Type: 				
4-Allylanisole (estragole)	$-CH_2\cdot CH:CH_2$	—H	$-OCH_3$	+
4-Propenylanisole (anethole)	$-CH:CH\cdot CH_3$	—H	$-OCH_3$	+
4-*n*-Propylanisole	$-CH_2\cdot CH_2\cdot CH_3$	—H	$-OCH_3$	+
3-*p*-Methoxyphenylpropanol	$-CH_2\cdot CH_2\cdot CH_2OH$	—H	$-OCH_3$	−
2-Allylphenol	$-CH_2\cdot CH:CH_2$	—OH	—H	−
4-Cumylphenol	$-C(CH_3)_2\cdot C_6H_5$	—H	—OH	−
4-Allyl-2-methoxyphenol (eugenol)	—OH	$-OCH_3$	$-CH_2\cdot CH:CH_2$	−
4-Propenyl-2-methoxyphenol (isoeugenol)	—OH	$-OCH_3$	$-CH:CH\cdot CH_3$	−
4-Methoxybenzyl alcohol	$-CH_2OH$	—H	$-OCH_3$	−
2-Hydroxy-4-methoxybenzaldehyde	—CHO	—OH	$-OCH_3$	−
2-Hydroxy-5-methoxybenzaldehyde	—OH	—CHO	$-OCH_3$	−
Anisole	$-OCH_3$	—H	—H	−
o-Methoxyphenol (guaiacol)	—OH	$-OCH_3$	—H	−
p-Isopropylbenzaldehyde (cuminaldehyde)	—CHO	—H	$-CH(CH_3)_2$	+
Allylbenzene	$-CH_2\cdot CH:CH_2$	—H	—H	+
β-Methylstyrene	$-CH:CH\cdot CH_3$	—H	—H	−
n-Propylbenzene	$-CH_2\cdot CH_2\cdot CH_3$	—H	—H	+
Allyl phenyl ether	$-O\cdot CH_2CH:CH_2$	—H	—H	+
Type: 				
Safrole	$-CH_2\cdot CH:CH_2$			+
Isosafrole	$-CH:CH\cdot CH_3$			+
Dihydrosafrole	$-CH_2\cdot CH_2\cdot CH_3$			+
Piperine (1-piperoylpiperidine)	$-CH:CH\cdot CH:CH\cdot CO\cdot N$ 			+
Piperonal	—CHO			−
Piperonyl alcohol	$-CH_2\cdot OH$			−

+ = Significant increase in the liver increment − = Little effect over the controls

*The dosages appear in Tables 1 and 2. Both 3-allylguaiacol and 4-allyl-2,6-dimethoxyphenol proved ineffective.

ively) and the parent compound, anisole. Of the monosubstituted compounds, stimulation was marked with allylbenzene and allyl phenyl ether, intermediate with *n*-propylbenzene and absent with β-methylstyrene. It must be emphasized that these findings pertain to the dosages screened (Table 1); more massive levels were not investigated. It was shown earlier (Gershbein, 1975) that the action of *n*-propylbenzene on the regenerating liver was borderline and that styrene and phenetole were without influence at the very high doses studied. It may be mentioned that the methylenedioxybenzene, piperine, which occurs in black pepper, was present in the pepper oil screened at too low a level to be effective (Table 2).

Toxicological studies on anisoles, allylbenzene and methylenedioxybenzenes have dealt with the determination of the oral LD_{50} (Jenner, Hagan, Taylor, Cook & Fitzhugh, 1964) and with the evaluation of microscopic changes, the most striking of which was hepatic cell enlargement (Hagan *et al.*, 1965; Taylor *et al.*, 1964). The increase in size of the hepatic cells varied from slight with safrole and isosafrole to moderate with dihydrosafrole. Other consistent alterations were minimal degrees of fatty metamorphosis, bile-duct proliferation and architectural irregularity. The degree of liver enlargement was slight with eugenol. Because of the short period of investigation used in the present study, morphological findings in the liver were not remarkable even though the doses administered were massive.

The effect on liver regeneration exerted by compounds with an azulene structure was ascertained in this study. Azulene as such has been reported to stimulate the restoration of liver weight when given by the oral and sc routes (Gershbein, 1975) and the current sc findings (Table 1) indicate that the fully aromatized structure is required for the activity. Azulenes occur in *Matricaria chamomilla* L. in cubeb, wormwood, guaiac wood and vetivert oils and in yarrow flowers, among others, and except for the blue chamomile oils and the tea, the findings were negative with these materials as well as with the pale oils with a low azulene content from *Anthemis nobilis* L. Whereas the hydro-derivative, guaiene, obtained from the sesquiterpene alcohol, guaiol, was ineffective, the aromatized molecule, guaiazulene, was very active by the sc route in an overall dosage of 1065 mg/kg (series 19E; Table 1) as were the Hungarian chamomile oils, H-4 and H-5. The latter should contain about 5% of the chamazulene, although some oils have been reported with levels up to 15%. It is thought that chamazulene does not occur in the plant as such but results from enzymatic action on the component sesquiterpenes (Ruhemann & Lewy, 1927) or from another derivative or precursor (Čekan, Herout & Šorm, 1954; Čekan, Procházka, Herout & Šorm, 1959; Koch, 1942). Given by the oral route, Haitian vetivert oil, the alcohol mixture vetiverol, and guaiene, like the Matricaria tea and oils, stimulated liver regeneration, although the Bourbon vetivert oil proved negative at a level of 0·35%. These findings indicate that significant aromatization of the hydroazulenes might occur in the gastro-intestinal tract.

With few exceptions, the stimulatory response engendered by several agents on the regenerating liver also extended to increases in wet and dry liver weights expressed in relation to body weight in intact rats. The results with intact and partially hepatectomized males were also checked in the female but no sex difference in the liver parameters could be discerned with the agents tested. The latter work was undertaken because of a report by one group (Homburger *et al.*, 1962) that fibrosis and ceroid deposits in the livers of female rats fed a 1% safrole diet were less marked than those observed in the male.

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PLACENTAL AND MAMMARY TRANSFER OF CHLORINATED FATTY ACIDS IN RATS

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Abstract—Oleic acid was chlorinated with ^{36}Cl and administered orally along with [^{3}H]oleic acid to both pregnant and lactating rats. There was about one-half as much ^{36}Cl as ^{3}H in foetal lipids on the day after dosing of the dams and the level of ^{36}Cl declined more rapidly than ^{3}H, indicating that the foetus may have the ability to dechlorinate fatty acids or selectively to eliminate them from the body. Greater amounts of chlorinated lipid crossed the placenta into the foetus as gestation advanced, reaching an average of 0·42% of the dose at day 19. Mammary transfer of ^{36}Cl from chlorinated oleic acid into the body lipids of 2-wk-old suckling rats was 15·9% in 24 hr compared with 34·0% for [^{3}H]oleic acid, while the corresponding transfer of ^{36}Cl-chlorinated linoleic and linolenic acids accounted for only 2·43 and 2·73% of the dose, respectively.

INTRODUCTION

Chlorine-bleached flour has been found to be toxic to rats and the lipids extracted from it were found to be particularly toxic (Cunningham, Lawrence & Tryphonas, 1977). The absorption and distribution of ^{36}Cl-chlorinated flour lipids, unsaturated fatty acids and triglycerides of unsaturated fatty acids have been studied in rats and compared with the metabolism of unchlorinated oleic acid labelled with tritium (Cunningham & Lawrence, 1976 & 1977a,b). These studies showed that although chlorination reduced the absorption and deposition of all lipids in tissues, the reduction was less severe with chlorinated oleic acid than with chlorinated linoleic or linolenic acid. Some tissues, including the brain, took up very little of the chlorinated lipids in comparison with adipose tissue, and excretion in the bile was negligible.

The present studies were conducted to determine the degree to which chlorinated fatty acids cross the placenta at various stages of development and the extent to which they are excreted into the milk.

EXPERIMENTAL

Materials. Chlorine-36 was purchased as 3·25 N-H^{36}Cl with a specific activity of 4·4 mCi/g from Atomic Energy of Canada Limited, Commercial Products, Ottawa and [9,10-^{3}H(n)]oleic acid (5·71) Ci/mmol) was purchased from New England Nuclear, Boston, Mass. Oleic, linoleic and linolenic acids (all 99·0% pure) were obtained from the Sigma Chemical Company, St Louis, Mo. These were labelled with ^{36}Cl and fully chlorinated with carrier chlorine as described previously (Cunningham & Lawrence, 1976 & 1977b). Each preparation included unchlorinated [^{3}H]oleic acid and was diluted with corn oil so that each ml contained 15 μCi [^{3}H]oleic acid and 50 mg of one of the chlorinated fatty acids labelled with 1·5 μCi ^{36}Cl.

Animal experiments

Experiment 1. This was designed to determine the length of time that would elapse between the dosing of a pregnant rat with chlorinated lipid and the occurrence of maximum levels in the foetus and to establish whether the foetus was able to eliminate chlorinated lipids from its body. Sixteen Wistar female rats weighing 270–370 g were obtained from Biobreeding Laboratories of Canada Ltd., Ottawa, Ontario, at days 12–14 of gestation and each was given orally 1 ml of the double-labelled oleic acid preparation described above. After 8 and 24 hr and 3 and 7 days, groups of four rats were anaesthetized with ether, blood samples were obtained by heart puncture and the rats were killed by an overdose of ether. The liver, placenta and foetuses were removed from each dam and the ^{3}H and ^{36}Cl activities of the lipids were determined by previously described methods (Cunningham & Lawrence, 1976).

Experiment 2. This was conducted to determine whether the amounts of chlorinated lipids transferred across the placenta increased as gestation advanced. In day 13, 16 or 19 of gestation, groups of four Wistar females (weighing 250–450 g) received the double-labelled oleic acid preparation by oral incubation and 24 hr later the rats were killed with an overdose of ether. The placenta, foetuses and intestine were removed from each dam and the carcass was passed repeatedly through a meat grinder. The ^{3}H and ^{36}Cl in the lipids of the placenta, foetuses and carcasses were determined as in Experiment 1.

Experiment 3. This was undertaken to determine the degree to which various chlorinated unsaturated fatty acids could pass into the milk. Twenty-four Wistar females, each with twelve 2-wk-old pups, were divided into three groups of eight and given orally the chlorinated oleic, linoleic or linolenic acid preparation in a dose of 1·0 ml/rat. In order to determine the excretion of the labelled compounds in the milk during the first 12 hr after dosing, the pups were removed immediately after dosing from four dams in each group, four of the pups being returned 3 hr later to each dam to suckle for 1 hr, after which they were killed with an overdose of ether. Four more pups from each litter were suckled for 1 hr between 7 and 8 hr after dosing and the remaining four between 11 and 12 hr. The excretion of labels in the milk between

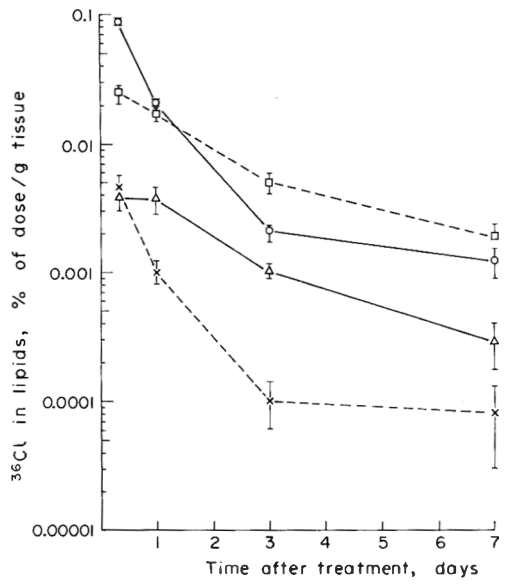

Fig. 1. Distribution of ^{36}Cl in the lipids of maternal liver (○), placenta (□), maternal blood (×) and foetuses (△) at intervals after giving pregnant rats an oral dose of 15 μCi [^{3}H]oleic acid and 50 mg chlorinated oleic acid (dichlorostearic acid) labelled with 1·5 μCi ^{36}Cl. Each point shows the mean ± SEM for four rats.

12 and 24 hr after dosing was determined with the other 12 litters. Only three of the 12 pups in each litter were left with the dam for the first 12 hr, after which they were killed. Three groups of three additional pups were then returned to suckle at 15, at 19 and at 23 hr, each of these groups being killed as soon as a 1-hr suckling period had been completed. The 12 dams that completed the 24-hr period were killed, their intestines were removed and their carcasses were ground as before. In each of the three original groups, the carcasses of the pups from each suckling period were pooled and these, too, were ground in the same manner. The ^{3}H and ^{36}Cl levels of the lipids of all carcass samples were determined.

RESULTS

Experiment 1

When females were given a dose of chlorinated lipid between day 12 and 14 of pregnancy, the concentration of ^{36}Cl in tissue lipids 8 hr later was highest in the liver (Fig. 1). The level of lipid ^{36}Cl was much lower in the blood of the dams than in the liver, but like the liver level, it declined quite rapidly until 3 days after dosing and then very slowly. The level of ^{36}Cl in foetal lipids was much lower than that in the placenta but both declined slowly and in parallel. The total percentage of the dose of ^{36}Cl appearing in the lipids of each tissue 8 hr after dosing was less than the percentage of the ^{3}H dose and declined more rapidly during the remainder of the experiment (Table 1). The ^{3}H from the unchlorinated oleic acid showed no appreciable decline in the foetus during the 7 days after dosing, while the ^{36}Cl from chlorinated oleic acid showed a gradual decline from day 1 to day 7.

Experiment 2

The data in Table 2 indicate that only small amounts of chlorinated lipids are deposited in the foetus even when the dam is dosed quite near parturition. There was a threefold increase in the concentration of ^{36}Cl in the foetal lipids following dosing on day 19 of gestation compared with that on day 13 and owing to the large increase in foetal material, this represented a sixtyfold increase in the total amount of ^{36}Cl transferred across the placenta into the foetal lipids. During the same period the total amount of lipid ^{36}Cl in the placenta showed only a fivefold increase. The concentration of chlorinated lipids in the 13-day old foetuses was just under 3% of that in the tissues of the dams 1 day after dosing and in the 19-day old foetuses the corresponding figure was about 15%. The total percentage of the dose present in all the foetal tissues 1 day after dosing was very small compared to that in the maternal tissues. It was interesting to find that while the levels of ^{3}H- and ^{36}Cl-labelled lipid increased in the foetus with advancing gestation, their levels in the dams declined. Very little of the lipid not retained in the carcasses could be accounted for in the placental or foetal tissues and the rest was probably metabolized to provide the extra energy required as gestation advanced.

Experiment 3

After the oral dosing of lactating rats with a ^{36}Cl-labelled fatty acid, considerably more of the label was

Table 1. *Distribution of radiosotopes in the tissue lipids of pregnant female rats at intervals up to 7 days after oral dosing between days 12 and 14 of gestation with 1 ml corn oil containing 15 μCi [^{3}H]oleic acid and 50 mg chlorinated oleic acid labelled with 1·5 μCi ^{36}Cl*

Time after dosing (days)	Isotope	Recovery of radioactivity (% of dose/tissue)* in			
		Maternal liver	Maternal blood	Placentae	Foetuses
0·33	^{3}H	3·330 ± 0·252	0·259 ± 0·074	0·064 ± 0·047	0·009 ± 0·003
	^{36}Cl	1·322 ± 0·018	0·097 ± 0·024	0·025 ± 0·003	0·006 ± 0·003
1	^{3}H	0·957 ± 0·262	0·050 ± 0·006	0·062 ± 0·003	0·031 ± 0·021
	^{36}Cl	0·332 ± 0·079	0·022 ± 0·005	0·018 ± 0·002	0·015 ± 0·010
3	^{3}H	0·145 ± 0·007	0·027 ± 0·005	0·051 ± 0·009	0·041 ± 0·009
	^{36}Cl	0·034 ± 0·005	0·002 ± 0·001	0·005 ± 0·001	0·008 ± 0·002
7	^{3}H	0·086 ± 0·025	0·017 ± 0·003	0·030 ± 0·011	0·039 ± 0·005
	^{36}Cl	0·018 ± 0·004	0·002 ± 0·001	0·002 ± 0·001	0·004 ± 0·001

*Values are means ± SEM for groups of four rats at each time.

Table 2. *Distribution of radioisotopes in the tissue lipids of pregnant female rats 24 hr after oral dosing at different stages of gestation with 1 ml corn oil containing 15 μCi[^3H]oleic acid and 50 mg chlorinated oleic acid labelled with 1·5 μCi ^{36}Cl*

Time of dosing (day of gestation)	Isotope	Recovery of radioactivity in Maternal carcass	Placentae	Foetuses
			% of dose/g tissue*	
13	^3H	0·160 ± 0·014^A	0·026 ± 0·003	0·005 ± 0·001^a
	^{36}Cl	0·070 ± 0·028	0·013 ± 0·001^a	0·002 ± 0·001
16	^3H	0·148 ± 0·047	0·033 ± 0·002	0·007 ± 0·001
	^{36}Cl	0·054 ± 0·017	0·020 ± 0·002^A	0·003 ± 0·001
19	^3H	0·081 ± 0·005^a	0·071 ± 0·032	0·012 ± 0·001^A
	^{36}Cl	0·041 ± 0·001	0·031 ± 0·004^A	0·006 ± 0·001
			% of dose/tissue*	
13	^3H	45·297 ± 2·865	0·080 ± 0·013^a	0·020 ± 0·005^a
	^{36}Cl	20·003 ± 4·015	0·040 ± 0·003^b	0·007 ± 0·001^b
16	^3H	39·182 ± 8·947	0·192 ± 0·017^A	0·151 ± 0·047^a
	^{36}Cl	14·139 ± 3·084	0·112 ± 0·013^B	0·062 ± 0·019
19	^3H	19·200 ± 2·632^a	0·502 ± 0·226^A	0·752 ± 0·111^A
	^{36}Cl	9·335 ± 1·762	0·219 ± 0·041^B	0·415 ± 0·150^B

*Means ± SEM for groups of four rats at each time. Statistical differences ($P < 0·01$) between values at different times for each tissue are indicated by different superscripts of the same letter (e.g. "A" is significantly greater than "a").

present in the body lipids of suckling rats when chlorinated oleic acid was given than was the case with chlorinated linoleic or linolenic acids (Fig. 2). A peak of 7·3% of the dose of ^{36}Cl from [^{36}Cl]oleic acid was found in the body lipids of rats that suckled in the period 7-8 hr after dosing. This declined to about 1·5% of the dose in each of the 4-hr intervals from 12–24 hr after dosing.

Table 3 shows that the total accumulation of ^{36}Cl from chlorinated oleic acid found in the body lipids of suckling rats was 14·33% of the dose during the first 12 hr with intermittent feeding of successive sets of rats and 11·78% when one set of rats was suckled for the entire 12-hr period. Very low levels of ^{36}Cl from chlorinated linoleic or linolenic acids were found in the body lipids of suckling rats during the first 12-hr period, but, unlike the findings with oleic acid, the continuous suckling tended to yield slightly higher accumulation than the intermittent feeding. The total accumulation of ^{36}Cl in the body lipids of rats suckled continuously for 12 hr and of their litter mates suckled intermittently for the following 12 hr was 15·90% of the dose for chlorinated oleic acid, 2·43% for linoleic acid and 2·73% for linolenic acid.

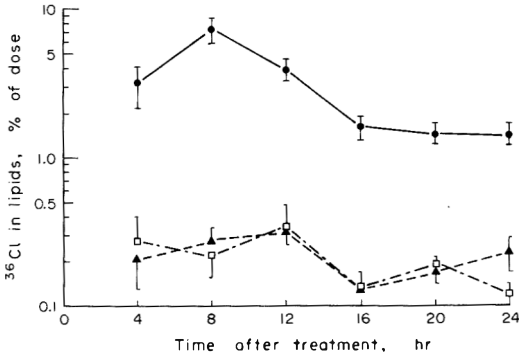

Fig. 2. Accumulation of ^{36}Cl in the body lipids of 2-wk-old suckling rats at intervals after the dams were dosed orally with 50 mg chlorinated oleic (●), linoleic (▲) or linolenic (□) acid labelled with 1·5 μCi ^{36}Cl. Each point shows the mean ± SEM for four rats from each of four litters for 0–12 hr and for three rats from each of four litters for 12–24 hr.

Considerably more of the ^3H label of unchlorinated oleic acid than of the ^{36}Cl label from the fatty acids was recovered in the bodies of suckling rats (Table 3). The ratio of ^3H to ^{36}Cl transferred via the milk showed very little variation from litter to litter but was considerably lower with ^{36}Cl-oleic acid (2·14) than with ^{36}Cl-linoleic acid (15.42) or ^{36}Cl-linolenic acid (13·50). These ratios were only slightly greater than those found in the carcass lipids of the respective dams.

DISCUSSION

It is well known that free fatty acids cross the placental membrane and from mid-pregnancy are rapidly taken up by foetuses (McBride & Korn, 1964). There is no transfer of chylomicron glycerides across the placental membrane but both free fatty acids and chylomicron glycerides are taken up from the plasma by mammary tissue (Linzell, 1968; McBride & Korn, 1964) and may appear in the milk without change in chain length (Lossow & Chaikoff, 1958). Iodinated fatty acids fed to cows have been detected in milk fat, in which their concentration was directly related to that in the neutral fat of the plasma (Garton, 1963).

Earlier work showed that the ^{36}Cl label of chlorinated oleic acid (dichlorostearic acid) fed orally to rats could be located in the lipids of all the major organs of the body (Cunningham & Lawrence, 1976) and therefore it is not surprising to find that it can cross the placenta in increasing amounts as pregnancy advances. It is particularly interesting to find that once they are taken up by the foetus, the ^{36}Cl-labelled lipids decline faster than the ^3H-labelled lipids. This may indicate that, like the adult (Cunningham & Lawrence, 1976), the foetus can dechlorinate chlorinated fatty acids or that it is capable of selectively recycling them back through the placenta.

Table 3. *Accumulation of ^{3}H and ^{36}Cl in the body lipids of suckling rats 12–24 hr after the dams were given orally 1 ml corn oil containing 15 μCi[^{3}H]oleic acid and 50 mg chlorinated oleic, linoleic or linolenic acids labelled with 1·5 μCi ^{36}Cl*

Radioactivity	Chlorinated fatty acid	Values for suckling periods (hr after dosing) of		
		0–12 (I)*	0–12 (C)†	0–24 (C/I)‡
In body lipids of suckling rats				
% of dose	Oleic	14·33 ± 1·40	11·78 ± 2·02	15·90 ± 2·81
	Linoleic	0·83 ± 0·27	1·93 ± 0·22	2·43 ± 0·26
	Linolenic	1·07 ± 0·35	2·39 ± 0·20	2·73 ± 0·06
^{3}H:^{36}Cl ratio	Oleic	2·07 ± 0·04	2·14 ± 0·05	2·14 ± 0·05
	Linoleic	15·40 ± 0·58	15·32 ± 1·92	15·42 ± 1·85
	Linolenic	15·68 ± 0·46	13·26 ± 0·23	13·50 ± 0·55
In maternal body lipids				
% of dose	Oleic	—	—	24·05 ± 9·61
	Linoleic	—	—	1·66 ± 0·29
	Linolenic	—	—	1·06 ± 0·20
^{3}H:^{36}Cl ratio	Oleic	—	—	1·82 ± 0·06
	Linoleic	—	—	9·65 ± 0·57
	Linolenic	—	—	11·23 ± 0·70

I = Intermittent C = Continuous

* Means ± SEM derived from cumulative totals of tracers in the body lipids of three sets of four rats, each set suckling for the last hour of each of three successive 4-hr periods.

†Means ± SEM derived from total tracers found in the body lipids of groups of three rats suckling continuously for 12 hr.

‡Means ± SEM derived from combined totals of tracers in the body lipids of three rats which suckled continuously for 12 hr and of three groups of three rats suckled for the last hour of three successive 4-hr periods.

The mammary transfer of chlorinated lipid was found to be considerably higher than placental transfer. In the case of oleic acid, 16% of the dose passed through the milk into the tissue lipids of suckling rats during the first 24 hr after dosing of the dam. Only very small quantities of the ^{36}Cl from chlorinated linoleic acid (2·43% of the dose) and chlorinated linolenic acid (2·73%) passed into the milk during the same period. Although milk normally contains less of these acids than oleic acid (Garton, 1963), chlorinated linoleic and linolenic acids are less digestible than chlorinated oleic acid (Cunningham & Lawrence, 1977b) and much less of the ^{36}Cl from them was found in the tissue lipids of the lactating dams. It is, therefore, difficult to determine which factor was more responsible for the differences in mammary transfer.

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EFFECT OF BENOMYL AND BENOMYL HYDROLYSIS PRODUCTS ON *TETRAHYMENA PYRIFORMIS**

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Abstract—The toxicity of the fungicide benomyl (methyl-1-(butylcarbamoyl)-2-benzimidazol-2-yl carbamate) was evaluated at the molecular level using the ciliated protozoan *Tetrahymena pyriformis* as a model eukaryotic cell system. Growth of *T. pyriformis* was inhibited in a dose-related manner by benomyl and two major hydrolysis products, methylbenzimidazol-2-yl carbamate (MBC) and butyl isocyanate (BIC). A minor degradation product, 2-aminobenzimidazole (2-AB) had no effect on growth. Light-microscopic observations of the organisms revealed a slowing of cellular motility and the development of rounded cells after exposure to 20 ppm benomyl or BIC in the incubation medium. After the cells became immobile, part of the cell membrane appeared to separate, swell and form a bubble along the side of the cell. Similar concentrations of MBC and 2-AB had no apparent morphological effect upon the cells. Increasing the concentration of benomyl decreased the incorporation of [^{14}C]acetate and [^{14}C]amino acids into protein and [^{3}H]uridine into RNA. The incorporation of [^{14}C]acetate into lipids and of [^{3}H]thymidine into DNA increased with benomyl concentrations of 5 ppm, but a decrease in incorporation occurred with higher concentrations.

INTRODUCTION

The systemic fungicide benomyl (methyl-1-(butylcarbamoyl)-2-benzimidazol-2-yl carbamate), the active ingredient in Benlate®, was first reported to be fungitoxic by Delp & Klopping (1968). Benomyl has been reported to be the most effective of the benzimidazole fungicides and has the widest spectrum of fungitoxic activity among the newer systemic fungicides (Erwin, 1973).

Benomyl is hydrolysed to methylbenzimidazol-2-yl carbamate (MBC) and butyl isocyanate (BIC) in aqueous solution (Fig. 1). In a model system of methanol–water (50:50, v/v), the half-life of benomyl was 4·28 hr (Calmon & Sayag, 1976). The MBC is hydrolysed in turn to 2-aminobenzimidazole (2-AB) and BIC is converted to *n*-butylamine (Fig. 1). Baude, Gardiner & Han (1973) reported that the principal residues on treated crop samples were benomyl, MBC and 2-AB.

The oral LD_{50} of benomyl in fasted rats was reported to be >10 g/kg body weight (Sherman, Culik & Jackson, 1975). When [^{14}C]benomyl was administered to rats in a single oral dose, 99% was excreted in the urine and faeces within 72 hr (Gardiner, Kirkland, Klopping & Sherman, 1974). The principal metabolites were the glucuronide and sulphate conjugates of 5-hydroxy-2-benzimidazolecarbamate (Douch, 1973; Gardiner, Brantley & Sherman, 1968; Gardiner *et al.* 1974). Sherman *et al.* (1975) reported no pathological tissue changes, teratogenic effects or mutagenic effects when benomyl was fed to rats at levels of 0·25 or 0·50% of the diet. However, when Chang cells were exposed to benomyl at a level of 10^{-5} M in the medium, metaphase arrest and cytochrome damage were observed (Styles & Garner, 1974). These mutation studies were initiated because of the structural similarity of benzimidazoles with purine bases. Sieler (1972) reported that MBC and 2-AB caused forward mutations in *Salmonella typhimurium*.

Tetrahymena pyriformis is a single-cell organism, which has nutritional requirements, organelles and biochemical pathways similar to those of mammalian cells (Hill, 1972). In addition, several drugs (e.g. cycloheximide, actinomycin D and triparanol) have molecular biological effects on *T. pyriformis* similar to their effects in higher species (Elliot, 1973). Since the data on benomyl toxicity are somewhat conflicting, the use of *T. pyriformis* offers an opportunity for the effects of benomyl to be studied in a simplified system.

EXPERIMENTAL

Materials. Analytical-grade benomyl and MBC were obtained from E. I. du Pont de Nemours & Co., Wilmington, Del., reagent-grade BIC and 2-AB from Eastman Chemical Co., New York, proteose peptone and yeast extract from Difco Laboratories, Detroit, Mich., Soluene 100 from Packard Instrument Co., Chicago, Ill., and sodium [2-^{14}C]acetate, ^{14}C-labelled reconstituted protein hydrolysate, [*Me*-^{3}H]thymidine and [5-^{3}H]uridine from New England Nuclear, Boston, Mass. All other reagents used were reagent grade.

Measurement of cell growth. T. pyriformis strain E was incubated at 23 ± 1°C in the dark in stationary 1-litre Roux flasks containing 200 ml autoclaved medium consisting of 2% (w/v) proteose peptone and

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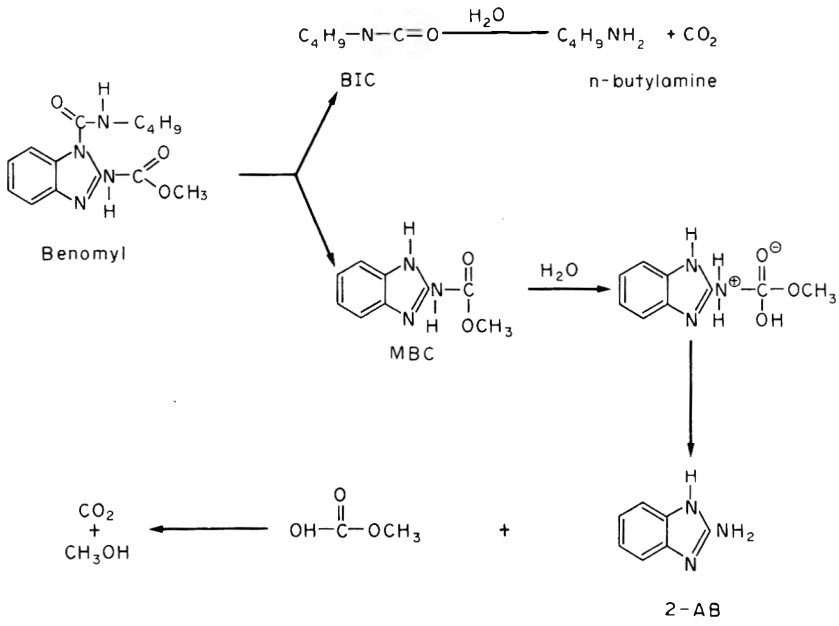

Fig. 1. Hydrolysis products of benomyl.

0·1% (w/v) yeast extract. For experimental purposes a 1% inoculum of early log growth cells was used. Benomyl, MBC, 2-AB and BIC were prepared as solutions of various concentrations in dimethylsulphoxide (DMSO) and were added to 20-hr-old 200-ml cultures, so that the final concentration of DMSO in the medium was 1%. This level of DMSO did not affect cell growth (Surak, Bradley, Branen & Shrago, 1976). Fresh solutions of the pesticide or derivative in DMSO were prepared immediately before their addition to the test culture. Growth of *T. pyriformis* was measured by drying a washed aliquot of cells to constant weight or by counting cell numbers with a Neubauer haemocytometer. The data presented are the means of five growth trials with four determinations at each concentration of the test compound in each trial.

Microscopic observations. DMSO solutions of benomyl, MBC, BIC and 2-AB were prepared and added to aliquots of early log growth cells to yield a final concentration of 20 ppm of test compound in the medium. After an initial incubation period of 1 min, aliquots of 0·1 ml were removed at various intervals for phase-interference microscopic observation through a Nikon SUR-KE research microscope equipped with a phase-interference unit.

Radioactivity measurements. Benomyl was added to 20-hr cultures, and incubated for 5 hr, after which radioisotopes were added for an additional 3 hr of incubation. The incorporation of sodium [2-^{14}C]acetate into lipid, protein and ^{14}C-labelled amino acids into protein, [5-^{3}H]uridine into RNA and [*Me*-^{3}H]-thymidine into DNA were measured. Cells were then harvested by centrifugation and washed three times with cold de-ionized water. The procedure of Shug, Elson & Shrago (1969) was used to fractionate cellular components and to determine the quantity of label from [^{14}C]acetate incorporated into protein and lipid. The procedure of Milner (1967) was used to determine the incorporation of [^{3}H]thymidine into DNA, [^{3}H]uridine into RNA and [^{14}C]amino acids into protein. The protein or nucleoprotein precipitate was dissolved in Soluene 100 prior to the determination of radioactivity. The samples were counted in scintillation fluid (toluene–PPO–POPOP, 3·5 litres: 10·5 g:350 mg) using a Packard Tri-Carb Liquid Scintillation Spectrometer Model 3375. The data presented are the means of three experiments with three replications at each concentration of benomyl.

RESULTS

Growth and morphology

The growth of *T. pyriformis* was inhibited by increasing concentrations of benomyl (Fig. 2). When benomyl was added to cultures in concentrations of 20 or 25 ppm, there was a decrease in cell count in the first 8–9 hr. At 12 hr, during the log growth phase, the benomyl dose effecting 50% growth inhibition (ED_{50}) was 9·10 ppm in the medium (Table 1). In addition, compared with benomyl-free control cultures there was a decrease in cell numbers in the stationary growth phase, with an ED_{50} at 36 hr of 8·04 ppm.

The initial reduction in cell number was detected 45 min after addition of 20 ppm benomyl to an early log growth culture. Microscopic examination showed swelling of cells and nuclei with an increase in cytoplasmic vacuoles and loss of pyriform shape (Fig. 3a). *T. pyriformis* then became non-motile with part of the cell membrane separating and forming a bubble along the cell (Fig. 3a). Cells so affected were never observed to recover.

T. pyriformis growth was inhibited also in the presence of BIC, the ED_{50} of which was 10·96 ppm at 12 hr (Table 1; Fig. 4). Addition of BIC to an early log growth culture resulted in an initial decrease in cell concentration, the decrease being proportional to

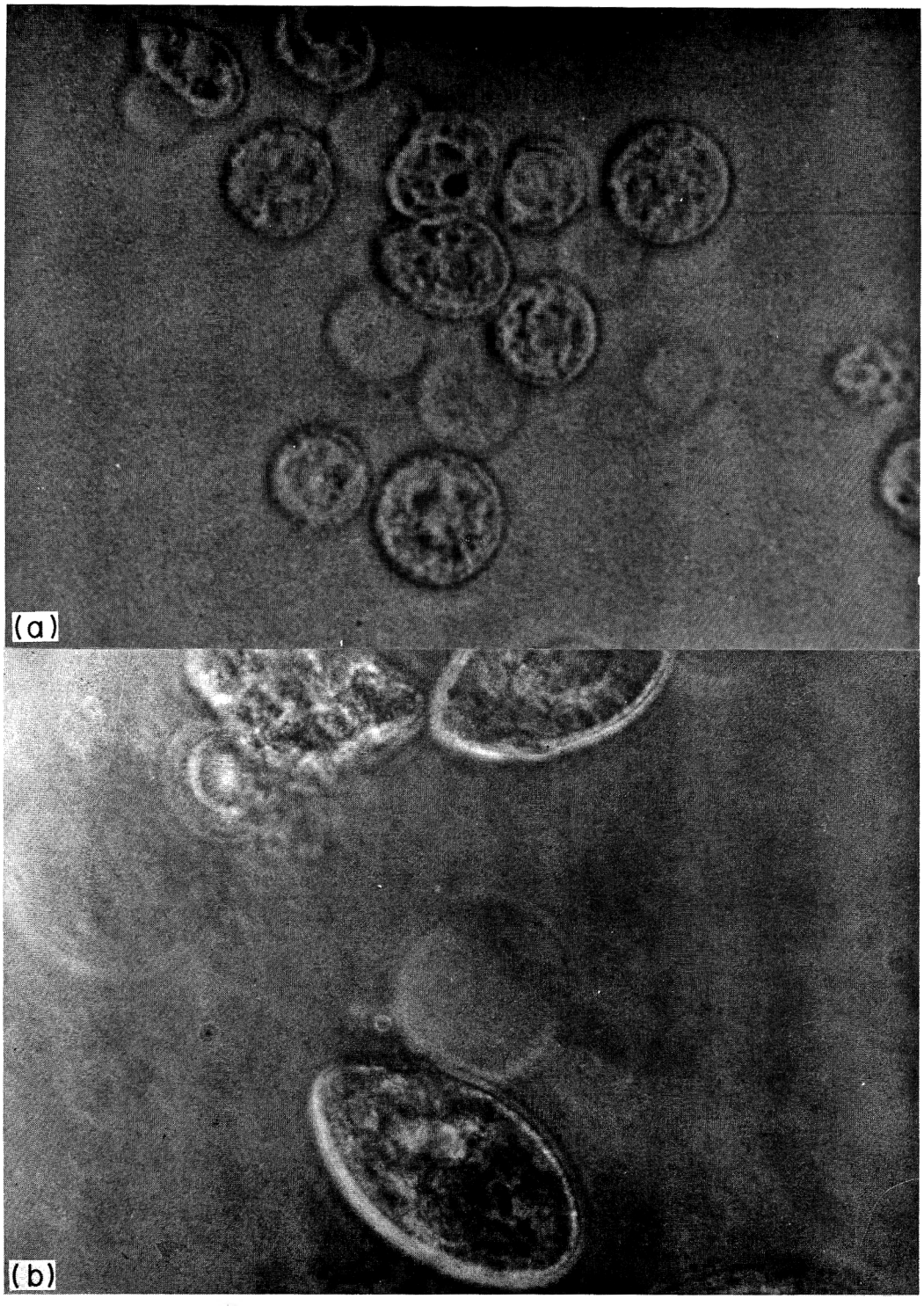

Fig. 3. Morphological changes in *T. pyriformis* demonstrated by phase-interference microscopy following exposure to a test compound, added to the culture medium in DMSO solution, and subsequent immobilization with methylcellulose; (a) after 45-min exposure to 20 ppm benomyl (×200) and (b) after 40-min exposure to 20 ppm BIC (×400).

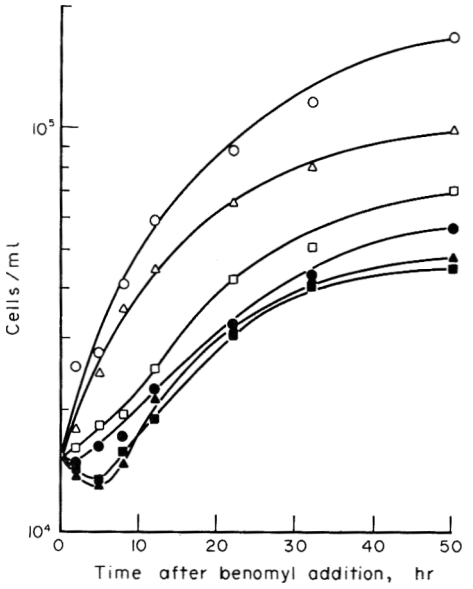

Fig. 2. Growth curves of *T. pyriformis* in media containing 0 (○), 5 (△), 10 (□), 15 (●), 20 (▲) or 25 (■) ppm benomyl added to an early log growth culture. Each point is the mean of 20 values (four replicates from each of five trials).

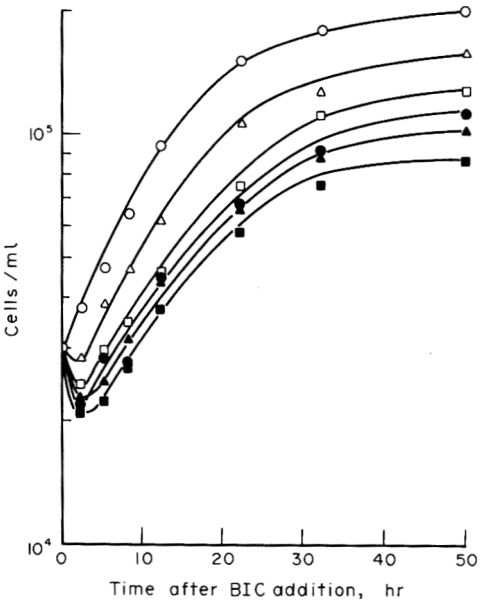

Fig. 4. Growth curves of *T. pyriformis* in media containing 0 (○), 5 (△), 10 (□), 15 (●), 20 (▲) or 25 (■) ppm BIC, added to an early log growth culture. Each point is the mean of four replicates from each of five trials.

the BIC concentration. Recovery to the initial cell concentration occurred within 10 hr. When compared to the control group, BIC decreased the cell density in the stationary growth phase, with an ED_{50} of 12·05 ppm at 36 hr (Table 1). Cellular changes similar to those induced by benomyl were observed microscopically with BIC (Fig. 3b). Within 40 min of the addition of 20 ppm BIC to an early log growth culture, the cells and nuclei swelled and there was membrane separation with bubble formation. As in the case of benomyl treatment, individual cells did not recover.

MBC inhibited the growth of *T. pyriformis* during both the log and stationary growth phases (Fig. 5), the ED_{50} values being 10·86 and 6·38 ppm, respectively. Cell populations in media containing 10 ppm MBC or more showed an initial decrease in cell density, which returned to the initial level in 7 hr. Within 25 min of the initial exposure of early log growth cultures to 20 ppm MBC, microscopic observations revealed an apparent increase in the motility of most cells in the culture. The few cells that became non-motile contained cytoplasmic granules.

Growth of *T. pyriformis* was not inhibited in media containing 15–25 ppm 2-AB (Fig. 6), and no morphological changes were observed when 20 ppm 2-AB was added to early log growth cultures.

Table 1. *Effective doses of benomyl and its hydrolysis products on the growth of* T. pyriformis

Chemical	ED_{50} (ppm) values at 12 hr	36 hr
Benomyl	9·10 ± 1·02	8·04 ± 0·89
BIC	10·96 ± 1·44	12·05 ± 2·31
MBC	10·86 ± 2·17	6·38 ± 1·10
2-AB	>25	>25

BIC = Butyl isocyanate
MBC = Methyl 2-benzimidazolecarbamate
2-AB = 2-Aminobenzimidazole

*Added as a concentrated solution dissolved in DMSO to an early log growth culture of *T. pyriformis*.

Means ± SEM were calculated as the concentration in ppm in the media needed to cause 50% inhibition of the growth of *T. pyriformis* (from Figs. 2, 4, 6 and 7).

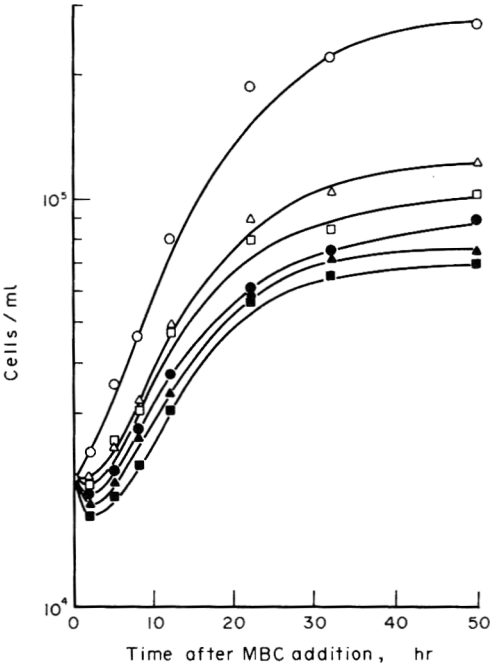

Fig. 5. Growth curves of *T. pyriformis* in media containing 0 (○), 5 (△), 10 (□), 15 (●), 20 (▲) or 25 (■) ppm MBC added to an early log growth culture. Each point is the mean of four replicates from each of five trials.

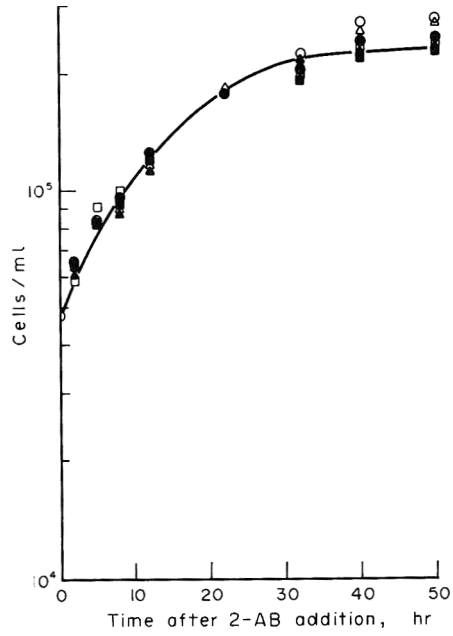

Fig. 6. Growth curves of *T. pyriformis* in media containing 0 (○), 5 (△), 10 (□), 15 (●) 20 (▲) or 25 (■) ppm 2-AB added to an early log growth culture. Each point is the mean of four replicates from each of five trials.

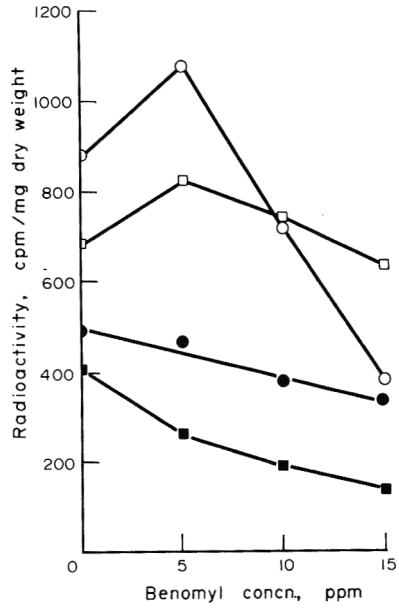

Fig. 7. Effect of benomyl (added to an early log growth culture of *T. pyriformis* and incubated for 5 hr prior to the addition of radioactive isotope) on the synthesis of macromolecules. Protein synthesis (●) and lipid synthesis (○) were determined from the amount of ^{14}C incorporated when [^{14}C]acetate (0·5 μCi) was added to the cultures and incubated for 3 hr. DNA synthesis (□) and RNA synthesis (■) were determined from the amount of ^{3}H incorporated when [^{3}H]thymidine or [^{3}H]uridine (5.0 μCi) was added to the cultures and incubated for 3 hr.

Radioisotope measurements

Benomyl added to an early log growth culture decreased the incorporation of [^{14}C]acetate into protein with 32% inhibition at 15 ppm (Fig. 7). The incorporation of [^{14}C]acetate into lipids increased with 5 ppm benomyl but decreased with benomyl concentrations of 10 ppm or more (Fig. 7). A decrease in the incorporation of [^{3}H]uridine at all levels of benomyl studied (Fig. 7) was paralleled by the incorporation of [^{14}C]amino acids into protein. However, 5 ppm benomyl stimulated DNA synthesis as well as lipid synthesis compared with the control level (Fig. 7). Addition of higher benomyl concentrations to the cultures decreased the incorporation of [^{3}H]thymidine into DNA.

DISCUSSION

Sherman *et al.* (1975) did not observe any pathological changes, teratogenic effects or mutagenic effects when three generations of rats were fed benomyl at dietary levels of 0·25%. However, mammalian cells in culture have been reported to be affected by benomyl. Styles & Garner (1974) reported ED_{50} values of 6·8 and 5·0 ppm for benomyl and MBC, respectively, in cultures of Chang cells. The data reported here are consistent with this finding, in that similar ED_{50} values were found for each of these compounds in cultures of non-replicating *T. pyriformis* (Table 1).

Benomyl and BIC have similar effects on the morphology of *T. pyriformis* (Figs. 3a,b), but the swelling and membrane changes were not observed with MBC. In addition, the primary toxic effect of benomyl appears to be an inhibition of protein synthesis and RNA synthesis (Fig. 7). These inhibitory effects were dose dependent; 15 ppm benomyl inhibited protein synthesis by 50%, and 10 ppm inhibited RNA synthesis by 50%. Synthesis of both lipids and DNA was increased by 5 ppm benomyl and inhibited by 15 ppm (Fig. 7). Hammerschlag & Sisler (1972) reported that the mode of action of benomyl differed from that of MBC. While 15 ppm benomyl inhibited DNA, RNA and protein synthesis, 10 ppm MBC inhibited DNA synthesis after exposure for 1 hr but only moderately inhibited RNA synthesis and protein synthesis after exposure for 4 hr. Later it was postulated that the difference could be attributed to the release of BIC (Hammerschlag & Sisler, 1973). However, these authors did not report the effect of 5 ppm benomyl on DNA synthesis in *Saccharomyces pastorians* (Hammerschlag & Sisler, 1972). Styles & Garner (1974) reported that benomyl and MBC can interfere with mitosis in Chang cells; DNA replication was unaffected by either compound, but microscopic observations showed that mother-daughter cells often failed to separate as a result of lagging anaphase bridges, producing an abnormal number of doublet cells. This phenomenon was not observed in *T. pyriformis* exposed to benomyl.

Results of experiments reported here indicate that 2-AB is not toxic to *T. pyriformis* under the conditions studied (Fig. 6). Toxicity of 2-AB to mammalian cells in culture has not been reported, and 2-AB is not toxic to fungi (Edgington, Khew & Barron, 1971).

These results form a basis for establishing the action of benomyl on a model cellular system.

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DETERMINATION OF POLYCHLORINATED DIBENZOFURANS IN TISSUES OF PATIENTS WITH 'YUSHO'

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Abstract— Tissues of patients with 'Yusho' and of persons not suffering from this disease were analysed for polychlorinated biphenyls (PCBs) and polychlorinated dibenzofurans (PCDFs) by column chromatography, gas chromatography, and gas chromatography–mass spectrometry. Both PCBs and PCDFs were identified in the tissues of patients with Yusho, while only PCBs were detected in the tissues of the other persons studied. In contrast to the PCBs, which were found far more in the adipose tissue than in the liver, PCDFs occurred in very similar concentrations in these two types of tissue. The persisting PCDFs in the tissues of Yusho patients were almost exclusively penta and hexachloro derivatives.

INTRODUCTION

Polychlorinated biphenyls (PCBs) are known to accumulate in the human body as a result of their ingestion in foods, mainly fish. Since polychlorinated dibenzofurans (PCDFs) have been reported to contaminate commercial PCB formulations (Bowes, Mulvihill, DeCamp & Kende, 1975; Bowes, Mulvihill, Simoneit, Burlingame & Risebrough, 1975; Nagayama, Kuratsune & Masuda, 1976; Nagayama, Masuda & Kuratsune, 1975; Roach & Pomerantz, 1974; Vos, Koeman, van der Maas, ten Noever de Brauw & de Vos, 1970) the human body may also be contaminated with PCDFs, which are more toxic than PCBs. PCBs are known to have been the cause of a mass food poisoning which affected more than 1000 persons in western Japan in 1968 (Kuratsune, Yoshimura, Matsuzaka & Yamaguchi, 1972; Tsukamoto *et al.* 1969). The disease is called 'Yusho' i.e. oil disease, because the patients were proved to have consumed a commercial rice oil contaminated with a large amount of Kanechlor-400 (KC-400), a Japanese PCB formulation. Recently, a sample of unused KC-400 and samples of the rice oil consumed by patients with Yusho were found to be contaminated with 20 and 5 ppm PCDF, respectively, indicating that the patients had ingested PCDFs with the PCBs (Nagayama *et al.* 1975 & 1976).

All these findings made it imperative to analyse tissues of unaffected persons and of patients with Yusho for their possible content of PCDFs. This paper describes the main results of such an investigation.

EXPERIMENTAL

Materials analysed. Samples of the liver, mesenteric adipose tissue and a few other organs of three Yusho patients who had died were supplied from the Department of Pathology, School of Medicine, Yamaguchi University, and the Department of Pathology, Faculty of Medicine, Kyushu University. These tissues had been preserved in 4% formaldehyde solution after resection. Cases 1 and 2 had died in 1969, the year after the outbreak of Yusho, while case 3 had died in 1972. From the severity of dermal lesions, case 2 had been identified as grade 4 according to the criteria established by Goto & Higuchi (1969) and case 3 as grade 3, while case 1 was judged to be grade 3 or 4 from an examination of his clinical records. The similarly preserved adipose tissue and livers of two persons without Yusho who died in accidents were also obtained for analysis from the Department of Forensic Medicine, Faculty of Medicine, Kyushu University.

Chemicals. *n*-Hexane, ethanol, potassium hydroxide, anhydrous sodium sulphate and silica gel, all reagent-grade, were purified by the methods described previously (Nagayama *et al.* 1975). Reagent-grade carbon tetrachloride and methylene chloride were fractionally redistilled. Alumina for column chromatography (about 300 mesh) was obtained from Wako Pure Chemical Industry, Ltd., Osaka, and activated by heating at 150°C for 12 hr before every use. PCDF for use as a standard was synthesized by chlorination of dibenzofuran (Nishizumi, Kuratsune & Masuda, 1975) and consisted mainly of tetra- and pentachlorodibenzofurans. 2,3,7,8-Tetrachlorodibenzofuran was kindly donated by Dr. J. G. Vos, National Institute for Public Health, Bilthoven, The Netherlands, and by Dr. I. H. Pomerantz, Food and Drug Administration, Washington, DC, USA. KC-500 was supplied by Kanegafuchi Chemical Industry, Japan.

Analytical procedure. The official standard analytical method for PCB established by the Ministry of Health and Welfare. Tokyo (Kawashiro *et al.* 1972) and the separation method for PCDFs and PCBs (Nagayama *et al.* 1975) were used to fractionate PCBs and PCDFs from tissue samples and to separate the PCBs and PCDFs in the fraction. Samples (about 5–180 g) were first homogenized with *n*-hexane (150–400 ml) and anhydrous sodium sulphate (20 g) in a Waring blender. The *n*-hexane solutions were pooled and evaporated to dryness to yield fatty residues, which were saponified with 1 N-KOH–ethanol

solution (50 ml). The *n*-hexane extract of the KOH–ethanol solutions was concentrated and then chromatographed on a column of silica gel (2 g) eluted with *n*-hexane (150 ml). The *n*-hexane eluate containing PCBs and PCDFs was further fractionated on a column of activated alumina (3 g) using *n*-hexane–carbon tetrachloride (4:1, v/v; 60 ml) and *n*-hexane–methylene chloride (4:1, v/v; 20 ml) as successive eluants. PCBs were expected to be contained in the former eluate and PCDFs in the latter. These eluates were evaporated, and the residues were redissolved in *n*-hexane and subjected first to gas chromatography (ECD–GC) in a Beckman GC 72-5 fitted with an electron-capture detector and a glass column (2 mm × 2 m) containing Chromosorb W AW-DMCS (100–120 mesh) coated with 2% SE-30 (column temp. 200°C, carrier gas helium, flow rate 30 ml/min) and subsequently to gas chromatography–mass spectrometry (GC–MS) using a type D-100 instrument (Japan Electron Optics Laboratory, Tokyo) and a column similar to that used for ECD–GC, the ionizing energy being 25 eV and the ionizing current 0·3 mA.

PCBs were estimated quantitatively by means of the official standard analytical method already mentioned (Kawashiro *et al.* 1972), while PCDFs were estimated by comparing the heights of the gas-chromatographic peaks with those of synthesized PCDF, the assumption being made that all the PCDF isomers isolated from tissue samples had the same peak height sensitivity regardless of the number and sites of chlorine substitution. This assumption can be justified on two counts. First we had found in quantitative studies of PCDF in Kanechlors and 'Yusho oil' that the use of this assumption gave figures very close to those obtained by the perchlorination method (Nagayama *et al.* 1975 & 1976). The latter method could not be used in the present analyses because the PCDF levels in the tissue samples were much lower than those in Kanechlors or 'Yusho oil'. Secondly, as will become apparent later, the patterns of gas-chromatographic peaks were similar for PCDFs isolated from a variety of tissues taken from the Yusho patients, indicating that a fairly accurate comparison could be made of PCDF levels in various tissue on the basis of this assumption.

RESULTS

Gas chromatograms of PCB and/or PCDF fractions of the liver of a patient (case 1) and of the toxic rice oil, before and after separation on an alumina column, are shown in Fig. 1, chromatograms A, B and C indicating that a good separation of PCB and PCDF was achieved.

Comparison of chromatograms C and D shows that there is a distinct difference in the peak pattern between PCDF obtained from the liver of the patient and that from the toxic rice oil. The former consisted almost exclusively of peaks 4′, 6′ and 7′, which coincided exactly in retention times with peaks 4, 6 and 7, respectively, in chromatogram D. Peak 3 of chromatogram D had exactly the same retention time as that of 2,3,7,8-tetrachlorodibenzofuran, but was hardly seen in chromatogram C. A similar lack of peaks corresponding to peaks 1, 2 and 5 was also noted in chromatogram C.

Each peak in chromatogram C was examined by GC–MS and the components of peaks 4′ and 6′ were identified as pentachlorodibenzofurans, and that of peak 7′ as hexachlorodibenzofuran. The mass spectrum of peak 6′ is shown in Fig. 2, together with the mass spectrum of a synthesized pentachlorodibenzofuran, which showed the same retention time as peak 6′. These two mass spectra are considered to be practically the same. The gas chromatograms of other tissue samples of Yusho patients were almost the same as chromatogram C in Fig. 1.

Table 1 summarizes the results of analyses of various tissues from patients and from accident victims. With the exception of the spleen of case 2, all the tissues taken for analysis from Yusho patients contained PCDFs. The adipose tissues and livers of the accident victims contained PCBs but did not contain any detectable amount of PCDFs (detection limit 0·05 ppb). Table 1 also shows another interesting fact

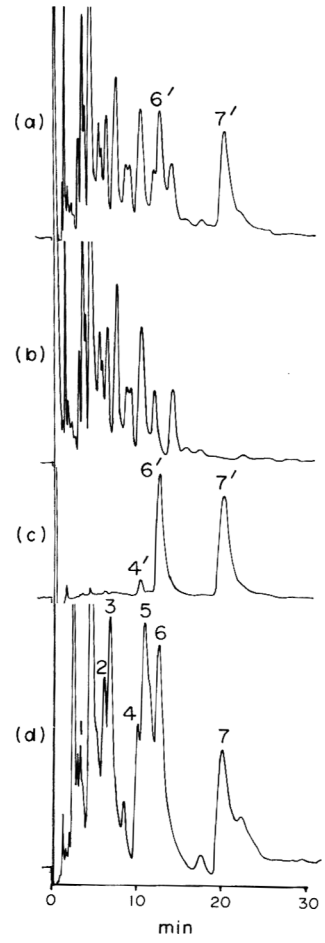

Fig. 1. Gas chromatograms of PCB and/or PCDF fractions obtained from the liver of a patient with Yusho (case 1) and from the toxic rice oil: (a) PCB + PCDF fraction from the liver of a patient with Yusho; (b) PCB fraction separated from A; (c) PCDF fraction separated from A; (d) PCDF fraction from the rice oil. The peaks were identified by GC–MS as (1) $C_{12}H_5OCl_3$; (2–3) $C_{12}H_4OCl_4$; (4–6, 4′, 6′) $C_{12}H_3OCl_5$; (7, 7′) $C_{12}H_2OCl_6$. Column, 2% SE-30; detector, ECD.

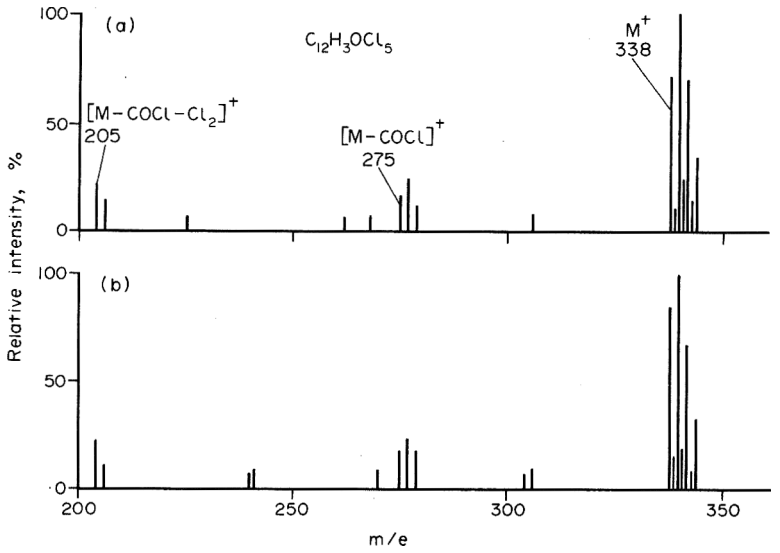

Fig. 2. Mass spectra of (a) gas-chromatographic peak 6′ of PCDF fraction from the liver of a patient (case 1) with Yusho and (b) peak 6 of synthesized PCDF.

in that in all three patients concentrations of PCDF in relation to PCB were much higher in the liver than in the adipose tissue. The concentrations of PCDF on whole basis were particularly high in the liver in cases 1 and 2. This pattern of distribution contrasted sharply with that of the PCBs which showed far higher concentrations in the adipose tissue than in the liver. Analysis of the kidneys from cases 2 and 3 showed that PCDF concentrations in this organ were very much lower than those found in the liver.

DISCUSSION

In contrast to the situation with PCBs (Burse, Kimbrough, Villanueva, Jennings, Linder & Sovocool, 1974; Curley, Burse, Grim, Jennings & Linder, 1971; Grant, Phillips & Villeneuve, 1971; Platonow, Liptrap & Geissinger, 1972), the tissue distribution of PCDFs has received little attention. This study seems to be the first to identify PCDFs in the tissues of patients with Yusho and to disclose the peculiarity of their tissue distribution, although no definite conclusions can yet be drawn from our findings because of the limited number of observations made. It is noteworthy, in this connexion, that polychlorinated dibenzo-*p*-dioxins, which are closely related to PCDFs in chemical structure, also have a definite tendency to accumulate in the livers of animals (Allen, Van Miller & Norback, 1975; Firestone, Flick, Ress & Higginbotham, 1971; Norback, Engblom & Allen, 1975; Van Miller, Marlar & Allen, 1976; Williams, Cunningham & Blanchfield, 1972). As indicated ear-

Table 1. *Concentrations of PCB and PCDF in the tissues of male patients with Yusho and of male accident victims not affected by the disease*

Case no.	Age (yr)	Time of death	Tissue	Conc (ppm) of PCB: In whole tissue	PCB: Calc on tissue fat	PCDF: In whole tissue	PCDF: Calc. on tissue fat	$\frac{\text{PCDF concn}}{\text{PCB concn}} \times 100$
				Yusho patients				
1	13	July 1969	Adipose	1·4	3·4	0·013	0·03	0·9
			Liver	0·05	4·7	0·025	2·3	50
2	25	July 1969	Adipose	1·3	8·5	0·006	0·04	0·5
			Liver	0·06	5·6	0·010	1·1	17
			Kidney	0·13	6·6	0·0005	0·03	0·4
			Spleen	0·02	8·5	ND	ND	—
3	46	May 1972	Adipose	1·2	2·1	0·007	0·001	0·6
			Liver	0·03	3·5	0·003	0·3	10
			Kidney	0·03	1·4	0·0002	0·007	0·7
			Spleen	0·02	1·9	TR	TR	—
			Lung	0·01	2·2	TR	TR	—
				Accident victims				
4	52	March 1975	Adipose	1·0	1.4	ND	ND	—
			Liver	0·08	1·3	ND	ND	—
5	58	March 1975	Adipose	0·40	0·7	ND	ND	—
			Liver	0·02	1·0	ND	ND	—

TR = 0·00005–0·0001 ppm ND = None detectable (< 0·00005 ppm)

lier, all the three cases analysed were in the early convalescent stage as far as the Yusho incident was concerned, and the current distribution of residual PCDFs and PCBs in the bodies of survivors more than 8 years after the poisoning may differ considerably from that observed in this study. It is highly desirable that the current distribution pattern should be clarified soon, because it is closely connected with the medical treatment of the surviving patients.

In contrast to cases 1 and 2, fairly accurate information is available concerning the consumption of toxic rice oil by case 3. From an earlier epidemiological study (T. Yoshimura, unpublished data, 1973), case 3 is known to have consumed approximately 560 ml of a canned rice oil containing about 1000 ppm PCB (KC-400) and 5 ppm PCDF over a period of 140 days. Therefore, the total amounts of PCB and PCDF ingested are estimated to have been approximately 560 and 2·8 mg, respectively. On the other hand, it is calculated that the whole liver of patient no. 3, weighing 2070 g at autopsy, contained 62 μg PCB and 6·2 μg PCDF. Thus, 0·22% of the PCDF ingested apparently remained in the liver of this patient when he died in May 1972, approximately 44 months after use of the toxic rice oil had been discontinued. The corresponding figure for PCB was 0·011%, much smaller than that for PCDF. At the present time, it is hard to comment with any certainty on the possible significance of these figures, because adequate information on the intestinal absorption rate and toxicity of PCDF in man and the possible metabolic conversion of PCBs to PCDFs is lacking. It may be said at least, however, that PCDFs deserve more attention in attempts to improve our understanding of Yusho.

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THE FATE OF OCHRATOXIN A IN RATS

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Abstract—The fate of ochratoxin A was studied in rats given a single ip injection of 1 mg labelled with ^{14}C. Ochratoxin A reached its highest levels in serum (accounting for up to 90% of the ^{14}C dose), liver (4·5%) and kidney (4·4%) 30 min after the injection and then decreased gradually. Disc-gel electrophoresis of rat serum revealed that the toxin was predominantly bound to the serum-albumin fraction *in vivo*. Ochratoxin A was excreted primarily in the urine, either as the unchanged toxin or its metabolites. Excretion in the faeces was less significant and was mainly in the form of unchanged toxin. The kinetics of the distribution and excretion of ochratoxin A and of *in vivo* binding of the toxin with serum albumin are described.

INTRODUCTION

Ochratoxin A (OA) is one of the most potent nephrotoxins among a series of ochratoxins produced by several species of Aspergillus and Penicillium.

The chemistry and biological and toxicological significance of ochratoxins have been reviewed (Applegate & Chipley, 1973; Chu, 1974a; Harwig, 1974; Steyn, 1971). Although the pathological changes induced by OA have been studied in some detail in several animal species, the fate of OA *in vivo* is not completely understood. It has been reported (Nel & Purchase, 1968; Purchase & Nel, 1967) that OA was found in the blood and liver as well as in the urine and faeces after rats had been given the toxin. In addition, ochratoxin α, the hydrolysed product of OA was found in the rat urine and faeces 74 hours after administration of the toxin. Rats excreted daily just over 10% of daily intubated doses of OA (Van Walbeek, Moodie, Scott, Harwig & Grice, 1969). More recently, OA has been found in the organs and tissues of slaughtered bacon pigs which had fed on cereal contaminated with OA (Krogh, Axelsen, Elling, Gyrd-Hansen, Hald, Hyldgaard-Jensen, Larsen, Madsen, Mortensen, Møller, Petersen, Ravnskov, Rostgaard & Aalund, 1974a; Krogh, Hald, Englund, Rutqvist & Swahn, 1974b). The fate of OA in rats given a single ip injection of [^{14}C]OA was investigated and the results are presented in this paper.

EXPERIMENTAL

Preparation of OA. The toxin was produced by *Aspergillus ochraceus* NRRL 3174 in rice and was purified as described previously (Chu & Butz, 1970). Ochratoxin α, obtained by acid hydrolysis of the purified OA, was coupled with [^{14}C]phenylalanine (466 mCi/mmol obtained from New England Nuclear, Boston, Mass.) as described elsewhere (Chang, 1975; Wei & Chu, 1974) to yield [^{14}C]OA with a specific activity of 420 mCi/mmol. All other reagents and chemicals were either chemically pure or reagent grade.

Animals and treatment. Sprague–Dawley male rats weighing around 100 g (from Sprague Dawley Co., Madison, Wis.) were used. Each rat received an ip injection of 1 ml OA solution containing 1 mg OA in 0·9 ml 0·85% sterilized $NaHCO_3$ and 0·1 ml [^{14}C]OA in ethanol (*c.* 220,000 cpm). Control rats were each given a mixture of 0·9 ml $NaHCO_3$ and 0·1 ml ethanol. All rats were housed separately in metabolic cages and given water and food *ad lib.* At intervals of 0·5, 2, 4, 8 and 24 hr after dosing, two rats were killed and their blood, livers and kidneys were collected. The rat serum was recovered by refrigerating the blood overnight and then centrifuging it at 3000 g for 10 min. The livers and kidneys were frozen immediately after the rats died. To study the *in vivo* binding of OA with serum protein, one group of 12 rats was given a dose of 4 mg unlabelled OA/kg body weight and a second, similar, group was given 8 mg OA/kg. The sera obtained from these rats were subjected to disc-gel electrophoresis.

Determination of OA in tissues and biological fluids

Extraction and preliminary treatment. The frozen livers and kidneys were sliced into small pieces and homogenized with 4 vols methanol–chloroform (1:4, v/v) in a Potter Elvehjem-type glass homogenizer with a Teflon pestle. The homogenate was centrifuged at 5000 g for 10 min and the supernatants were collected in a round-bottom flask, while the residues were re-extracted twice with the same solvent. The supernatants were pooled, evaporated to dryness *in vacuo* and redissolved quantitatively in a small amount of methanol for radioactivity counting and thin-layer chromatography (TLC). The concentration of OA in serum was determined either by a combination of TLC and radioactivity counting or by disc-electrophoretic analysis.

Urine samples were collected at appropriate times and the cages and collection funnels were washed with 0·85% $NaHCO_3$ from a wash bottle. The combined solutions were acidified with 1 N-HCl and extracted with chloroform. The chloroform extract was concentrated, redissolved in methanol quantitatively and subjected to analysis. The faeces were

extracted with methanol-water (55:45, v/v), in which they were stirred until completely dispersed. The suspensions were filtered through filter paper into suction flasks until no radioactive filtrate emerged. The filtrates were then combined and concentrated to a small volume, usually less than 5 ml. The concentrated extract was spotted directly onto a TLC plate, and the OA radioactivity was counted.

Thin-layer chromatography. The plate was generally coated to a thickness of 0·25 mm with Adsorbosil-5 (Applied Science Laboratory, State College, Penn.). Ochratoxins were identified by comparison with the chromatograms of standard toxins and their reported R_F values, the plates being developed with benzene–methanol–acetic acid (12:2:1, by vol.). The OA spot on the TLC plate was scraped off and transferred to a counting vial, to which 10 ml Bray's solution (Bray, 1960) was also added, for radioactivity counting.

Determination of radioactivity. Except where otherwise specified, an appropriate amount of extract (generally less than 0·5 ml) or a radioactive spot scraped from a TLC plate was mixed with 10 ml Bray's solution. The solution was subjected to counting in a Packard Tri-carb Model 5017 liquid scintillation spectrometer for at least 10 min.

Disc-gel electrophoresis. The disc-gel electrophoresis was carried out in a Canalco Model 12 (300 B) apparatus, except that quartz tubes (7 × 0·5 cm) were used instead of regular glass tubes. In order to prevent the destruction of OA by light, ammonium persulphate (0·14%) was used to initiate gel polymerization in the sample gel preparation. The method of Davis (1964) was used in the polyacrylamide gel preparation. The sample gel was prepared by mixing 150 μl of gel solution (2·5%) with 1–20 μl of sample in a rubber disc cap, after which the quartz tube was connected and the mixture was left to stand for 20 min for polymerization to occur. Another 50 μl of double-strength sample gel solution (5%) was layered on the polymerized sample gel and was left to stand for another 20 min for polymerization, after which the developing gel was added. The final developing gel concentration was 7% in Tris–glycine buffer, pH 8·3 (6·0 g Tris, 28·8 g glycine and 20 ml mercaptoethanol in one litre distilled water; a 1:10 dilution of this solution was used).

Electrophoresis was carried out at 3 mA/tube at pH 8·3 (Tris–glycine buffer) for less than 1 hr. After electrophoresis, the tubes were transferred immediately to a specially designed tube holder and the fluorescence intensity was determined. This was followed by staining the proteins with coomassie blue according to the method described by Chrambach, Reisfeld, Wyckoff & Zaccari (1967).

Determination of OA fluorescence in polyacrylamide gel. A special aluminium plate (20 × 20 cm) with two rows of windows (0·5 × 6·5 cm) was designed (Chang, 1975). After electrophoresis, the tubes containing the gels were laid directly on the open windows of the plate, which was then placed on the TLC autoscanning attachment for fluorescence measurement in an Aminco–Bowman spectrophotofluorometer (Chu & Butz, 1970). The activation and emission maxima for OA in the gel at pH 8·3 in the presence of bovine serum albumin (BSA) or serum were 390 and 450 nm, respectively, and were used throughout the experiments.

Determination of OA in serum by electrophoresis. In order to determine the OA concentration in serum, a standard curve was established by electrophoresis of known quantities of OA in the presence of excess amounts of BSA. A solution containing 20 μg OA/ml and 0·65 mg BSA/ml in 0·1 M-sodium phosphate buffer at pH 7·2 was incubated at 37°C for 1 hr. An appropriate amount (1–30 μl) of the incubation mixture was withdrawn, mixed with the sample gel and subjected to electrophoresis. The OA concentration varied from 10 to 600 ng/tube. After electrophoresis, the fluorescence intensities of fluorescent bands in individual tubes were determined. Under these conditions, OA migrated with BSA to form a sharp fluorescent band. A standard curve was prepared by plotting OA concentrations against the fluorescence intensities (in terms of relative area). When the concentration of OA in the unknown sample was high, sample dilution was necessary so that the fluorescence intensity of the unknown would fall within the range of the standard curve. In cases where OA concentrations in the samples were high or the albumin concentration in the serum was low, two fluorescent bands were observed, one representing the free OA (faster moving) and the other the bound OA. In this case, the standard curve (BSA–OA) was used for calculating the concentrations of both the bound and free OA.

RESULTS

Levels of OA in rat serum

The levels of radioactivity and of OA in rat serum are given in Table 1. The radioactivity in the serum reached a maximum 30 min after injection of the toxin with a concentration equivalent to about 90% of the dose, assuming a serum volume of 4 ml/rat. Analysis of the OA in serum-protein samples by TLC revealed that in the first 8 hr 70–80% of the radioactivity consisted of unaltered OA, while in the 24-hr samples, only 56% of the radioactivity was counted as OA. During the first 8 hr, OA concentrations in serum were approximately 0·1–0·2 mg/ml.

Binding of OA by serum albumin

The *in vivo* interaction of OA with serum albumin was demonstrated by disc electrophoresis of serum obtained from rats given 4 or 8 mg unlabelled OA/kg. In general, two fluorescent bands were detected. One band, associated with albumin, was considered as bound OA; and the other band, a faster moving component, was considered as free OA. A typical fluorometric scanning pattern of the gel is shown in Fig. 1. No fluorescent band was observed in serum obtained from control rats. The kinetics of distribution of OA in rat serum, as determined by the gel electrophoresis and fluorometric method, are given in Table 2. The results are similar to those obtained from the radioactivity and TLC analyses. The total OA concentration in the serum reached a maximum 30 min after the injection and decreased thereafter, and the concentration of bound OA was much higher than that of free OA in the early stages. With the low dose (4 mg/kg), more than 80% of the toxin

Table 1. *Levels of OA in serum 0·5–24 hr after ip injection of rats with [^{14}C]OA*

Time after injection (hr)	Total radioactivity in serum (cpm × 10^{-4}/ml)	Radioactivity as OA		Concn of OA (μg/ml)
		cpm × 10^{-4}/ml	% of total	
0·5	4·8	3·9	81	178
2	3·8	3·0	78	134
4	3·6	2·7	74	121
8	3·3	2·4	73	109
24	1·3	0·7	56	34

OA = Ochratoxin A

The injection of 1·0 mg OA/rat was labelled with ^{14}C in the phenylalanine moiety ($2·2 \times 10^5$ cpm/dose).

existed in the bound form during the first 24 hr after injection but the proportion had decreased to 20% or less in the 48-hr samples. Similar effects were observed with the higher dose, except that the free-toxin concentration was considerably higher than that seen with the lower dose, even in the 30-min sample, and the bound form decreased to less than 5% in the 24-hr sample.

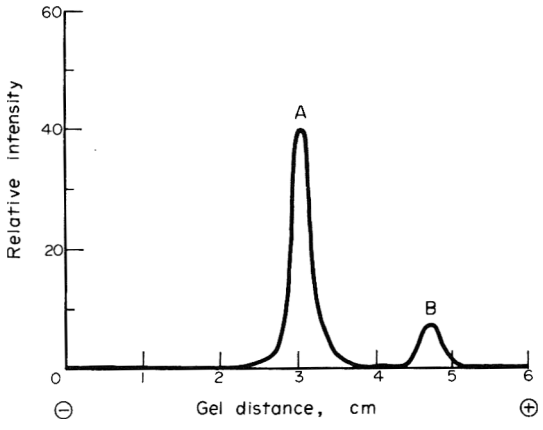

Fig. 1. Polyacrylamide-gel electrophoresis pattern of OA in rat serum (5 μl). Electrophoresis was carried out in Tris–glycine buffer at pH 8·3 and 3 mA/tube for 40 min and the gel was scanned by Aminco–Bowman spectrophotofluorometer. The sensitivity was set at 0·03 and the activation and emission wavelengths were set at 390 nm and 450 nm, respectively. The leading small peak (B) and the major peak (A) are the free and bound OA, respectively.

Accumulation of OA in rat liver and kidney

The levels of OA that accumulated in rat liver and kidney after a single ip injection are given in Table 3. Each organ contained approximately 3–5% of the injected dose 30 min after the injection. Thereafter the levels in both organs began to decrease and in the 24-hr samples, only 1–2% of the injected radioactivity was found in the liver or kidney. Although most of the radioactivity (60–80%) in samples of both tissues obtained before 8 hr was detected as unchanged OA, only a small amount (27%) of that recovered from the 24-hr samples of the liver was in the unchanged form. At this time, however, the level of unchanged OA in the kidney was still relatively high (57·7% of the total radioactivity).

Excretion of OA

The excretion of OA from rats in the urine and faeces is shown in Table 4. Excretion proceeded at a constant rate during the first 8 hr, during which some 50% of the dose was excreted, and then the rate decreased. Examination of the urine samples by TLC revealed that not all of the radioactivity was in the form of OA. In addition to the radioactivity in the OA position (R_F 0·6), a non-radioactive blue-fluorescent spot (R_F 0·25) was identified as ochratoxin α, and a radioactive spot (accounting for 10–20% of the applied radioactivity) remained at the origin. Although over 50% of the total injected radioactivity was excreted in the urine during the first 24 hr, only 45% was OA. Approximately 25–40% of the radioactivity in the urine did not migrate in the TLC system,

Table 2. In vivo *binding of OA by serum albumin in the rat*

Time after injection (hr)	Levels of OA in serum (μg/ml) after dose of					
	4 mg/kg			8 mg/kg		
	Bound*	Free*	Total	Bound*	Free*	Total
0·5	38 (83)	8 (17)	46	92 (65)	50 (35)	142
1·0	35 (81)	8 (19)	43	69 (59)	69 (59)	116
4·0	25 (83)	5 (17)	30	—	—	—
12·0	25 (81)	6 (19)	31	—	—	—
24·0	16 (80)	4 (20)	20	5 (4)	28 (96)	33
48·0	5 (19)	14 (79)	19	—	—	—

OA = Ochratoxin A

*With percentage of total serum OA in parentheses.

Table 3. *Accumulation of OA in rat liver and kidney after a single ip injection of [^{14}C]OA*

Time after injection (hr)	Total organ weight (g/rat)	Total radioactivity in organ		Radioactivity as OA		OA content*	
		cpm × 10^{-3}	% of dose	cpm × 10^{-3}	% of total RA	μg/rat	μg/g tissue
			Liver				
0·5	4·6	9·9	4·5	7·1	70·7	32	7·1
2	4·5	7·8	3·2	5·5	79·1	25	5·6
4	4·6	6·5	3·0	5·3	81·5	24	5·3
8	4·7	4·0	1·8	3·0	75·0	14	3·0
24	4·4	3·7	1·7	1·0	27·0	4·5	1·0
			Kidney				
0·5	2·2	9·7	4·4	6·8	70·1	30	13·6
2	2·4	6·0	2·7	4·4	73·3	20	8·3
4	2·2	4·4	2·0	2·9	65·9	13	6·14
8	2·2	3·4	1·5	2·2	64·5	10	4·5
24	2·2	2·6	1·2	1·5	57·7	7	3·18

OA = Ochratoxin A

*Analysed by TLC.

Table 4. *Cumulative excretion of OA in urine and faeces of rats given a single ip injection of [^{14}C]OA*

Time after injection (hr)	Total radioactivity		Radioactivity as OA		Content of OA* (μg)	Non-migrating metabolites (R_F = 0)*		
						Radioactivity		Content calc. as OA (μg)
	cpm × 10^{-4}	% of dose	cpm × 10^{-4}	% of total		cpm × 10^{-4}	% of total	
				Urine				
2	3·6	16·4	1·0	28	45	1·4	40	64
4	4·1	18·6	1·4	25	64	2·3	42	106
8	9·0	40·9	3·2	38	146	3·0	33	134
24	11·7	53·2	4·9	43	223	3·10	26	139
				Faeces				
8	2·19	10·0	1·4	66	64	—†	—	—
24	3·79	17·3	2·9	77	133	—†	—	—

OA = Ochratoxin A

*Analysed by TLC.
†Not determined.

but since our main purpose was to determine the rate of excretion of OA from rats, no attempt was made to identify these substances. One of them would have been [^{14}C]phenylalanine, because ochratoxin α was demonstrated in the urine sample. However, our data cannot rule out the possibility that other metabolites or conjugates may have remained at this position.

Only 13% of the injected radioactive OA was excreted in the faeces during the first 24 hr and 77% of this was identified as unaltered OA. Ochratoxin α was also found in the faeces.

DISCUSSION

The overall fate of OA in rats is indicated in Fig. 2. There was a clear parallel between OA concentrations in the serum and the amounts of the toxin present in the liver and kidney. There was a considerable difference between the half-life of OA in the serum of rats given the toxin by ip injection (12–18 hr; present study) and that of rats given a single oral dose (68 hr; Galtier, 1974). In the latter case, the serum-OA level reached its maximum only after 8 hr

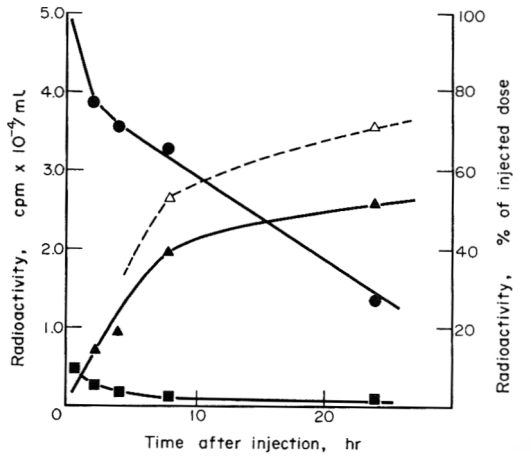

Fig. 2. The fate of OA in rats [^{14}C]OA after a single ip dose (1 mg/rat) of 2·2 × 10^5 cpm [^{14}C]OA, labelled in the phenylalanine moiety. The concentration of OA in serum (●) is expressed as cpm/ml, and the amounts in liver and kidney (■), in urine (▲) and in total excreta (△) are expressed as a percentage of the injected [^{14}C]OA.

(Galtier, 1974), and thus reflected the gradual process of absorption of the toxin. The biological half-life of OA in serum is comparable to that of warfarin (Yacobi & Levy, 1974).

It has been shown that OA interacts strongly with serum albumin *in vitro* (Chu, 1971 & 1974b), and this interaction was confirmed in the present *in vivo* study. Both free and bound forms of OA were found in the rat serum. Whereas OA existed primarily (>80%) in the bound form in rats given a dose of 4 mg OA/kg, considerable amounts of OA were found as the free toxin when the dose was doubled (8 mg/kg). At the higher dose, the total OA bound to albumin (92 μg/ml) was comparable to the 70 μg/ml reported by Galtier (1974). However, Galtier (1974) demonstrated that approximately 95·6% of the toxin present was in the bound form, whereas we found that only about 60% bound to albumin. Since smaller and younger rats were used in our study (weighing about 100 g compared with the 250-g rats used in the French study), the discrepancy may have been due to the differences in the age and size of the rats. It has been noted that plasma concentrations of unbound drugs are frequently higher in neonates than in adults (Chignell, Vesel, Starkweather & Berlin, 1971) and this has been attributed to the presence of endogenous substances, such as fatty acids and bilirubin, which compete for the binding sites (Gillette, 1973). In addition, since equilibrium in the OA-serum albumin interaction depends upon the concentrations of OA and albumin, an alteration in the concentration of one could alter the binding pattern. The total OA concentration had decreased appreciably 24 hr after injection. A decrease in the albumin concentration could possibly have been responsible for the increase in free OA at 48 hr, especially since Szczech, Carlton & Tuite (1973) reported that ochratoxicosis in beagles resulted in the loss of serum albumin.

Blood concentrations of OA decreased exponentially in the first 8 hr and the toxin was excreted in the urine partly as unchanged OA but to a considerable extent also as its metabolites or conjugates. Our finding of relatively small proportions of ochratoxin α in rat urine agrees with the results of Van Walbeek *et al.* (1969) and of Nel & Purchase (1968) but differs from the findings in cows, in which ochratoxin α was the main excretion product in the urine (Still, 1973).

Although the total amounts of OA in the liver and kidneys were similar, the peak concentration of OA in the kidney (13·6 μg/g tissue) was nearly twice that in the liver (7·1 μg/g). Van Walbeek *et al.* (1969) also found that the concentration of OA was greater in the kidney than in the liver (6·2 and 2·0 μg/g, respectively) after rats had been given 500 μg OA/day by intubation for 5 days. Since OA has been shown to be a potent nephrotoxin, further investigations on the relation of the levels of free or bound OA in the kidney to the degree of nephropathy and to kidney function, and on renal concentrations of OA following acute dosage, could provide information on the mechanism of OA intoxication.

The problem of transmittance of OA in animal organs is clearly demonstrated in the present study. Krogh *et al.* (1974a) found that OA was present at levels up to 67 ppb (0·067 μg/g) in the meat and organs of pigs suffering from mycotoxic nephropathy. In our study, tissue levels varied considerably with the sampling time, being at a maximum in the organs studied 30 min after the toxin was administered and subsequently decreasing, although even at 24 hr, the samples still contained as much as 1 μg OA/g. The meat and organs of animals suffering from mycotoxic nephropathy should be rejected for food use, therefore, or should be analysed for possible contamination with OA or other mycotoxins, such as citrinin which also causes mycotoxic nephropathy.

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METABOLISM AND TOXICITY OF HALOGENATED CARBANILIDES: ABSORPTION, DISTRIBUTION AND EXCRETION OF RADIOACTIVITY FROM 3,4,4′-TRICHLORO[^{14}C]CARBANILIDE (TCC) AND 3-TRIFLUOROMETHYL-4,4′-DICHLORO[^{14}C]CARBANILIDE (TFC) IN RATS

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Abstract—The absorption, distribution and excretion of [^{14}C]TCC (3,4,4′-trichlorocarbanilide) and [^{14}C]TFC (3-trifluoromethyl-4,4′-dichlorocarbanilide) were evaluated in rats given a single oral, iv or dermal dose of radioactive material. With either compound and with each route of administration, more than 65% of the absorbed radioactivity was eliminated in the bile during the 72 hr after dosing. The organ distribution at 72 hr was liver > kidneys ≫ lungs ≃ testes, with the liver containing < 1% of the absorbed dose. There were enough similarities between iv, oral and dermal exposure with respect to elimination routes and tissue distribution to justify the conclusion that oral exposure could be used in toxicity evaluations and still be relevant to dermal exposure. Enterohepatic circulation was evaluated using rats with bile ducts cannulated in tandem. Between 30 and 60% of the material eliminated into the gastro-intestinal tract was reabsorbed. The tissue distribution and the relative contribution of the bile and urine to elimination of this reabsorbed radioactivity was very similar to that found in rats given an oral dose of radioactive TCC or TFC. Both TCC and TFC are probably extensively metabolized, but evidence was obtained showing that cleavage of the C–N bond is not involved to a detectable extent.

INTRODUCTION

3,4,4′-Trichlorocarbanilide (TCC) and 3-trifluoromethyl-4,4′-dichlorocarbanilide (TFC), shown in Fig. 1, have been used in soap products to diminish the bacterial population of the skin surface. The chemistry of TCC (Roman, Barnett & Blaske, 1958) and its disposition after dermal application to man, guinea-pigs and rats (Black, Howes & Rutherford, 1975; Howes & Black, 1976; Rutherford & Black, 1969; Scharpf, Hill & Maibach, 1975) have been partially investigated. No such information has been published for TFC.

The purpose of the studies reported here was to define the absorption, distribution and excretion of these antibacterial agents in the rat after a single oral, iv or dermal exposure. Such studies help to establish the suitability of oral exposure studies as models for assessing the toxicity of a compound normally applied to the skin. They are a first step in the process of judging whether the rat is an appropriate model for predicting risk in man. In addition, the studies provide an extension of our knowledge on the effect of chemical modification (e.g. substitution of a $-CF_3$ group for a –Cl atom) on the biological properties of compounds.

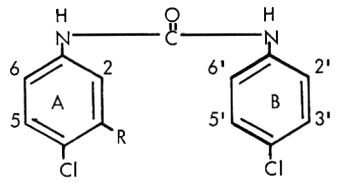

Fig. 1. Structure of TCC (R = Cl) and TFC (R = CF_3).

EXPERIMENTAL

Chemicals. TCC labelled with ^{14}C was a gift of Monsanto Chemical Co., St. Louis, Mo. TCC uniformly labelled in the 4′-chlorophenyl ring (43 μCi/mg) was found by high-pressure liquid chromatography (HPLC; Jeffcoat, Handy, Francis, Willis, Wall, Birch & Hiles, 1977) to be > 98% radiochemically pure. TCC labelled in the carbonyl carbon (20 μCi/mg) was only 93% pure and most of the impurity co-eluted from the HPLC column with 4,4′-dichlorocarbanilide. Radioactive [^{14}C]TFC was a gift of Ciba-Geigy Chemical Co., Greensboro, N. Carolina, and was labelled either in the 4′-chlorophenyl group (69 μCi/mg) or in the carbonyl group (33 μCi/mg). In both cases the compounds were > 97% radiochemically pure according to HPLC analysis. Non-radioactive TCC and TFC were production-grade chemicals and were judged to be > 97% pure according to HPLC. The soap used in dermal experiments was production-grade tallow/coconut (50/50) flakes containing no perfumes or colouring agents. Corn oil used in the oral experiments was USP grade.

Animals. Male Sprague–Dawley rats (from Sprague–Dawley, Madison, Wisc.) weighing 200–300 g

were used in all experiments. They were allowed free access to water, but were deprived of food overnight before dosing. Rats with externally cannulated bile ducts were prepared 12–24 hr before dosing by the surgical technique described by Lambert (1965). Pairs of animals bile-duct-cannulated in tandem were used to evaluate enterohepatic circulation and were prepared surgically by the procedure of Michael (1968). In this a bile-duct cannula from the 'bile donor' runs into the distal portion of the bile-duct of the rat acting as the 'bile receiver'. A second cannula runs from the bile duct (proximal to the liver) of the 'bile receiver' into a collection flask. A ligature separates the two cannulas in the 'bile receiver'.

Oral dosing. Corn oil solutions of TCC or TFC were prepared to give each animal 5–10 μCi of radioactivity in a 1-g dose of oil. The ^{14}C-labelled and non-radioactive carbanilides were dissolved together in acetone or ethanol; the corn oil was added to this solution and the volatile solvent was removed in a stream of nitrogen directed at the surface of the solution. Animals were intubated by means of a syringe and rubber tube, and the actual dose was determined by the difference in weight of the dosing apparatus before and after dosing. The final concentration of TCC or TFC in the corn oil was 5·5 mg/ml.

Intravenous dosing. Lymph was collected from rats that had been fitted with a thoracic-duct cannula (Lambert, 1965). They were given a fatty meal (Mattson & Volpenhein, 1962) and drinking-water containing 0·9% sodium chloride. The lymph was passed through a filter (pore size 8 μm) and then an acetone solution of TCC or TFC was added to it dropwise. The acetone was removed under a stream of nitrogen and the lymph was again filtered to remove any precipitated carbanilide. This preparation could be diluted 20-fold with rat plasma without precipitation of the TCC or TFC. The volume of the iv doses was about 2 ml/animal (5–10 μCi), the TCC concentration being 40–60 μg/ml of lymph and the TFC concentration 150–170 μg/ml. The dose was administered over a period of approximately 1 min by the method of Salem, Grossman & Bilbey (1963).

Dermal dosing. Each animal was fitted with two protective dermal dosing cells as described by Rice & Ketterer (1977) to give an exposure area of 10 cm^2. A 10% soap solution was prepared and the carbanilide, dissolved in acetone, was added to it. The acetone was removed under a stream of nitrogen. The final concentration of carbanilide was 1·5 mg/g of solution. Each animal received 13–15 μCi of ^{14}C, which was administered and spread over the clipped dosing area with a rubber-tipped syringe, the actual doses given being determined by the change in weight of the dosing syringe.

Sample collection and analysis. After administration of the single radioactive dose, urine, faeces and bile were collected separately during three 24-hr periods. When collected, CO_2 was trapped in a 6% NaOH solution. Animals were killed 72 hr after dosing by exsanguination using heparinized equipment. In the oral and iv experiments, the gastro-intestinal tract, liver, lungs, kidneys, testes and remaining carcass were taken as separate samples. In the dermal experiments, the skin of the dosing site and the non-dosed skin (all of the skin except that to which the dose was applied) were also taken as separate samples. Skin samples were extracted twice with boiling acetone. The extract of the skin from the site of application and the extracted skin from this site were used for radiochemical analysis, while in the case of the non-dosed skin, only the extract was analysed for radioactivity.

Radiochemical (^{14}C) content was determined by liquid scintillation spectrophotometry (Packer Liquid Scintillation Spectrophotometer, Model 3375, Packard Instruments, DesPlaines, Ill) with automatic external standardization for quenching correction. Urine, bile and NaOH–CO_2 samples were placed directly in scintillation fluid. Standards and all other-samples were burned in a stream of oxygen, the resulting CO_2 was trapped and its ^{14}C content was determined as above. Evaluation for statistical differences was made by Student's t test.

Solvent distribution. The distribution of TCC and TFC between water and 1-octanol was measured by the method of Hansch, Maloney & Fujita (1962). A solution of 10 mg radioactive compound dissolved in 100 ml water-saturated 1-octanol was mixed vigorously with 100 ml 1-octanol-saturated water. Samples of each layer were centrifuged and the relative distribution between the two solvents was determined by liquid scintillation counting.

RESULTS

In a pilot study, animals were dosed iv with TCC or TFC using the lymph solution described in this paper. The ^{14}C content of the bladder, pancreas, spleen, testes , adrenals, kidneys, liver, thymus, heart, lungs, thyroid and parathyroid, brain, blood cells, plasma, lymph nodes, adipose tissue, muscle and bone was determined 72 hr after dosing. Liver, kidneys, lungs and testes were the only organs that contained more than 0·01% of the dosed ^{14}C, and these organs were therefore examined in subsequent studies. More than 90% of the radioactivity administered was found in the faeces, suggesting that biliary elimination was important. Therefore animals, with cannulated bile ducts were used in most subsequent studies.

A summary of the experiments, including dosing methods, recovery of administered radioactivity, calculated absorption and total systemic load during the 72-hr experimental period, is presented in Table 1. The percentage of the recovered dose that was absorbed was determined by summing the percentage of the ^{14}C recovered in the bile, urine and tissues after oral dosing, while in studies of iv and dermal exposure, the amounts found in the faeces were also included. It can be seen from this table that the systemic exposure varied greatly with the different dosing methods. Since one aim of this work was to compare the disposition of the radioactivity absorbed after the various dosing methods rather than to determine the fate of the total material administered, tissue and excretion data were compared in terms of percentages of absorbed (systemic) radioactivity.

Figure 2 shows the relative importance of the bile and urine in the elimination of the systemic radioactivity in the three 24-hr periods after dosing. After oral or iv dosing only 4–6% of the systemic load was eliminated in the urine in 72 hr while the bile con-

Table 1. *Experimental summary*

Test compound and labelling	Vehicle and method of dosing	No. of animals	Dose of TCC or TFC (μmol/kg)	Recovery of ^{14}C (% of dose)*	Absorption of ^{14}C (% of recovered ^{14}C)*	Total systemic load of TCC or TFC (μmol/kg)*
Rats with cannulated bile duct						
[ClPh-U-^{14}C]-†						
TCC	Lymph—iv	4	1·52	91·3 ± 3·8	100	1·51 ± 0·12
TFC	Lymph—iv	5	3·58	91·8 ± 3·4	100	3·61 ± 0·22
TCC	Corn oil—oral	3	69·94	100·6 ± 7·0	43·01 ± 3·40	30·03 ± 5·27
TFC	Corn oil—oral	7	61·60	89.8 ± 3·0	38·63 ± 2·48	23·63 ± 2·29
TCC	Soap—dermal	4	6·86‡	86·0 ± 1·6	7·79 ± 1·07	0·51 ± 0·05
TFC	Soap—dermal	3	4·53‡	98·0 ± 6·0	7·54 ± 0·67	0·36 ± 0·08
Bile ducts cannulated in tandem						
TCC	Corn oil—oral	2 × 2	66·64	100 (97·6–103)	See p. 207	See p. 207
TFC	Corn oil—oral	2 × 2	62·51	119 (115–124)	See p. 207	See p. 207
Intact rats						
[Carbonyl-^{14}C]-						
TCC	Corn oil—oral	3	53·71	104 ± 2·5	—	—
TFC	Corn oil—oral	3	84·95	93·17 ± 2·2	—	—

TCC = Trichlorocarbanilide TFC = Trifluoromethyldichlorocarbanilide

*Results are expressed as means ± SEM for the numbers of animals stated, or the mean and range of results.

†4′-Chloro-U-^{14}C-phenyl labelling.

‡TCC dose equivalent to 0·14 μmol/cm^2; TFC dose equivalent to 0·11 μmol/cm^2.

tained 86–89%. After dermal exposure, 13–16% of the absorbed radioactivity was found in the urine in 72 hr and 77–82% was in the bile. A difference was seen also in the time course of elimination when iv and oral exposure were compared with dermal exposure. After dermal exposure, approximately one-third of the excreted radioactivity was found in each of the three 24-hr collection periods for the urine and bile, while after oral or iv dosing most (> 85%) of the absorbed dose was eliminated in the first 24-hr period. The data thus support the argument that dermal absorption over the 72-hr exposure period was continuous but slow enough for the skin surface pool not to change significantly.

In both iv and dermal studies in bile-duct-cannulated animals, some of the dose was found in the faeces (Table 2). Since the bile duct was ligated between the cannula and the intestines and since, in the dermal studies, the applied dose was enclosed in a cell, it is probable that this faecal radioactivity represented a small amount of compound eliminated directly into the gastro-intestinal tract. Therefore, the amount in the faeces was considered to have been systemic at one time and not a contaminant. Obviously, there was no way to correct for direct gastro-intestinal tract elimination in oral experiments.

The tissue distributions of radioactivity calculated as percentages of the absorbed dose remaining 72 hr after iv, oral or dermal dosing of TCC or TFC are shown in Table 2. For all three routes of administration, the same relative order of tissue residues was seen, i.e. carcass > liver > kidneys > lungs ≃ testes. The carcass always contained more of the absorbed dose after oral dosing than after iv or dermal exposure, while the carcass levels were very similar in the iv and dermal experiments. The liver and kidney values were higher after dermal exposure than after iv or oral dosing, a finding consistent with the previously mentioned hypothesis of continuous absorption. Table 2 shows also that very little of the dermally applied radioactivity strayed from the dosing cell to the 'dermal dosing non-application site skin'.

Because much of the absorbed radioactivity was excreted in the bile, the potential for enterohepatic circulation in the rat was evaluated using tandem bile-duct cannulation (Table 3). The bile-donor rat received a single oral dose of TCC or TFC (Table 1). The bile from this animal flowed into the distal portion of the common duct of the bile-receiver rat. From the bile-receiver rat, bile was collected from a second cannula placed in the bile duct proximal

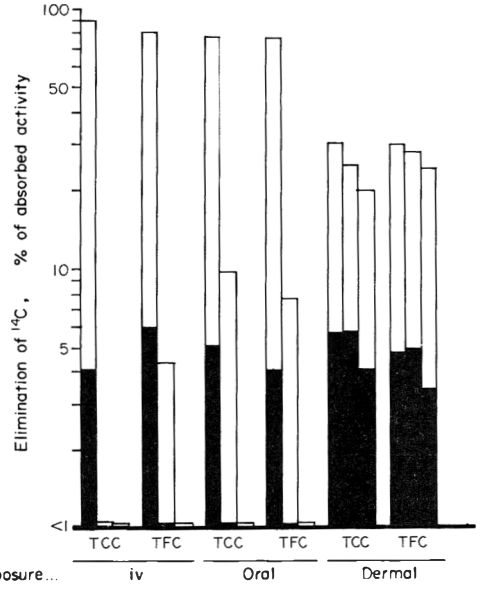

Fig. 2. Elimination of ^{14}C in the bile (□) and urine (■) of rats with cannulated bile ducts, after a single iv, oral or dermal exposure to [^{14}C]TCC or [^{14}C]TFC. From left to right the bars in each group represent the 0–24-, 24–28- and 48–72-hr collection periods.

Table 2. *Distribution and excretion patterns of ^{14}C 72 hr after iv, oral or dermal exposure of rats with cannulated bile ducts to [^{14}C]TCC or [^{14}C]TFC*

Body fluid, organ or tissue — Test compound† ...	Amount of radioactivity (% of absorbed ^{14}C)* following administration					
	Intravenously		Orally		Dermally	
	TCC (4)	TFC (5)	TCC (3)	TFC (7)	TCC (4)	TFC (3)
Urine	4·32 ± 0·71[l]	6·70 ± 1·85[n]	6·46 ± 1·5[m]	5·02 ± 0·56[o]	15·60 ± 1·24[l,m]	13·11 ± 3·18[n,o]
Faeces	4·77 ± 0·44	5·97 ± 1·16	—	—	2·42 ± 0·52	2·06 ± 0·09
Bile	89·96 ± 1·24[j]	86·22 ± 2·80	88·11 ± 1·75[k]	85·94 ± 1·58	77·24 ± 1·81[j,k]	82·15 ± 3·86
Cage and gut washings	0·12 ± 0·07	0·30 ± 0·04	—	—	2·06 ± 0·45	1·20 ± 0·44
Liver	0·072 ± 0·009[b]	0·161 ± 0·029	0·078 ± 0·002[c]	0·095 ± 0·010[d]	0·673 ± 0·324[b,c]	0·406 ± 0·082[d]
Kidneys	0·017 ± 0·004[e]	0·053 ± 0·015	0·016 ± 0·002[f]	0·025 ± 0·002[p]	0·236 ± 0·067[e,f]	0·241 ± 0·069[p]
Lungs	0·004 ± 0·001[g,h]	0·027 ± 0·015[g]	0·021 ± 0·011	0·008 ± 0·001	0·022 ± 0·006[h]	0·009 ± 0·004
Testes	0·005 ± 0·002[q]	0·017 ± 0·006	0·005 ± 0·001	0·013 ± 0·004	0·016 ± 0·002[q]	<0·001
Carcass	0·844 ± 0·478	1·100 ± 0·063	5·32 ± 2·12	8·52 ± 1·46[i]	0·918 ± 0·316	0·698 ± 0·170[i]
Non-application site skin	—	—	—	—	0·84 ± 0·20	0·18 ± 0·05

TCC = Trichlorocarbanilide TFC = Trifluoromethyldichlorocarbanilide

*Values are means ± SEM for the numbers of rats shown. Those carrying the same superscript differ significantly at $P \leq 0{\cdot}03$.

†With numbers of treated rats in parentheses.

to the liver. A ligature separated the two cannulas in this animal. The bile donors absorbed 56–67% and 31–36% of the oral dose of TCC and TFC, respectively. These values were calculated by summing the percentage of the dose found in the tissues and urine of both animals and in the bile and faeces of the bile receiver 72 hr after dosing. The total dose to the bile receiver was determined by summing the percentage of the dose found in the tissues, urine, bile and faeces of that animal. The percentage of this dose absorbed by the bile receiver was calculated from the amount in the tissues, urine and bile at 72 hr. The bile receiver used in the TCC experiments absorbed 27–34% of the ^{14}C to which they were exposed and the TFC animals absorbed 29–57% of the radioactivity.

Elimination and distribution of the radioactivity in the donor and receiver rats were calculated for each as a percentage of the absorbed dose and are presented in Table 3. The relative roles of the bile and urine in elimination of the radioactivity and the tissue distribution 72 hr after dosing were almost identical between the donor and receiver rats and were very similar to those reported for orally dosed rats (Table 2).

The structure of TCC and TFC suggest that metabolic cleavage of the carbon–nitrogen bonds might form chloroanilines. Hydrolysis at either side of the nitrogen would leave a carbamate, which on further hydrolysis to the chlorinated aromatic amine would also produce CO_2. The potential for the occurrence of this reaction, either in the animal or by the gut flora, was evaluated by giving carbonyl-labelled TCC or TFC orally to intact rats. The CO_2 produced during the 72 hr after dosing was trapped and found to contain only 0·47 ± 0·05 and 0·40 ± 0·04% of the total radioactivity recovered from the TCC and TFC, respectively.

In all experiments, the degree of absorption, rate of elimination and tissue distribution were almost identical for TCC and TFC when the same dosing method was used.

The distribution of TCC and TFC between equal parts of water and 1-octanol was greatly in favour of the 1-octanol, and was similar for both compounds. For TCC, the concentrations were 0·123 and 99·88 μg/ml, in the water and 1-octanol, respectively. For TFC these values were 0·118 and 99·88 μg/ml respectively.

DISCUSSION

In any metabolic evaluation of a compound, the chemical properties of the material and their potential effect on its disposition must be considered. Affinity for lipids rather than water is one of these considerations. Both TCC and TFC are almost insoluble in water. The solubilities of TFC in acetone, dimethylformamide and propylene glycol have been reported, respectively, as 68, 58 and 4 g/100 g solvent (vender production information, Ciba-Geigy Co.), while for TCC these values are 4, 35 and 1 g/100 g solvent (Roman *et al.* 1958). We found that TFC was also more soluble in the chylomicrons of lymph than was TCC, as indicated by the fact that each iv-dosed animal received 2 ml of a lymph solution saturated with compound (Table 1). However, when the lipo-hydrophilic character of TCC and TFC was evaluated using the biological partition model of water and 1-octanol (Hansch & Fujita, 1964) it was apparent that the compounds have very similar distributions. Thus, any of their biological responses that depend on their lipo-hydrophilic nature, such as diffusion through the skin or absorption from the gut, should be very similar. Our data show that both compounds were absorbed to the same extent within a given dosing mode (dermal or oral).

Moreover, it was found that the elimination pattern and tissue distribution were not significantly changed when a $-CF_3$ group was substituted for one of the chlorines. Similar observations have been noted in connexion with the diuretic effect of chlorothiazide and flumethiazide (Smith, 1973a) and with the absorption, distribution and excretion of the chloro- and trifluoromethyl-analogues of a piperazine-substituted dihydrobenzoxazepine psychoactive agent (Dreyfuss, Ross, Shekosky and Schreiber, 1971; Dreyfuss, Shaw, Ross, Wang, Wong and Schreiber, 1973).

It has been shown by autoradiography and scintillation counting of skin sections obtained from guinea-pigs treated with a soap solution of [^{14}C]TCC that most of the TCC remained on the outer layers of the stratum corneum (Rutherford & Black, 1969). This skin distribution resulted in a low degree of absorption through guinea-pig skin as was shown by Black *et al.* (1975). From a skin-surface concentration of 2·46 nmol/cm^2 (20 cm^2 total area), 16–27 nmol was found in the excreta of these guinea-pigs. Assuming a constant rate of absorption over the 48-hr period,

Table 3. *Distribution and excretion of ^{14}C 72 hr after oral administration of TCC or TFC in rats with bile ducts cannulated in tandem*

Body fluid or tissue	Radioactivity (% of absorbed ^{14}C load)* in pairs of rats treated with			
	TCC		TFC	
	Bile donors	Bile receivers	Bile donors	Bile receivers
Urine	6·72 (5·71–7·73)	10·78 (9·99–11·58)	3·96 (3·05–4·87)	6·34 (4·47–8·21)
Bile	91·38 (89·90–92·86)	87·75 (86·31–89·19)	88·87 (84·34–93·40)	91·64 (90·17–93·10)
Liver	0·089 (0·087–0·091)	0·037 (0·036–0·037)	0·062 (0·052–0·072)	0·060 (0·040–0·060)
Kidneys	0·015 (0·012–0·018)	0·009 (0·007–0·010)	0·022 (0·019–0·025)	0·029 (0·012–0·046)
Lungs	0·006 (0·005–0·006)	0·001	0·007 (0·006–0·008)	0·009 (0·006–0·012)
Testes	0·002 (0·001–0·003)	0·003 (0·001–0·005)	0·007 (0·006–0·008)	0·061 (0·024–0·098)
Carcass	1·87 (1·33–2·40)	1·42 (0·78–1·33)	7·07 (3·48–10·66)	1·90 (1·46–2·34)

*Means of two determinations (with range).

the flux can be calculated to have been 0·016–0·028 nmol/cm^2/hr. Thus the dose to flux ratio was 88–154 in the guinea-pig. The total concentrations of TCC and TFC used in our rat experiments were 141 and 110 nmol/cm^2, respectively. Absorption occurred at a constant rate (zero order) over the 72-hr exposure and the flux was calculated to be 0·15 and 0·12 nmol/cm^2/hr for TCC and TFC, respectively. The dose to flux ratio was 959 for TCC and 932 for TFC in the rat. Since there was a visually detectable stacking of material (soap plus compound) in the rat experiments, it is felt that the flux in rats represents a maximum absorption rate in these models from this soap vehicle and this sanitizer concentration. The addition of more dosing solution, as might occur in a dermal toxicity study, would not be expected to result in a greater flux or greater systemic exposure. However, Howes & Black (1976) have shown that an increase in systemic exposure can be obtained by increasing the concentration of the chemicals in the dermal dosing medium.

The major route of elimination was found to be in the bile, which explains the high faecal level of ^{14}C observed by Howes & Black (1976) in intact rats following exposure to [^{14}C]TCC.

The sanitizers, as indicated by radioactivity, were found mainly in the liver, kidneys, lungs and testes at the 72-hr autopsy after iv, dermal or oral exposure. In general, the relative degree of accumulation (liver > kidneys > lungs $\simeq$ testes) was the same for both compounds and all modes of administration. The undefined tissues of the carcass contained the bulk of the residual radioactivity. These observations imply that the tissues exposed to the compounds or their metabolites in an oral toxicity study would be the same as those exposed after dermal application.

Since very little dermally applied radioactivity strayed from the dosing cell to the surrounding area (non-application site skin), there was little potential for contamination of the urine in dermal studies. The finding of a higher proportion of the absorbed radioactivity in the urine in the dermal than in the iv or oral studies indicated that the kidneys played a greater role in the elimination of TCC and TFC after dermal exposure than after oral dosing. That this was not due to an effect of portal absorption and first-pass metabolism was shown by the results of iv dosing, following which the relative amounts eliminated in the bile and urine were the same as after oral dosing. With some allowance for speculation, it could be explained by an easily saturable metabolic process. Both oral and iv doses would have resulted in high blood levels over a short time, while the blood levels from dermal exposure would have been very low over a long period of time. Scharpf *et al.* (1975) observed in man a ratio of faecal to urinary radioactivity of 2·75 and 1·60, respectively, after iv and dermal dosing of TCC.

Several pieces of information imply that both TCC and TFC are highly metabolized once they are absorbed. First, despite the highly lipophilic character of the compounds, very little residue was found in fatty tissues in the iv pilot study. Secondly, it has been observed that acidic compounds of molecular weight greater than 400 are extensively eliminated in the bile of rats (Smith, 1973b), and for TCC or TFC to be eliminated to an extent greater than 95% and fit the above criterion, they would have had to undergo conjugation with acidic groups (sulphate and glucuronide). Finally the high level of enterohepatic circulation observed with TCC and TFC is normally associated with hydrolysis of acid conjugates by gut flora followed by reabsorption (Mandel, 1971). The studies of Howes & Black (1976) also support the theory of extensive metabolism. Additional studies have confirmed these preliminary observations (Jeffcoat *et al.* 1977).

The extremely low level of expired $^{14}CO_2$ recovered from the compound labelled on the bridge carbon supports the conclusion that the metabolic changes involving cleavage of the C–N bond would be very minor. Thus, one would expect chloroanilines, if formed at all, to be only minor metabolites of TCC or TFC.

In conclusion, the absorption, distribution and excretion studies have shown that both TCC and TFC are absorbed, distributed and excreted in a similar fashion that the assessment of toxicity in the rat by oral exposure should be relevant to dermal exposure, and that both TCC and TFC are probably extensively metabolized.

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PULMONARY DEPOSITION, TRANSLOCATION AND CLEARANCE OF INHALED NEUTRON-ACTIVATED TALC IN HAMSTERS

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Abstract—To determine pulmonary deposition, translocation and clearance of inhaled talc, 10-wk-old hamsters received a single 2-hr nose-only exposure to neutron-activated talc. Over a period of 4 months, the hamsters were killed in groups of four. Lungs, liver, kidneys, ovaries, skinned carcass and 2-day and 4-day excreta were collected for γ-ray analysis. The isotope ^{60}Co was used to calculate the quantity of talc in all samples; in addition, the isotope ^{46}Sc was used in a number of samples to check the validity of ^{60}Co as a tracer for talc. From 20 to 80 μg talc, an estimated 6–8% of the inhaled quantity, was deposited in the alveoli. The biological half-life of the talc deposited in the alveoli was 7–10 days. Alveolar clearance was essentially complete 4 months after exposure. No translocation of talc to liver, kidneys, ovaries or other parts of the body was found. Several hundred micrograms of talc were found in the faecal samples. Results of a leaching study to be described elsewhere suggest that the picogram quantities of ^{60}Co found in the urine probably represented leached ^{60}Co absorbed in the gastro-intestinal tract.

INTRODUCTION

The objective of this study was to supplement the results of a recently completed talc-inhalation study in Syrian golden hamsters (Wehner, Zwicker, Cannon, Watson & Carlton, 1977) by investigating pulmonary deposition, translocation and clearance of the inhaled talc in the hamster. Since information on the fate of an inhaled material is essential for the correct interpretation of the results of an inhalation study, we had originally intended in the inhalation study to analyse known quantities of lung and other tissues for talc, using atomic absorption spectrophotometry. This would have enabled us to estimate the quantities of talc deposited and retained in the lungs and the quantity that translocated to other organs and tissues as a function of time, and to correlate these findings with histological changes. However, the elements present in talc in sufficient amounts to be analysed by atomic absorption spectrophotometry were also naturally present at relatively high and varying levels in the hamsters, and therefore small increases in these elements in the hamster organs due to the presence of talc could not be detected. To circumvent this analytical problem, we conducted this separate pulmonary deposition, translocation and clearance study using neutron-activated (radioactive) talc and γ-ray counting of the tissues of the exposed animals. The radioactively labelled talc is easily measured against a very low background in tissue samples from control animals.

EXPERIMENTAL

Neutron activation of talc. Johnson's Baby Powder®, lot 228p, subsequently referred to as talc, was exposed to an integrated neutron flux of about 7×10^{16} n/cm^2, which produced radionuclides by (n,γ) reactions on the major and minor elements in the talc. The neutron-activated talc was allowed to cool for about 30 days so that the short-lived radionuclides could decay. After this decay period, the talc was subjected to γ-ray analysis to determine the long-lived activities that could be used as tracers of the talc. Results (Fig. 1) showed the major activities to be the (n,γ) products ^{46}Sc (83.8 days), ^{51}Cr (27.8 days), ^{59}Fe (45 days) and ^{60}Co (5·2 yr), and the fast neutron reaction products ^{54}Mn (312 days) and ^{58}Co (71 days) from (n,p) reactions on Fe and Ni, respectively. The figures in parentheses give the half-lives of the radionuclides. Counts were made on both a solid-state Ge(Li) detector and a NaI(Tl) γ–γ coincidence multiparameter system. The induced activities of ^{46}Sc, ^{51}Cr, ^{59}Fe and ^{60}Co (in dpm/μg talc $\pm$ SEM for four randomly collected samples) were calculated to be 0·065 $\pm$ 0·001, 0·860 $\pm$ 0·016, 0·262 $\pm$ 0·005 and 0·260 $\pm$ 0·004 ^{59}Fe, respectively, all counts (standards and samples) having been decay-corrected to a standard time, 31 October 1975, 00·00 hr.

Aerosol exposure system. Exposure of the hamsters to the neutron-activated talc took place in our radioactive aerosol exposure laboratory (Fig. 2). The 20-litre aerosol exposure chamber (Fig. 3) was constructed of Lucite with exposure ports for nose-only exposures arranged in seven tiers each of 11 ports. For exposure, the animals were placed in soft-drink bottles that had the bottom and part of the top removed so that the noses of the animals were close to the open tops of the bottles; the bottles were then inserted through neoprene stoppers into the ports of the exposure chamber. The animals were maintained in this position by wadding pushed against their backs and neoprene stoppers taped to the bottoms

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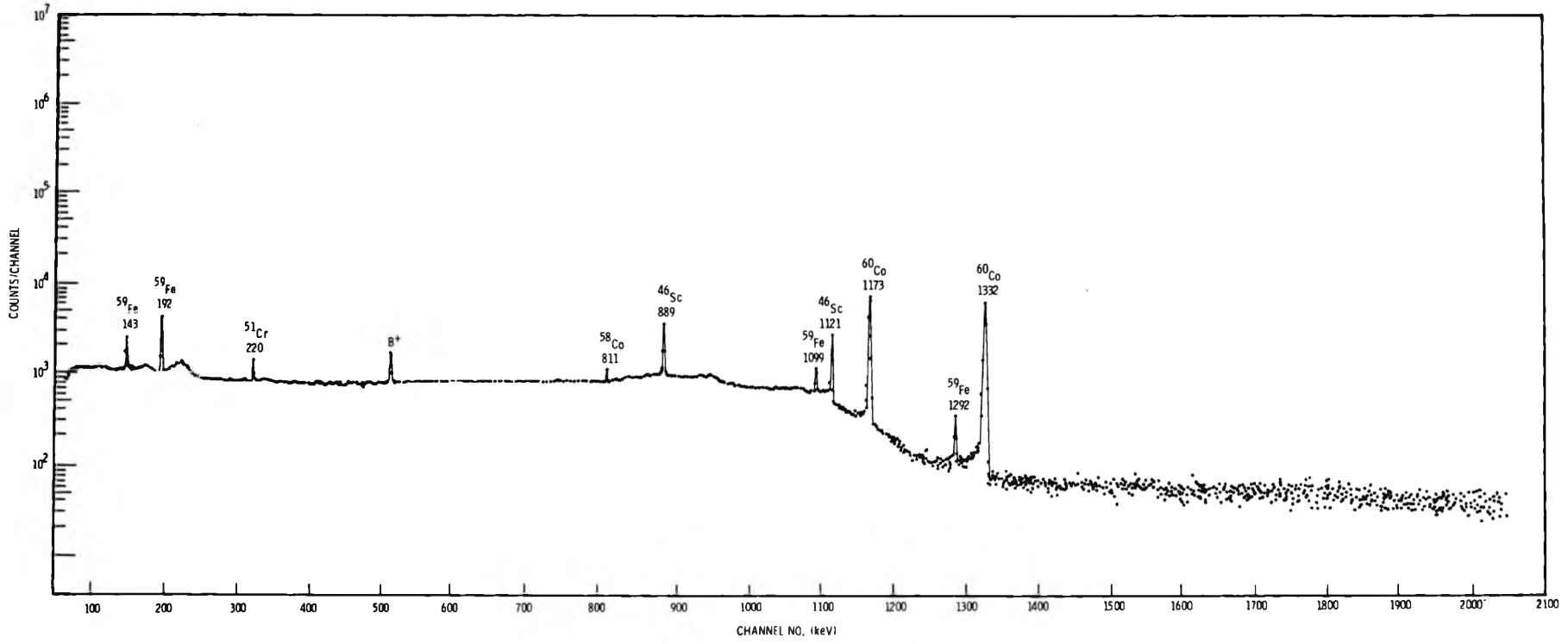

Fig. 1. Gamma-ray spectrum of radioisotope activity in neutron-activated sample of Johnson's Baby Powder.

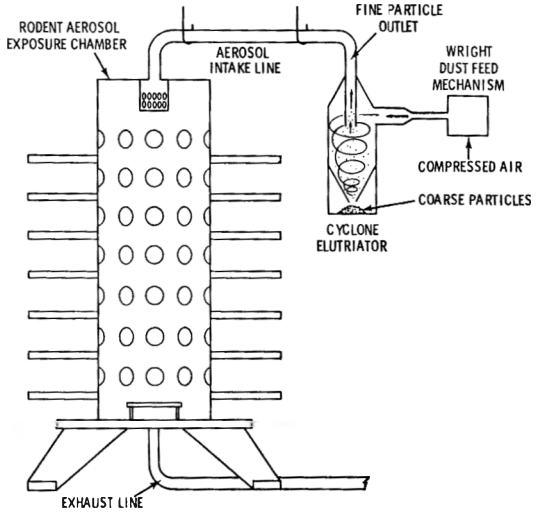

Fig. 3. Scheme of system for exposing rodents to radioactive aerosols.

of, and sealing, the bottles. For exposure, the hamsters and four aerosol samplers were positioned at the ports of the upper five tiers, the ports not occupied by animals or aerosol samplers being closed with neoprene stoppers.

The talc aerosol was generated by a Wright Dust Feed Mechanism (Wright, 1950) and then passed through a cyclone elutriator for removal of particles larger than approximately 10 μm aerodynamic equivalent diameter (AED). The aerosol exposure chamber, aerosol generating equipment and aerosol sampling devices were contained in a Lucite glove box with slight negative pressure relative to the exposure room.

Aerosol characterization. Aerosol concentration in the exposure chamber was determined by γ-ray counting of open-face filter paper samples (25-mm diameter Millipore HAWP, with a mean pore size of 0·45 μm), sampled through ports on tiers 1, 3 and 5. The samples were collected for two periods during the 2-hr exposure, from 15 to 30 and from 60 to 90 min, at a sampling rate of 0·4 litres/min. The air-flow rate through the chamber was 208 litres/min.

Particle-size distribution was determined using a Mercer cascade impactor (Mercer, Tillery & Newton, 1970) and a sampling rate of 0·6 litres/min. The impactor was positioned on the fourth tier from the top. It contained seven glass slides (impaction stages) sprayed with Dow Corning antifoam spray, and a Millipore back-up filter as the eighth stage. The cascade impactor fractionates the aerosol particles into eight size ranges. The quantity of material on each stage was measured in the manner described by Craig & Buschbom (1975). A computer program (NEWCAS) was used to fit the data to a log-normal distribution, calculating a median aerodynamic diameter (MMAD or AMAD, depending upon whether mass or radioactivity was measured) and a geometric standard deviation (GSD). These quantities are identical if the radioactivity is uniformly distributed throughout the mass of the material.

Exposure and serial killings of the hamsters. A group of 44 10-wk-old female Syrian golden hamsters (Mesocricetus auratus, outbred E1a:ENG strain, obtained from Engle Laboratory Animals, Farmersburg, Ind.) received a 2-hr nose-only exposure to neutron-activated talc aerosol in the aerosol exposure system described above. The animals were of the same strain and were obtained from the same source as those used in the previously conducted talc inhalation experiment (Wehner *et al.* 1977).

To our knowledge there is no evidence to suggest a significant effect of sex on pulmonary deposition and clearance. In view of the findings reported by Henderson, Joslin, Turnbull & Griffiths (1971), we chose females for this study in order to investigate whether talc translocated from the lungs to the ovaries in this animal model.

After exposure, the animals were killed in groups of four, and tissues and excreta were collected according to the schedule shown in Table 1. For the first eight serial killings, one animal from each of tiers 1–4 was randomly selected; for the last three, animals were selected from tiers 2–5 as no survivors from tier 1 remained. The lungs, including mediastinal tissue, and the liver, kidneys, skinned carcass, ovaries, urine and faeces were analysed for radioactive isotopes by

Table 1. *Serial killing and sampling schedule for the pulmonary deposition, translocation and clearance study*

Time after exposure*	Samples taken from exposed animals						
	Lungs	Liver	Kidneys	Skinned carcass	Ovaries	Urine	Faeces
15 min	×	×	×	–	–	–	–
100 min	×	×	×	–	×	–	–
4 hr	×	×	×	×	–	–	–
21 hr	×	×	×	×	–	×†	×†
2 days	×	×	×	×	–	–	–
4 days	×	×	×	×	–	×‡	×‡
8 days	×	×	×	×	×	–	–
19 days	×	×	×	×	–	–	–
36 days	×	×	×	–	×	–	–
68 days	×	×	×	×	–	–	–
132 days	×	×	×	×	×	–	–

*Four hamsters were killed at each of the 11 times.
†Combined 0–21 hr excreta from each of four hamsters only.
‡Combined 21 hr–4 day excreta from each of the same four hamsters.

γ-ray spectrometry, and the γ-ray counts were compared with those obtained from nine unexposed control hamsters, four of which were killed on the day the test animals were exposed while the remaining five were killed 132 days later. The lungs, liver, kidneys, skinned carcass and ovaries from all these control animals were analysed, together with urine and faeces samples collected over the previous 2 days from the four hamsters killed on day 0.

Analyses were carried out on the lungs to determine pulmonary deposition and clearance, on liver and kidneys to show whether, during translocation (if any) and clearance of pulmonary deposits of talc, any of the material would pass through and/or accumulate in these organs, and on the ovaries in view of the findings of Henderson *et al.* (1971). The remainder of the skinned carcass was analysed for talc with no attempt at further differentiation of translocation sites. The skin was removed from the carcass to reduce the chance of sample contamination. Analysis of excreta as part of a translocation and clearance study sheds light on the excretion rate of the material.

Analysis of samples. Tissue samples for γ-ray counting were preserved either in formalin or by freezing. Small tissue samples (such as the lung, kidney and ovaries), preserved in formalin, were placed under a heat lamp to reduce the volume and then placed in 7·4-ml polyethylene vials for analysis. Small frozen tissue samples and faecal and urine samples were transferred directly to vials for counting. Large samples, such as the carcass, required high-temperature ashing to reduce the volume for analysis. Previous work in our laboratory has shown that this does not cause volatilization resulting in losses of the radionuclides considered in this study (L. A. Rancitelli, unpublished data 1975).

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

The isotope ^{60}Co was used in all cases to calculate the talc in samples since it has a long half-life (5·2 yr as compared to 83·8 days for ^{46}Sc) and easily detectable coincident γ-rays. For the period of this study, the decay correction for the ^{60}Co activity was only 6% or less. In addition, ^{46}Sc activity was used to estimate the quantity of talc in a number of samples for purposes of comparing the ^{46}Sc-based with the ^{60}Co-based talc values (Table 2). The advantage of using a pair of radioactive tracers to monitor translocation of talc lies in observing two elements with different dissolution and transfer coefficients in biological systems. If the ^{60}Co:^{46}Sc ratio in a biological system is identical to the ratio in talc, it strongly suggests that the radioisotopes were transferred together by particulates. An alternative situation that would produce the same ^{60}Co:^{46}Sc ratio in tissue as was observed in the talc would require that these radionuclides be leached and transferred in identical fashion. This latter possibility is not supported by past experience in our laboratory, which suggests that biological systems take up Co in preference to Sc (L. A. Rancitelli, unpublished data 1975). To obtain additional information on the degree of leaching of radionuclides from talc in body fluids, a leaching study was conducted (C. L. Wilkerson, A. P. Wehner & L. A. Rancitelli, unpublished data 1976).

The isotopes ^{59}Fe and ^{51}Cr were unsuitable as tracers because of the unfavourable characteristics of their radioactive decay schemes and their shorter half-lives.

Table 2. *Quantity of talc in samples, calculated from ^{60}Co and ^{46}Sc data*

Sample	Time of kill after exposure	Exposure position (tier)	Talc content (μg*)		
			^{60}Co (X)	^{46}Sc (Y)	(X/Y ± $S_{X/Y}$)
Lung	15 min	1	77·5	69·6	1·11 ± 0·08
		3	17·8	14·7	1·21 ± 0·28
	100 min	1	42·7	42·6	1·00 ± 0·15
	21 hr	1	21·9	18·5	1·18 ± 0·36
		2	20·1	20·5	0·98 ± 0·25
	2 days	4	10·3	5·3	1·94 ± 1·74
	4 days	3	14·6	13·9	1·05 ± 0·34
	8 days	4	5·3	4·5	1·18 ± 1·21
Carcass	2 days	1	213	146	1·46 ± 0·13
		2	104	100	1·04 ± 0·12
		3	106	95	1·12 ± 0·13
Faeces	21 hr	1	39·5	31·3	1·26 ± 0·27
		2	152	131	1·16 ± 0·07
		3	137	109	1·26 ± 0·05
	4 days	1	186	115	1·62 ± 0·41
		2	243	198	1·23 ± 0·04
		3	781	653	1·20 ± 0·03
		4	172	141	1·22 ± 0·26
Urine	21 hr	1	13·1	15·4	0·85 ± 0·25
	4 days	3	0·9	0	Undefined

*Corrected for control values.

Table 3. *Aerosol data*

Sampling period during 120-min exposure (min)	Aerosol concn* (μg/litre)			AMAD† (μm)	GSD
	Mean	SD	SEM		
15–30	39·7	24·3	14·1	6·4	3·1
60–90	74·7	32·1	18·5	6·9	2·8

AMAD = Activity median aerodynamic diameter
GSD = Geometric standard deviation
*Determined by means of filter-paper samples positioned at tiers 1, 3 and 5, and by a cascade impactor positioned at tier 4.
†Determined by means of a cascade impactor.

RESULTS

Quantities of talc referred to in this text have been calculated from ^{60}Co data corrected for decay, as stated above. It was assumed that the Co concentration in the talc particles was independent of particle size and that ^{60}Co measured in tissue samples reflected talc particles rather than leached ^{60}Co. The latter assumption is supported by the results of the leaching study mentioned above and by the ^{60}Co:^{46}Sc ratios shown in Table 2 for lungs, carcasses and faeces. Most of these ratios are close to 1, suggesting that the tracers ^{60}Co and ^{46}Sc were measured together in talc particles and not as leached radionuclides.

Instead of defining the mean of the controls as zero and correcting the γ-ray counts from the exposed animals to this value, the γ-ray counts from the controls are generally expressed as the μg talc equivalent. Showing the variations in the control values in this manner rather than as a zero point facilitates a more meaningful comparison of data when the values for the exposed animals are approaching control levels.

The aerosol data are summarized in Table 3. The mean aerosol concentrations during the two sampling periods (from 15 to 30 and from 60 to 90 min in the 2-hr exposure period) were 40 and 75 μg/litre, AMADs were 6·4 and 6·9 μm, and the GSDs were 3·1 and 2·8, respectively.

The pulmonary deposition and clearance data are illustrated in Fig. 4 and presented according to the tier position of the exposed animals in Table 4. At times these data varied considerably among animals killed at the same time. While these variations, which were probably due to variations in aerosol concentration at the different tier levels and to differences in individual breathing patterns, may make it difficult to determine absolute pulmonary deposition accurately, reasonable approximations can be made, and the variations do not interfere with the estimation of the biological half-life of talc in the lung and the time required for pulmonary clearance of the talc. More specifically, while data for animals killed at 15 min vary from less than 20 μg for tiers 2–4 to about 80 μg for tier 1 (Table 4), the results, both in the high-deposition animals from the upper tier and in the lower-deposition animals from the lower tiers, indicate that the biological half-life of the talc deposited in the lung is 7–10 days. There is no statistically significant difference ($P > 0{\cdot}05$) between the γ-ray counts from the lungs (including mediastinal tissue) of the animals killed 132 days after exposure and those from the five controls killed on the same day (note that the bars in Fig. 4 show the standard error of the mean, not the 95% confidence intervals). This indicates that in the hamster, pulmonary clearance of talc is complete within approximately 4 months.

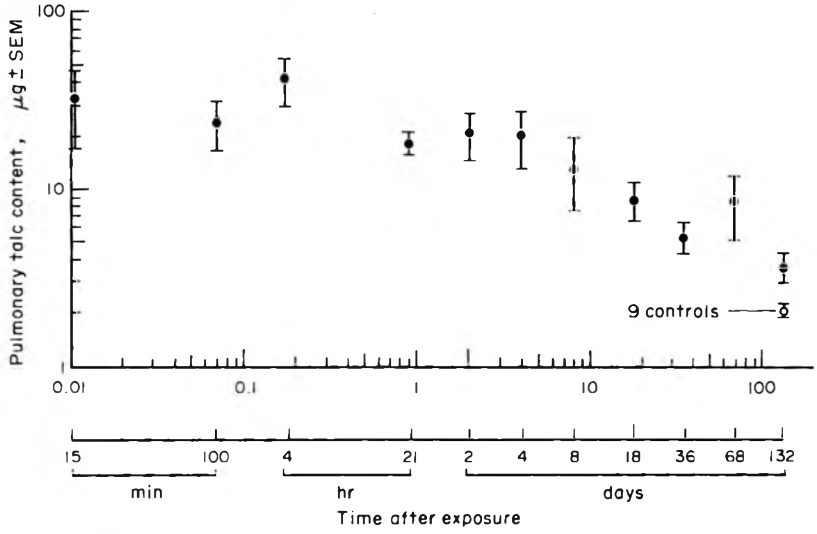

Fig. 4. Pulmonary deposition and clearance of inhaled talc in hamsters, with points and bars indicating means ± SEM for groups of four animals killed at each time.

Table 4. *Pulmonary talc burdens as a function of time after talc exposures*

	Pulmonary talc content (μg†) in											
	Day-0 control	Animals killed after exposure at intervals of										Day-132 control
Tier level*	group	15 min	100 min	4 hr	21 hr	2 days	4 days	8 days	18 days	36 days	68 days	132 days	group
1	2·1	79·6	44·8	70·2	24·0	40·6	43·5	31·0	6·4	—	—	—	2·6
2	1·8	16·4	24·6	20·6	22·2	17·8	11·0	14·8	9·8	8·0	5·8	2·5	2·0
3	1·4	19·9	16·1	22·4	12·7	15·4	17·5	2·2	14·8	2·9	19·8	5·5	2·1
4	1·8	16·4	10·8	57·6	16·1	11·4	12·1	7·4	4·8	4·6	5·9	2·6	1·9
5	—	—	—	—	—	—	—	—	—	6·6	4·2	4·2	2·9
Mean...	1·78	33·08	24·08	42·70	18·75	21·30	21·03	13·85	8·95	5·53	8·93	3·70	2·30
SD...	0·29	31·06	14·94	25·03	5·26	13·13	15·25	12·55	4·42	2·24	7·29	1·43	0·43
SEM...	0·14	15·53	7·47	12·51	2·63	6·57	7·63	6·27	2.21	1·12	3·65	0·72	0·19

*Top tier = no. 1. Tier numbers are not applicable to controls.

†To facilitate a more meaningful comparison, the γ-ray counts from the tissues of the exposed hamsters are not corrected for control values and the γ-ray counts from the tissues of the controls are expressed as μg talc equivalents.

Table 5. *Mean talc burdens in livers, kidneys and ovaries of talc-exposed hamsters*

Values	Organ content of talc (μg‡) in												
	Animals killed at post-exposure intervals of											Controls	
	15 min	100 min	4 hr	21 hr	2 days	4 days	8 days	18 days	36 days	68 days	132 days	0 days	132 days
Liver													
No. of samples	4	4	4	4	4	4	4	3	4	4	4	4	5
Mean	2·55	2·13	1·43*	3·08	2·95	2·55	3·05	3·23	3·58*†	4·10†	2·63	2·93	2·08
SD	1·11	2·26	0·64	1·62	1·11	0·39	1·11	0·74	0·22	0·91	0·42	0·29	0·69
SEM	0·55	1·13	0·32	0·81	0·56	0·19	0·55	0·43	0·11	0·46	0·21	0·14	0·31
Kidneys													
No. of samples	2	1	3	1	1	2	2	2	3	3	4	1	5
Mean	1·70	1·00	1·70	1·00	1·90	2·10	1·90	1·65	2·07	2·00	1·95		2·34
SD	0·57		0·66			0·42	0·14	1·20	0·35	0·17	0·84		0·69
SEM	0·40		0·38			0·30	0·10	0·85	0·20	0·10	0·42		0·31
Ovaries													
No. of samples	—	4	—	—	—		4	—	4	—	4	2	5
Mean		2·00					1·88		1·43		3·05	2·20	2·18
SD		1·14					0·32		0·38		0·60	0·14	0·62
SEM		0·57					0·16		0·19		0·30	0·10	0·28

‡To facilitate a more meaningful comparison, the γ-ray counts from the tissues of the exposed hamsters are not corrected for control values, and the γ-ray counts from the tissues of the controls are expressed as μg talc equivalents.

Means marked * or † differ significantly ($P < 0.05$) from the 0-day and/or 132-day control value, respectively.

The mean γ-ray counts from the livers, kidneys and ovaries of the exposed and control animals, expressed in μg talc, are shown in Table 5. If the individual mean liver values of the exposed animals are compared with the 0-day or 132-day control means, one finds some means that are significantly higher or lower ($P < 0.05$). More specifically, the 4-hr value is significantly lower than the 0-day control value, the 36-day value is significantly higher than both the 0-day and 132-day control values, and the 68-day value is significantly higher than the 132-day control value. However, the important questions are whether or not the means are different from the controls when the data from all kills are considered rather than data from individual kills and whether there is a significant trend in the means as a function of time. To answer these questions, the data were analysed by the Kruskal-Wallis test (Kruskal & Wallis, 1952). The results showed that there was no significant difference among the means (0- and 132-day control means included) for the liver, kidney and ovary data, and consequently that there was no significant trend.

Considerable quantities of talc were found in the carcasses of the first groups killed (Table 6). At 4 hr (the first time at which carcasses were analysed), the values ranged from 193 to 941 μg, with a mean of 455 μg. Within 4 days the mean carcass burden had decreased to 23 μg, about 5% of the original level. By day 8 the γ-ray counts had reached background levels.

Values for the excreta are shown in Table 7. The faecal samples contained relatively high quantities of talc, the mean γ-ray count for the 4-day samples being about 3·5 times higher than that for the 21-hr samples. Five of the six urine samples analysed from either the 21-hr or 4-day collections showed relatively uniform values, ranging in μg talc equivalents from 2·4 to 4·5, all being significantly higher ($P < 0.05$) than the control values. The value for the sixth sample (14·6 μg) was high enough to suggest the possibility of contamination with faeces.

Table 7. *Excreta γ-ray counts expressed as μg talc equivalents*

Hamster no.	γ-Ray counts (μg talc equivalents)* for Exposed animals Faeces	Exposed animals Urine†	Controls Faeces	Controls Urine
	21-hr sample			
1	157	14.6 (838)	6·7	1·7
2	142	4·3 (179)	5·7	1·5
3	81	3·5 (128)	3·4	1·4
4	44	—	3·2	—
Mean...	106	7·5	4·8	1·5
SD...	53	6.2	1·7	0·2
SEM...	26	3·6	0·9	0·1
	4-day sample			
1	786	4·5 (192)		
2	248	3·0 (96)		
3	191	2·4 (58)		
4	177	—		
Mean...	350	3·3		
SD...	292	1·1		
SEM...	146	0·6		

*To facilitate a more meaningful comparison, the γ-ray counts from the excreta of the controls are expressed as μg talc equivalents and the data for the exposed animals have not been corrected for control values, with the exception of the Co values (in parentheses).

†Figures in parentheses are pg Co translocated to urine. The values are corrected for control values and are based on a Co concentration in Johnson's Baby Powder of 64 ppm.

DISCUSSION

Examination of Table 4 shows that the lung data fall essentially into two categories, a high-level category for the animals on tier 1 and a low-level category for the animals on the remaining (lower) tiers. These differences are most likely to have been due to differences in aerosol concentration. With the exception of 3 'odd' values (57·6 μg at 4 hr 2·2 μg at 8 days and 19·8 μg at 68 days), the 36 pulmonary deposition values for the animals from tiers 2–5 are in good agreement.

Apparently mucociliary clearance of talc deposited in the hamsters in this study was essentially completed by the time the first group of animals was killed, i.e. 15 min after exposure. This statement is based on the observation that, disregarding occa-

Table 6. *Talc burdens in the carcasses of talc-exposed hamsters*

Tier level†	Carcass* content of talc (μg) in Animals killed at post-exposure intervals of 4 hr	21 hr	2 days	4 days	8 days	18 days	68 days	132 days	Controls 0 days	132 days
1	941	319	216	26	2·8	3·3	—	—	4·0	3·6
2	424	213	107	32	3·8	3·8	3·1	2·2	4·0	2·7
3	262	116	103	28	4·1	3·1	3·5	1·5	3·8	2·7
4	193	67	176	4·9	3·3	4·4	4·1	2·0	1·8	2·5
5	—	—	—	—	—	—	3·3	2·2	—	1·7
Mean...	455	179	150	23	3·5	3·6	3·5	2·0	3·4	2·6
SD...	338	111	55	12	0·6	0·6	0·4	0·3	0·1	0·7
SEM...	169	56	28	6	0·3	0·3	0·2	0·2	0·5	0·3

*Skinned carcass minus lungs, liver and kidneys, and minus ovaries as shown in Table 1. To facilitate a more meaningful comparison, the γ-ray counts from the carcasses of the exposed hamsters are not corrected for control values, and the γ-ray counts from the carcasses of the controls are expressed as μg talc equivalents.

†Not applicable to controls.

Table 8. *Distribution of talc following inhalation in hamsters killed 4 days after exposure*

	Talc content of								
	Lung		Carcass		Faeces*		Urine*		
Tier level	μg	% of total	μg	% of total	μg	% of total	μg†	% of total	Total (μg)
1	41·2	4·1	23	2·3	933	92·1	16·1 (1030)	1·6	1013
2	8·7	2·1	29	6·9	380	90·0	4·3 (275)	1·0	422
3	15·2	5·0	25	8·2	262	85·9	2·9 (186)	1·0	305
4	9·8	4·4	1·9	0·8	211	93·8	2·3	1·0‡	225
Mean....		3·8		4·0		90·9		1·3	

*Combined 21-hr and 4-day samples.
†The figures in parentheses are picograms of Co translocated to urine. The values are based on a Co concentration in Johnson's Baby Powder of 64 ppm.
‡Estimated value.
All figures are corrected for control values.

sional odd values, the lung burdens remained essentially unchanged through the first 4–6 kills (Table 4), a finding characteristic of the slow alveolar clearance.

Table 8 shows material balance calculations for the hamsters killed 4 days after exposure, according to which approximately 4% of the talc accounted for was retained in the lung. It is reasonable to assume that an unknown fraction of the total quantity of talc accounted for was deposited on or about the nose and nares during the 2-hr nose-only exposure, rather than inhaled. To obtain an approximation of the quantity of talc deposited in the lungs of the hamsters in our previous inhalation study (Wehner *et al.* 1977), it is necessary to estimate alveolar talc deposition as a fraction of inhaled talc in the hamsters of the present study, on the basis of aerosol concentration and minute volume. The mean aerosol concentration for the 2-hr exposure period can be estimated from the data shown in Table 3. The minute volume of adult Syrian golden hamsters at rest is 60 ml (Altman & Dittmer, 1974). Mauderly & Tesarek (1973) investigated the effect of aerosol exposure restraint tubes on the minute volume of Syrian golden hamsters of about the same weight as our hamsters and found that it increased from 50 ml at rest to about 80 ml after 15 min, about 90 ml after 30 min and about 100 ml after 45 and 60 min of restraint.

At an assumed minute volume of 100 ml, based on the results of Mauderly & Tesarek (1973), each hamster inhaled approximately 12 litres of air during the 2-hr exposure period. At an average aerosol concentration of about 60 μg/litre at tier levels 2–4 during the 2-hr exposure period, and assuming that roughly 50% of the aerosol particles (AMAD from 6·4 to 6·9 μm; Table 3) were of respirable size, it can be estimated that the hamster at these tier levels inhaled an average of about 360 μg talc. The initial alveolar talc deposition in these animals was approximately 20 μg (Table 4), or roughly 6% of the inhaled amount. At an average aerosol concentration of 95 μg/litre under otherwise similar conditions, it can be estimated that the hamsters at the tier 1 position inhaled about 570 μg. In these animals the initial alveolar deposition was about 45 μg, i.e., about 8% of the inhaled amount. The estimated percentage alveolar deposition values for the different exposure (tier) levels, namely 6 and 8%, are in good agreement, as are the half-life values of 7–10 days.

The carcass values probably reflect talc deposited on or about the nose and nares during the nose-only exposure plus talc passing through the gastro-intestinal tract after mucociliary clearance, rather than material originally deposited in the alveoli and subsequently translocated to other tissues or organs. This conclusion is based on several observations. The talc quantities found in the carcasses of the hamsters positioned in tiers 1 and 2 and killed 4 hr after exposure, considerably exceeded the estimated total quantity of inhaled talc. For the hamsters in tiers 3 and 4, the carcass values account for more than half of the estimated quantity of talc inhaled. If the faecal talc values are added to the carcass values. the values for the hamsters of all four tiers far exceeded the estimated quantity of talc inhaled. Obviously, more talc cannot be deposited in the hamster respiratory system than is inhaled. Furthermore, in a typical translocation of relatively insoluble material from the lung to other sites, the tissue burden at the translocation site increases slowly as a function of time and as the alveolar burden decreases. In our case, high 'body burdens' decreased to about 5% of the original level within 4 days, and to background levels within 8 days. Finally, in the case of alveolar deposition and subsequent rapid translocation to other sites, one would expect to find certain quantities of such material in the liver and, perhaps, in the kidneys. However, the γ-ray counts from these organs did not differ significantly from those of the controls.

The faecal values reflect talc that had been deposited on the ciliated part of the respiratory tract, probably together with a fraction from licking (nose, lips, paws, etc.).

The relative uniformity of five of the six urine values suggests that they probably represented leached ^{60}Co (C. L. Wilkerson, A. P. Wehner and L. A. Rancitelli, unpublished data 1976). The remaining urine value (14·6 μg, uncorrected for control value; Table 7) was approximately four times greater than the mean of the other five, and can be shown to have been due probably to contamination of the sample with faeces during the separation of excreta in the metabolism cage. Urine contaminated with

talc-containing faeces would be expected to contain ^{60}Co and ^{46}Sc in the same ratio as is found in talc, i.e. approximately 1, while uncontaminated urine should contain only leached ^{60}Co with an undefined ^{60}Co:^{46}Sc ratio, since $^{46}Sc = 0$. The urine data in Table 2 reflect such a difference in ^{60}Co:^{46}Sc ratios. The high 21-hr urine value (13·1 μg in Table 2, corrected for controls and equal to 14·6 μg in Table 7, not corrected for controls) has a ^{60}Co:^{46}Sc ratio of 0·85 ± 0·25, i.e. approximately 1 (Table 2). The 4-day urine value (0·9 μg, Table 2) is representative of the five uniform low values and its ^{60}Co:^{46}Sc ratio is undefined as no ^{46}Sc activity was present. It can thus be concluded that the single high urine value was probably due to contamination with faeces.

In relating the results of this study to the earlier inhalation study (Wehner *et al.* 1977), the conditions of the two studies have to be compared. In the inhalation study, unrestrained male and female hamsters received whole body exposures, from the age of 4 wk for the 30-day exposures or from 7 wk for the 300-day exposures. In the present study, 10-wk-old restrained female hamsters received one 2-hr nose-only exposure to an aerosol, the concentration and AMAD of which ranged from about 40 to 75 μg/litre and from 6·4 to 6·9 μm, respectively, during the two measuring periods. By comparison, the concentrations and MMADs of the aerosols used for the 30- and 300-day exposures were about 37 μg/litre and 5 μm and 27 μg/litre and 6 μm, respectively. The biological half-life of talc in the lung and the pulmonary lung clearance are probably largely independent of these differences. Comparison of the AMAD with the MMADs of the previous study shows that the particle size distribution of the aerosol used in this study was very similar to those used in the inhalation study, especially to that in the 300-day exposures. While the minute volume has relatively little effect on the fractional deposition of a given aerosol in the respiratory tract, it controls the quantity of dust inhaled and deposited (Task Group on Lung Dynamics, 1966). The minute volume is generally a function of size (age) and, in addition, depends on the circumstances, notably the degree of rest, exercise and stress, such as restraint, respiratory irritants etc.).

Alveolar talc deposition in each exposure of the hamsters in the inhalation study can be estimated from the results of the present study, using the minute volume data for restrained hamsters of similar weight provided by Mauderly & Tesarek (1973) and the measured aerosol concentrations (Table 9). For the 300-day-exposure groups, which started exposures at the age of 7 wk and continued through most of their adulthood, a minute volume of 60 ml has been assumed. For the 30-day-exposure groups, exposed from 4 to 9 wk of age, a minute volume of 30 ml has been assumed. As a guideline for selecting this value, the relationship between the mean body weights at the middle of the 30 daily exposures (approximately 70 g) and those of adult hamsters (approximately 140 g) was used. Since the mean body weight of the 30-day hamsters was half that of adult hamsters, half of the minute volume of adult hamsters was used for these groups. The estimated alveolar deposition ranged from 0·04 to 0·06 μg talc for a 3-min exposure of the 30-day-exposure group to roughly 4–6 μg for a 150-min exposure of the 300-day-exposure group (Table 9).

Table 9. *Estimated alveolar deposition per exposure in hamsters exposed to talc aerosol in an earlier inhalation study**

Exposure time (min)	Alveolar deposition (μg)†
30-day exposure	
3	0·04–0·06
30	0·4–0·6
150	2–3
300-day exposure	
30	0·86–1·15
150	4·3–5·8

*Wehner *et al.* (1977). The calculations are based on a respirable aerosol concentration of 8 μg/litre and on an estimated alveolar deposition of 6–8% of the inhaled quantity of talc.

†Based on assumed minute volumes of 30 ml for the group exposed for 30 days and 60 ml for that exposed for 300 days.

The results of this study permit the conclusion that each exposure in the inhalation study resulted in the alveolar deposition of microgram quantities of talc in groups exposed for 300 days and in the group exposed for 150 min/day for 30 days. Deposition in the groups exposed for only 30 or 3 min/day for 30 days was proportionally less.

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SHORT PAPER

MUTAGENIC ACTIVITY OF HYMENOVIN, A SESQUITERPENE LACTONE FROM WESTERN BITTERWEED

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Summary—Hymenovin, the major toxic constituent of the poisonous range plant *Hymenoxys odorata*, was shown to be mutagenic in the Ames *Salmonella typhimurium* test for mutagens and potential carcinogens. An *in vitro* rat-liver metabolizing system did not alter the mutagenic activity significantly. Tenulin and helenalin, two other sesquiterpene lactones found in range plants, were inactive in the test.

Introduction

Numerous sesquiterpene lactones occurring in plants belonging to the family Compositae have been characterized (Geissman & Irwin, 1970), and their occurrence in common range plants has been of interest due to incidents of poisoning by the plants in sheep, goats, horses and cattle (Adams & Herz, 1949; Ivie, Witzel, Herz, Kannan, Norman, Rushing, Johnson, Rowe & Veech, 1975a; Ivie, Witzel, Herz, Sharma & Johnson, 1976; Ivie, Witzel & Rushing, 1975b; Kingsbury, 1964; Radeleff, 1970). The cytotoxicity of various structural types of sesquiterpene lactones has been investigated, and it has been postulated that a high degree of cytotoxicity is associated with the presence of an α-methylene-γ-lactone moiety, which is believed to function as an alkylating group (Lee, Huang, Piantadosi, Pagano & Geissman, 1971). Since a wide variety of alkylating lactones are carcinogenic as well as cytotoxic (Ciegler, Detroy & Lillehoj, 1971), it was decided to determine the mutagenic potential of three sesquiterpene lactones with differing functional groups using the Ames Salmonella/microsome mutagenicity test (Ames, McCann & Yamasaki, 1975), in which a high correlation between mutagenicity and carcinogenic activity in animals has been established (McCann, Choi, Yamasaki & Ames, 1976).

Experimental

Test compounds. Hymenovin, helenalin and tenulin (Fig. 1) were kindly supplied by Dr. G. W. Ivie, US Department of Agriculture. Details of their preparation and structures have been described (Adams & Herz, 1949; Ivie *et al.* 1975a,b). Hymenovin contains an α-methylene-γ-lactone group, the presence of which is correlated with a high degree of cytotoxicity (Lee *et al.* 1971). Tenulin does not contain the α-methylene-γ-lactone group, but does contain an α,β-unsaturated ketone group, a group present in many naturally occurring toxins and carcinogens (Ciegler *et al.* 1971). Helenalin contains both of these functional groups. All three compounds are found in bitterweed or sneezeweed, range plants which have been responsible for extensive losses of livestock in over-grazed pastures (Adams & Herz, 1949; Ivie *et al.* 1975a,b; Ivie *et al.* 1976; Kingsbury, 1964; Radeleff, 1970).

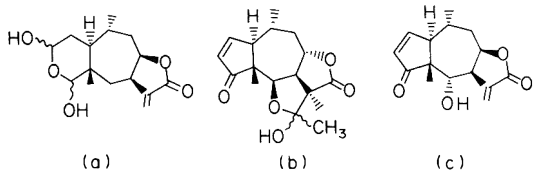

Fig. 1. Structures of the test compounds (a) hymenovin, (b) tenulin and (c) helanalin.

Test procedure. The histidine-requiring *Salmonella typhimurium* mutagenesis tester strains, TA100, TA98, TA1535 and TA1537, developed by Dr. B. N. Ames (Ames *et al.* 1975) were used. The plate test for reversion to histidine prototrophy, media, the oxidative-enzyme metabolizing system (S-9, a 9000-**g** supernatant of Aroclor 1254-induced rat liver) and the tests for the identity of the tester strains (*rfa* character, R factor and reversion specificity) have been described (Ames *et al.* 1975). Mutants obtained were shown to breed true by subculturing on mutagen-free, biotin-supplemented, minimal-glucose agar without histidine. Positive and negative controls were included in each experiment.

Results and Discussion

The results obtained are summarized in Table 1. Hymenovin was mutagenic in strains TA100 and TA98, the former being slightly more sensitive than the latter. The revertant frequency of strain TA100 was significantly increased by 3–10 μg hymenovin/plate. The mutagenic activity was not altered significantly by the *in vitro* metabolizing system. Increased

Table 1. *Reversion of* Salmonella typhimurium *strains TA100, TA98, TA1535 and TA1537 following exposure to hymenovin, helenalin and tenulin*

	Dose (μg/plate)	No. of mutants/plate*†							
		TA100		TA98		TA1535		TA1537	
		No S-9‡	+S-9‡	No S-9	+S-9	No S-9	+S-9	No S-9	+S-9
Hymenovin	1	18	(8)	(7)	5	8	4	2	(7)
	3	6	33	10	15	9	3	(1)	(3)
	10	69	101	7	8	6	3	(3)	(6)
	30	198	280	35	41	5	2	(1)	(2)
	100	c.1000	c.1000	124	153	3	(1)	(2)	(5)
	300	>1500	>1500	426	493	5	1	(1)	0
	1000	+	+	75+	11+	+	+	+	+
Helenalin	1	(4)	(3)	0	2	1	3	1	(7)
	3	(13)	3	3	0	6	(2)	(2)	1
	10	(11)	0	(9)	(2)	1	1	0	(2)
	30	(31)	(8)	8	10	7	3	(5)	0
	100	(38)	(23)	(6)	(6)	(8)	1	1	(5)
	300	+	+	(6)+	+	(6)+	(4)+	(6)+	(5)+
	1000	+	+	+	+	+	+	+	+
Tenulin	1	(3)	6	(4)	(10)	7	2	(3)	(3)
	3	0	(8)	8	(2)	5	0	(1)	0
	10	(2)	(10)	(7)	(5)	0	2	(2)	(6)
	30	(6)	7	5	11	2	(1)	(1)	(4)
	100	3	(3)	(3)	(8)	5	0	(2)	–
	300	(22)	0	7	7	0	(2)	(3)	(1)
	1000	21	4	(9)	(9)	2	0	0	(8)

*Values are numbers of revertants minus spontaneous values. Each value is based on a minimum of two determinations. Values in parentheses were less than the spontaneous value. Spontaneous values were approximately: TA100 75–110, TA98 25–40, TA1535 10–20 and TA1537 5–15. The mean differences between replicate control plates ±1 SD, without and with S-9, respectively, were TA100 8 ± 6 and 12 ± 7, TA98 5 ± 6 and 8 ± 6, TA1535 5 ± 4 and 5 ± 3, and TA1537 3 ± 3 and 3 ± 2 (calculated from 7–10 independent experiments in each case). Underlined values exceed the mean difference between replicate control plates plus 3 SD while + indicates growth inhibition of the background lawn.

†Positive controls. TA100: 1 μg aflatoxin B_1 (+S-9), 0.05 μg 4-nitroquinoline 1-oxide, 0·02 μg furylfuramide (AF-2) and 500 μg methylmethanesulphonate each gave >500 revertants: TA98: 1 μg aflatoxin B_1 (+S-9) gave approximately 1000 revertants; TA1535: 10 μg *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine gave >1000 revertants; TA1537: 100 μg 9-aminoacridine gave approximately 1000 revertants.

‡S-9 denotes the metabolizing mixture described by Ames *et al.* (1975) and used in a concentration of 50 μl liver supernatant/plate.

revertant frequencies were not observed in strains TA1535 and TA1537. The response pattern with respect to tester strain specificity thus resembles that of the carcinogens aflatoxin B_1, 4-nitroquinoline 1-oxide, furylfuramide and methylmethanesulphonate, which exhibit a much higher level of activity in the R factor-containing strains (TA100, TA98) than in those lacking this factor (McCann, Spingarn, Kobori & Ames, 1975).

Tenulin and helenalin were not mutagenic in the strains used (Table 1), even though helenalin contains two functional alkylating groups, the α-methylene-γ-lactone moiety and an α,β-unsaturated ketone group. Helenalin was, however, the most toxic of the three sesquiterpene lactones to the Salmonella tester strains.

In a study of the effects of hymenovin and tenulin on the growth of *Bacillus thuringiensis*, Norman, Johnson, Mollenhauer & Meola (1975) observed mutant colonies in streaks from cultures grown in the presence of hymenovin, and they have suggested that hymenovin may be mutagenic in this organism. Their results, however, could also be explained in terms of selective growth inhibition without mutagenesis. These workers also reported that hymenovin, but not tenulin, appeared to enhance bacteriophage production in this organism (Norman *et al.* 1975).

Lee *et al.* (1971) have shown helenalin to be the most cytotoxic of 18 different sesquiterpene lactones tested in three different human cell lines, a finding consistent with the relatively high toxicity of helenalin to the *Salmonella typhimurium* strains used in the present study. The lack of any mutagenic effect of helenalin, however, indicates that the mere presence of a reactive lactone grouping and a high degree of cytotoxicity does not necessarily correlate directly with mutagenic potential.

The widespread occurrence of sesquiterpene lactones in range plants and the present demonstration that at least one member of this family produces the same pattern of reversion in the Ames test as do several known potent carcinogens indicate that the mutagenic and possible carcinogenic properties of this class of compound should be thoroughly investigated. Both economic factors related to livestock production and the possible human health aspects should be considered. Mutagenic changes in the reproductive cells of livestock ingesting these compounds could cause long-range economic losses due to breeding failure or to the accumulated induction of undesirable traits,

adding to the present estimates of losses based on the acute toxic effects of these compounds (Ivie *et al.* 1975a,b & 1976). Very little is known about the possibility of transmitting these compounds to the human diet via livestock, although tenulin has been considered an economic problem to the dairy industry because it is transmitted into the milk of cattle grazing on *Helenium amarum* at levels sufficient to impart a bitter taste (Ivie *et al.* 1975b). Since more than 170 sesquiterpene lactones of related structure are known to occur among the genera of the family Compositae (Geissman & Irwin, 1970), it is likely that at least low levels of these compounds could enter human food via livestock. Attention should be given, therefore, to the question of the possible carcinogenicity and mutagenicity of these and other sesquiterpene lactones.

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PRELIMINARY COMMUNICATION

TRANSPLACENTAL CHRONIC TOXICITY TEST OF CARBARYL WITH NITRITE IN RATS*

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Summary—Pregnant female rats were given by gavage a total of 300 mg carbaryl during a 10-day period. No malignant tumours were induced in the dams or their offspring during their natural lifespan. Pregnant and non-pregnant female rats were given a suspension of carbaryl in sodium nitrite solution on several successive days, a total of 90 mg carbaryl and 120 mg sodium nitrite being administered to each adult rat. There was no significant incidence of malignant tumours in the dams or their offspring as a result of the treatment.

Introduction

Carbaryl, the 1-naphthyl ester of *N*-methylcarbamic acid, is a widely used insecticide which is permitted as a residue on crops, a tolerance level having been set for it by the Food and Drug Administration. Carbaryl itself is of interest as a possible environmental contaminant, and as a member of the large group of *N*-methylcarbamate esters which are used as insecticides. Several of them have been tested for toxicity and appear to be relatively non-toxic in mammals; neither do they show mutagenic activity in bacterial systems. However, because they can react with nitrous acid to form *N*-nitroso derivatives (Elespuru & Lijinsky, 1973), another aspect of their presence in the environment must be considered, namely the possible adverse biological effects of the nitroso compounds so formed.

It has been demonstrated that nitrosocarbaryl, which can interact with human DNA *in vitro* (Regan, Setlow, Francis & Lijinsky, 1976), is a potent bacterial mutagen (Elespuru, Lijinsky & Setlow, 1974), as are the nitroso derivatives of several other methylcarbamate insecticides (Lijinsky & Elespuru, 1976). Nitrosocarbaryl is also a fairly potent carcinogen when administered orally to rats (Eisenbrand, Schmähl & Preussman, 1976; Lijinsky & Taylor, 1976) and, because formation of nitrosocarbaryl from ingested carbaryl and nitrite is the most likely mode of human exposure, it was decided to investigate its formation in this way in rats. Since the amount formed in a rat's stomach was likely to be small, the transplacental route of exposure for the rats was chosen. This is considered the most sensitive mammalian test system, and has responded to very small doses of nitroso compounds formed in this way *in vivo* (Ivankovic & Preussmann, 1970).

Experimental

Technical-grade carbaryl ('Sevin', kindly supplied by Union Carbide Corp., Bound Brook, NJ) was crystallized from acetone, after insoluble dark material had been filtered off, colourless crystals, m.p. 143–144°C being obtained. These were finely powdered in a mortar and mixed with water to give a fairly uniform suspension which would pass through the needle of a small syringe. The suspensions given to the animals by gavage contained carbaryl (30 mg/ml) in water or in sodium nitrite solution (40 mg $NaNO_2$/ml).

Twenty-four female Sprague–Dawley rats, 12–14 wk old, were mated by leaving them with males for a week-end, in which time the majority would normally be expected to become pregnant. They were divided into three groups of eight animals. Those in group 1 were given 1 ml of a suspension of 30 mg carbaryl in water on each of 10 days between day 4 and 18 of gestation (weekends omitted). One animal died during the treatment and three of the remainder gave birth to a total of nine female and 13 male rats. Group II rats were given 1 ml of a suspension of 30 mg carbaryl in 4% sodium nitrite solution on days 4, 5 and 6 of gestation (first trimester). At that time six of the eight animals died of nitrite poisoning and the treatment was discontinued. Only one of the remaining animals gave birth, to a litter of seven females and three males. Group III rats were given only 0·6 ml of the suspension given to group II, to avoid nitrite poisoning. Treatment was given on each of days 14–18 of gestation (last trimester). No animals died and six gave birth to litters totalling 32 females and 32 males. To provide additional numbers for the significance of tumour incidences in the animals given carbaryl with nitrite to be assessed, an additional

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Table 1. *Survival of rats treated with carbaryl or carbaryl and nitrite*

Group no.	Treatment	Route	Time of exposure*	No. of rats/group	No. of animals surviving at week										
					60	70	80	90	100	110	120	130	140	150	160
I	Carbaryl	Intragastric	4–18	7F	7	7	7	5	3	2	2	1	1	0	
I	Carbaryl	Transplacental	4–18	9F	9	9	9	9	7	6	3	1	0		
				13M	12	12	11	10	6	5	2	0			
II, III, IV	Carbaryl + $NaNO_2$	Intragastric	14–18†	22F	22	20	20	18	13	9	7	0			
II	Carbaryl + $NaNO_2$	Transplacental	4–6	7F	7	7	7	6	5	4	2	2	1	1	0
				3M	3	3	3	3	3	2	1	0			
III	Carbaryl + $NaNO_2$	Transplacental	14–18	32F	30	28	28	26	23	15	10	5	3	1	0
				32M	32	32	31	27	24	16	5	2	0		

*Days of gestation on which test solution was administered to dams. Non-pregnant females of group IV were treated on an equivalent no. of days.
†Or days 4–6 (group II).

Table 2. *Tumours in rats treated with carbaryl or carbaryl and nitrite*

Group no.	Treatment	Route	Time of exposure*	No. of rats/ group	No. of animals with tumours of						
					Breast	Pituitary	Adrenal	Pancreas	Liver	Uterus	Other tissues
I	Carbaryl	Intragastric	4–18	6F	4	3	0	0	0	0	Lymphosarcoma (1) Leukemia (1)
I	Carbaryl	Transplacental	4–18	9F	7	7	2	1	0	0	Adenocarcinoma (1)
				13M	0	2	4	1	0	0	
II, III, IV	Carbaryl + $NaNO_2$	Intragastric	14–18†	22F	13	11	2	0	3	2	Lymphosarcoma (1) Oesophageal papilloma (1) Lymphangioma (1)
II	Carbaryl + $NaNO_2$	Transplacental	4–6	7F	5	2	1	0	0	0	Lymphosarcoma (1) Myeloid leukaemia (1) Thyroid carcinoma (1)
				3M	1	1	1	1	0	0	Stomach papilloma (1)
III	Carbaryl + $NaNO_2$	Transplacental	14–18	32F	22	17	4	1	2	1	Lymphosarcoma (1) Mesothelioma (1) Myeloid leukaemia (2)
				32M	4	4	6	6	0	0	Osteoadenocarcinoma of gut (1) Adenocarcinoma of gut (1) Lymphosarcoma (1) Myeloid leukaemia (1) Mesothelioma (1) Zymbal gland carcinoma (1) Skin trichoepithelioma (1)

*Days of gestation on which test solution was administered to dams. Non-pregnant females of group IV were treated on an equivalent no. of days.
†Or days 4–6 (group II).

twelve rats (group IV) were given the same treatment as group III on five successive days.

All the adults and offspring, were observed until natural death except a few that were killed when moribund. Complete autopsy was performed and all tumours and other lesions were fixed for histological examination, together with most grossly normal major organs.

Results and Discussion

As seen in Table 1, survival of all of the rats was good (with the exception of those killed by nitrite poisoning) and there was little difference between the groups when account was taken of the longer period of experimental observation of the offspring which were treated *in utero*. In each group at least half of the animals were alive after 2 years and a number survived beyond 2·5 years. Therefore, the dose of carbaryl given, with or without nitrite, had no apparent life-shortening effect.

The distribution of tumours in the rats of the various groups is shown in Table 2. The common tumours in all groups were those normally seen in Sprague–Dawley rats, notably mammary fibroadenomas and tumours of the pituitary (predominantly in females) and adrenal tumours (mostly cortical adenomas). There were a few islet-cell adenomas of the pancreas and some hepatocellular carcinomas. The distribution of these tumours did not suggest any treatment-related effect on their incidence.

In the females treated with carbaryl alone, and in their offspring, there were very few tumours other than those mentioned, and it appears that the dose of carbaryl administered, 300 mg or 1·5 mmol was without tumorigenic effect. Although this is by no means an exhaustive test of the carcinogenicity of carbaryl, the lack of response of the transplacentally exposed rats suggests that the tumorigenic effect of carbaryl, if any, is exceedingly weak. There is a small possibility that the compound does not cross the placenta, but it has been shown to be readily absorbed from the stomach (Casper, Pekas & Dinusson, 1973).

The offspring of the females receiving carbaryl plus nitrite in the latter part of pregnancy gave no indication of a transplacental carcinogenic effect of the nitrosocarbaryl formed in the stomach. Nitrosocarbaryl is a fairly potent carcinogen when administered to rats, producing squamous carcinomas of the stomach when given intragastrically (Eisenbrand *et al.*, 1976; Lijinsky & Taylor, 1976) and sarcomas when given sc (Eisenbrand, Ungerer & Preussmann, 1975), and can be formed to some extent *in vitro* even at low concentrations of carbaryl and nitrite (Eisenbrand *et al.* 1975). We conclude that either insufficient nitrosocarbaryl was formed in the rats *in vivo* during the 5 days of treatment with 18 mg carbaryl plus 24 mg sodium nitrite, or that unlike methylnitrosourea and ethylnitrosurea a significant proportion of the nitrosocarbaryl formed does not cross the placenta of rats. While transplacental treatment of rats with larger quantities of nitrosocarbaryl might establish this point, the results of these experiments demonstrate no overt transplacental carcinogenic hazard from ingestion of carbaryl and nitrite together. Neither do they establish conclusively the safety of a combination of carbaryl and nitrite when ingested by humans, who might be a more sensitive species to nitrosocarbaryl (received directly or transplacentally). However, an experiment of this kind with its negative results does give a little more comfort than results that are clearly positive.

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Review Section

SHORT REVIEW

QUANTITATIVE ASPECTS OF HUMAN EXPOSURE TO NITROSAMINES

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Summary—A method is proposed for the quantitative evaluation of the relative risk of environmental carcinogens and is used to demonstrate that evaluation of the potential dangers of toxic substances on the basis of concentration levels alone may lead to erroneous conclusions. This concept is used to suggest a method of estimating the likelihood that certain environmental carcinogens may cause significant cancer incidence. Finally, these ideas are applied to the problem of nitrosamines in food and the conclusion is drawn that those present in the highest concentrations may not pose the greatest risk.

It has become apparent that a large proportion of cancers in man are caused by chemicals in the environment (Cairns, 1975; Fraumeni, 1975). Although several environmental carcinogens have been detected and identified, eliminating human contact with them has proved to be extremely difficult because of the complex social, economic and political factors that are superimposed on the scientific aspects of the problem. Before expensive efforts are undertaken to remove a specific chemical from the environment, it is desirable to establish, first of all, the likelihood that the substance is carcinogenic to man and, secondly, that the actual environmental concentrations of the compound are high enough to pose a real hazard. Answers to these questions are, of course, rarely available, except in known cases of human exposure to industrial chemical carcinogens.

Quantitative animal data are available for a large number of chemical carcinogens, especially such well-studied series as the nitrosamines (Druckrey, Preussmann, Ivankovic & Schmähl, 1967) and the polycyclic aromatic hydrocarbons (Arcos & Argus, 1974). Since it is impossible to obtain such data for humans experimentally, we must develop rational and credible methods for extrapolating from animal data to man in order to assess explicitly the potential hazards of a known chemical carcinogen in the environment. This situation is currently exemplified by the well-publicized presence of low levels of nitrosamines in a variety of food products (Lijinsky & Epstein, 1970; Scanlan, 1975; Wogan & Tannenbaum, 1975; Wolff & Wasserman, 1972).

In the following discussion, we examine the nitrosamine problem in order to estimate both the relative hazards of the various nitrosamines present in food and the actual hazard posed by the ingestion of nitrosamine-contaminated food.

Table 1 summarizes typical high and low values of the three major nitrosamines found in processed meats, including bacon. Table 2 illustrates the carcinogenic potencies of the same three nitrosamines in the BD rat; these data are particularly valuable, since very similar daily doses (approximately 0·05 mmol/kg) were used in this feeding study for each of the three nitrosamines. The relative carcinogenic potencies of these compounds can be compared conveniently by taking the reciprocal of the mean total carcinogenic dose (D_{50}) followed by normalization (Wishnok & Archer, 1976). As shown in the table, dimethylnitrosamine (DMN) and diethylnitrosamine (DEN) are six to seven times more carcinogenic than nitrosopyrrolidine (NP) on a molar basis in the BD rat. When these data on relative carcinogenic potency are combined with information on the presence of the nitrosamines in food, a relative risk factor can be calculated: $R = PC$, where R = relative risk, P = relative molar potency and C = concentration in (mol/g of food) $\times 10^{10}$. This relative risk factor is based on the assumption that the relative carcinogenic potencies of the compounds are the same in man as in the rat. (This assumption must be made in the absence of human data, although, as compara-

Table 1. *Concentration of nitrosamines in processed meats, including bacon*

	Typical high values*		Typical low values*	
Nitrosamine	ppb	mol × 10^9/g food	ppb	mol × 10^9/g food
DMN	25	0·34	3	0·04
DEN	12	0·12	2	0·02
NP	50	0·50	5	0·05

DMN = Dimethylnitrosamine
DEN = Diethylnitrosamine
NP = Nitrosopyrrolidine

*The values are from the summary and compilation of current data made by Scanlan (1975); b = 10^9.

Table 2. *Carcinogenic potency of various nitrosamines in the BD rat**

Nitrosamine	Daily dose (mmol/kg)	D_{50} (mol/kg)	$1/D_{50}$	Relative carcinogenic potency
DMN	0·05	0·0054	185	7
DEN	0·05	0·0065	154	6
NP	0·05	0·039	26	1

*The values are taken from the extensive review of nitrosamine carcinogenicity by Druckrey *et al.* (1967).

tive biochemical information becomes available, more accurate extrapolations from rodents to man may become possible.) In Table 3, the relative risks have been calculated for the three nitrosamines; the risk of ingesting food contaminated with 5 ppb NP is defined as 1. It thus becomes apparent that, in terms of risk, a low intake of DMN (3 ppb) is comparable with a high intake of NP. This result is important in view of the current tendency to assume arbitrarily that concentrations of DMN and DEN in the lower parts-per-billion range are not hazardous, but to regard 50 ppb NP as potentially dangerous.

In addition to these considerations, Druckrey *et al.* (1967) have pointed out that nitrosamine carcinogenesis is an 'accelerated process'. The median induction time for tumour formation (t) and daily dosage of carcinogen (d) are related by the equation: dt^n = constant. For *N*-nitroso derivatives, n varies from 1·4 to 4, depending on structure. No data are available for DMN, but DEN and NP have n values of 2·3 and 1·4, respectively. These values indicate that, for similar daily doses, DEN carcinogenesis accelerates at a greater rate than NP carcinogenesis, which again suggests that, for similar daily doses, DEN may pose a greater hazard than NP.

Several recent reports have described calculations and estimates of 'no-effect levels' for the dose-response relationship between cells and chemicals (Claus, 1974; Claus, Krisko & Bolander, 1974; Dinman, 1972). Such estimates suggest a threshold for biological activity within an individual cell of about 10^4 atoms of any chemical. (Of course, it is possible that, unlike other types of biological response to chemicals, one molecule of a carcinogen in a cell may be all that is needed to elicit a response.) Table 4 illustrates the number of molecules of DMN, DEN and NP per cell resulting from the ingestion of 50 g of contaminated food by a 70-kg man, assuming an even distribution of the nitrosamine throughout the cells of the body, several studies (Magee, 1956; Stewart, Swann, Holsman & Magee, 1974) having indicated that this assumption may be at least partially correct. The numbers fall well short of the value of 10^4 molecules per cell, even if the amounts of individual nitrosamines consumed are added together.

Table 3. *Relative carcinogenic risk of nitrosamine ingestion*

Nitrosamine	Relative risk/g food	
	High intake*	Low intake*
DMN	48	6
DEN	14	2
NP	10	1

*See Table 1.

This analysis suggests several important conclusions. The firmest of these is that the potential hazard of environmental carcinogens should not be represented by simple concentration data. Regardless of the confidence with which animal data can be quantitatively extrapolated to man, there can be little doubt that some carcinogens are more potent than others for man. Low levels of highly active carcinogens may, in fact, be more hazardous than much higher levels of less active carcinogens. In many cases, there are sufficient data in the literature to allow at least some estimation of relative potencies among closely related groups of carcinogens.

When the intake data for nitrosamines are computed on a molecule-per-cell basis, the levels of total nitrosamines consumed daily are well below 10^4 molecules per cell, the level that has been suggested as a threshold value for intracellular biochemical interactions. If this threshold level is relevant to the process of carcinogenesis, it is clear that the levels of nitrosamines found in food are not significantly hazardous. The following points, however, must be considered:

(1) The effects of various chemical carcinogens may be additive or even synergistic, particularly when they have the same organ specificity (Argus & Arcos, 1976; Montesano, Mohr, Magee, Hilfrich & Haas, 1974; Schmähl, 1970). Thus it may not be reasonable to consider only a single class of compounds from a single source.

Table 4. *Daily intake of nitrosamines*

Nitrosamine	High intake* (molecules/ cell/day)	Low intake* (molecules/ cell/day)
DMN	72	9
DEN	26	4
NP	107	11
Total	205	24

DMN = Dimethylnitrosamine
DEN = Diethylnitrosamine
NP = Nitrosopyrrolidine

*Values calculated from an assumed ingestion of 50 g contaminated food, a figure of $1·4 \times 10^{14}$ for the number of cells in an adult human body (Claus *et al.* 1974), Avogadro's number (6×10^{23}) and the high and low nitrosamine concentrations given in Table 1.

(2) The effects of individual chemical carcinogens may be cumulative, even though the daily dose is below the threshold value estimated for other biochemical responses.
(3) If sufficient time elapses between doses of a carcinogen, repair of the lesions produced in DNA may render the effects of the doses non-cumulative (Swann, Magee, Mohr, Reznik, Green & Kaufman, 1976).
(4) Man may be more susceptible to a particular chemical carcinogen than the rodent in which its activity was demonstrated. It has also been suggested that safety factors as high as several thousand should be used when the hazards of chemical carcinogens are evaluated (Schneiderman, 1970; Weil, 1972). In this context, the levels of nitrosamines in food may be sufficiently high to warrant concern.

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REVIEWS OF RECENT PUBLICATIONS

IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Man. Some Naturally Occurring Substances. Vol. 10. International Agency for Research on Cancer, Lyon, 1976. pp. 353. Sw.fr. 38.00*.

IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Man. Cadmium, Nickel, Some Epoxides, Miscellaneous Industrial Chemicals and General Considerations on Volatile Anaesthetics. Vol. 11. International Agency for Research on Cancer, Lyon, 1976. pp. 306. Sw.fr. 34.00*.

The first volume cited above is devoted to the evaluation of the carcinogenicity of some metabolic products of plants and fungi. Four of the substances or groups of substances considered, namely the aflatoxins, cycasin, sterigmatocystin and safrole and its related compounds, figured in the first volume in this series, published in 1972 (*Cited in F.C.T.* 1974, **12,** 243). The carcinogenicity of the aflatoxins and cycasin to various species is now more firmly established and new epidemiological data support the earlier suspicions that the aflatoxins are carcinogenic to man. Newly acquired data have also confirmed the production of liver tumours by safrole in rats and mice and additional metabolites have been identified in several species.

The eleventh volume in the series also includes some up-dated monographs, those on cadmium and nickel having appeared first in Volume 2 (*ibid* 1974, **12,** 244). Most of the new information supports the earlier observations on the carcinogenicity of these two metals. Both induce tumours in experimental animals when administered subcutaneously, and nickel has been shown to be carcinogenic also in inhalation studies. While epidemiological evidence indicates that some forms of nickel are carcinogenic to man, the evidence on cadmium is much less convincing—in fact many workers consider it to be of little value. Volume 11 also presents monographs on various epoxide compounds and on a miscellaneous group of chemicals of importance chiefly in connexion with industrial exposure, and makes some general comments on volatile anaesthetics. The Working Group responsible for the latter contribution considered that the environment of the operating theatre presented some carcinogenic hazard but did not ascribe responsibility to any specific anaesthetics.

Some of the miscellaneous industrial chemicals considered in this volume are undoubtedly carcinogenic to animals, but although the epoxides are both industrially important and biologically reactive, the main evidence for their carcinogenic activity rests on studies involving repeated subcutaneous injection in rats and mice. The induction of local sarcomas after repeated injection features prominently also in many of the monographs in Volume 10, including those on actinomycins, parasorbic acid, patulin, penicillic acid and mitomycin C. It is true that major attention is paid to the effects of administering materials by routes more relevant to actual human exposure (ingestion, inhalation and skin contact) and that the reader is referred to an introductory comment questioning the validity of the subcutaneous sarcoma as an index of carcinogenicity, but no assessment of the significance of such data appears in the individual monographs. Similarly, no attempt has been made to distinguish, in terms of relative risk to man, between compounds that induce tumours (particularly of the liver) only when administered in very high doses (e.g. coumarin) and others, like aflatoxin, that are tumorigenic in doses of only a few micrograms.

These points illustrate what some would consider to be a major deficiency in this series. The monographs collect together from scattered sources a great deal of information of considerable value to toxicologists and others faced with the task of assessing substances in terms of their carcinogenic risk to man. In this way they perform a very useful function, but for the most part the experts involved in their compilation give little guidance on the significance of the data presented. The user of these monographs must not be misled by the title of the series, as he will have to depend upon his own judgement as far as any evaluation of probable human carcinogenicity is concerned.

Report of the Government Chemist 1975. Department of Industry: Laboratory of the Government Chemist. HMSO, London, 1976. pp. iv + 158. £2.10.

The Laboratory has for many years been involved in sampling foodstuffs for pesticide residues, and periodically this activity has been extended to a calculation of pesticide intakes in the total daily diet. The last comprehensive survey in 1970–71 showed that levels of dieldrin and DDT and its degradation products had decreased since the first survey in 1966–67, whereas γ-BHC had remained relatively constant and malathion had increased markedly (*Cited in F.C.T.* 1974, **12,** 747). A third survey conducted during 1974–75 has now revealed a dramatic fall in the levels of malathion, to below even that recorded in the initial survey, accompanied by further slight declines in the levels of organochlorines (including γ-BHC). The average daily intakes calculated were 4·4 μg for γ-BHC, 1·9 μg for dieldrin, 12 μg for DDT, TDE and DDE combined and 7 μg for malathion, the last contrasting with a peak of 53 μg during 1970–71. In all cases, levels were only a fraction of the ADIs allocated by FAO/WHO.

*Available in the UK from HMSO.

Other work on pesticides during the year included the development of analytical methods for dithiocarbamates and their degradation products, including ethylene thiourea, which may possibly be limited in the future in specifications for formulated products. In connexion with the fact that chlorophenol fungicides in wood shavings used as litter may give rise to taint in broiler chickens, the Laboratory has been investigating techniques of extraction and clean-up and limits of detection for chlorophenols in poultry meat. This investigation was requested by a subgroup of the Steering Group on Food Surveillance, set up to report on the "effect on wholesomeness of food of the use of wood litter in poultry management".

In the realm of animal feeding stuffs, the Laboratory has been engaged in the development of methods for the determination of goitrogenic thioglucoside derivatives, to provide substitutes for the somewhat unsatisfactory method at present prescribed in the relevant EEC directive. A method for the determination of uric acid has been developed and forwarded to MAFF for possible inclusion in the Fertilizer and Feeding Stuffs Regulations, should it be decided that the use of dried poultry waste in feeds should be controlled. The Laboratory has also participated in a collaborative study of the determination of copper, iron, manganese and zinc in feeding stuffs by atomic absorption spectrophotometry, as a result of which the method used was deemed acceptable for publication.

In human foods the range of trace elements monitored under the total diet study was extended in 1975 to include arsenic, copper, iron, manganese, nickel and zinc. Representative dietary items were also analysed for arsenic on behalf of the MAFF Working Party on the Monitoring of Foodstuffs for Heavy Metals, a survey which should provide information useful for the intended review of the Arsenic in Food Regulations 1959. Work on the revision of the nutritional tables for *The Composition of Foods* reached an advanced stage, and a new edition should be published in 1977. Although analysis of dry tea leaves has suggested that tea may be a rich source of nutrient metals, a study of tea infusions revealed that a major part of the metal content does not pass into the brew. In a representative sample more than 95% of the iron content was found to be retained by the leaf, together with 85% of the copper, 70% of the manganese, 65% of the zinc and 55% of the nickel, while on the benefit side the toxic metals cadmium and lead were retained to the extent of 65 and 45%, respectively.

Routine examination of drinking-water brought to light several cases where the WHO limits for lead and copper were exceeded, and one case in which the cadmium concentration, at 0·02 mg/litre, was twice the WHO limit, necessitating corrective action. Lead (28–33 mg/litre) released by two silver-plated tankards subjected to the usual extraction test with 4% acetic acid was apparently the result of extraction from solder through the nickel–silver plating. However, new pewter articles conforming to BS 5140:1974 yielded insignificant amounts of soluble lead. The Laboratory also collaborated in developing methods of sample preparation for inclusion in a proposed European standard on the safety of toys, which will extend the toxic-metal limits now specified for paints to other materials such as plastics and printed paper and card.

Following complaints of discomfort to the eyes and nose in a Department of the Environment building where textiles treated with urea-formaldehyde resin were stored, tests were conducted for atmospheric formaldehyde, but all levels were well below the TLV of 2 ppm. A similar complaint that eye irritation had resulted from the use of a foam-backed ironing-board cover led to the setting up of an experiment in which the air was analysed continuously for hourly periods above the covered ironing board, on which a mechanically-driven iron was made to function in a closed compartment. Concentrations of toluene diisocyanate were found to reach a maximum of one third of the TLV, and it was concluded that in a particularly sensitive person this could have produced the reported symptoms.

After the tragic death from cyanide poisoning of a small child, who had thrown a teddy bear filled with acrylic fibre on to an electric fire, it was found that even at 200°C the fibre could yield up to 5 mg HCN/g and at 300° up to ten times this amount could be produced. As alternative fillings such as polyurethane crumb are more flammable and can also yield toxic fumes, further research into the problem is now in progress at the Fire Research Station.

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BOOK REVIEWS

Methyl Chloroform and Trichloroethylene in the Environment. By D. M. Aviado, S. Zakhari, J. Simaan and A. G. Ulsamer. CRC Press, Cleveland, Ohio, 1976. pp. xv + 89. $19.95 (outside USA add $2.00 per copy).

The number of organic solvents available to industry, and the nature and extent to which they are used, have increased greatly in recent years. A systematic assessment of the safety of these materials requires an understanding of the pharmacological and toxicological response obtained not only in laboratory animals but from controlled or accidental human exposure. The integration of such data is no simple task and the advent of the series *Solvents in the Environment* is therefore to be welcomed.

The choice of methylchloroform (1,1,1-trichloroethane) and trichloroethylene as subjects for the first monograph is apposite, for they are present in a range of household products. The format, which is to be used in subsequent volumes, is novel and effective, combining a summary of the literature with the results of original investigations conducted by the senior author and his colleagues into the cardiotoxicity of these compounds. The research chapters contain much detailed information, while the review sections are likely to prove of only limited value to those who are not familiar with the subject or who do not have ready access to alternative reference books. This disadvantage could have been avoided had a more critical approach been adopted by the individual authors.

Unfortunately this volume contains many errors, some trivial, some humorous, but all irritating and likely to confuse the unwary. It is to be hoped that the success of this new venture will not be prejudiced by such inattention to detail. These defects apart, the monograph should prove valuable to those who have had to rely over the years on van Oettingen, Patty and Ethel Browning.

Progress in Drug Metabolism. Vol. 1. Edited by J. W. Bridges and L. F. Chasseaud. John Wiley & Sons Ltd., Chichester, Sussex, 1976. pp. xiii + 286. £9.80.

This book is the first volume of a new series intended to provide critical reviews in various areas of xenobiotic metabolism. A somewhat similar series entitled *Foreign Compound Metabolism in Mammals* (published by the Chemical Society) is already in existence, but while the Chemical Society series (*Cited in F.C.T.* 1972, **10,** 693; *ibid* 1973, **11,** 1116; *ibid* 1976, **14,** 52) is designed to provide an extensive coverage of the literature on xenobiotic metabolism, with each volume covering a 2-year period, the aim of *Progress in Drug Metabolism* is to provide more readable reviews in distinct subject areas. The present volume consists of five chapters, each with its own reference list. In addition, both author and subject indexes are provided for the entire work.

The first chapter, by B. J. Millard, deals with the use of mass spectrometry in the identification of xenobiotics and their metabolites. The basic principles of this important technique are considered, together with many examples of its application in metabolic studies. The second chapter, by T. A. Connors, considers the use of cytotoxic agents in cancer chemotherapy. These agents are designed to be activated *in vivo* to form highly toxic metabolites which, for a number of reasons, are more toxic to tumour cells than to most other types of cell. In recent years it has been discovered that a wide variety of xenobiotics, including some therapeutic agents, may be metabolized to epoxide compounds, and the formation of epoxides, some of which have either mutagenic or carcinogenic properties, is discussed in Chapter 3 by R. C. Garner.

Chapter 4, by J. Hunter and L. F. Chasseaud, is concerned with the clinical aspects of the induction of xenobiotic-metabolizing enzymes in man. Compounds known either to induce or to inhibit the human metabolism of foreign compounds are listed, and several examples are considered in detail. Attention is also devoted to the problems of assessing the levels of xenobiotic metabolism in the human liver by current methods which rely on drug half-lives in the plasma, needle-biopsy studies and urinary excretion of such compounds as D-glucaric acid or 6β-hydroxycortisol. As prolonged microsomal-enzyme induction is known to affect the metabolism of endogenous substances such as vitamin D, folic acid and various steroids, this review is clearly of great interest.

The last chapter, by J. W. Bridges and A. G. E. Wilson, deals with the interaction of drugs with serum proteins. Such interactions are extremely important in determining the absorption, distribution and excretion characteristics and the biological activity of drugs. The authors review both the principles involved in the binding of foreign compounds to protein and the methods available for studying such interaction. As albumin is the major serum protein involved, the structure and nature of the binding sites of this protein are reviewed. Finally, the authors consider the clinical implication of plasma-protein binding.

Thus the editors have assembled, in this one volume, five excellent reviews of different areas of xenobiotic metabolism. Each review provides adequate theoretical background, considers recent literature and, where appropriate, evaluates experimental techniques. In addition, the author and subject indexes are excellent, and it is to be hoped that these will be of a cumulative nature in subsequent volumes. In a few words, this book can be very highly recommended.

The Immune System: A Course on the Molecular and Cellular Basis of Immunity. By M. J. Hobart and I. McConnell. Blackwell Scientific Publications, Oxford, 1975. pp. xxiii + 357. £5.

There is no doubt that toxicologists, whether operating in political, contractual or experimental fields, should be aware of the immunological implications involved in any day-to-day toxicological exercise. Unfortunately, no currently available book presents immunology in a form likely to be directly beneficial to toxicologists in general, but *The Immune System* goes some way towards filling this gap.

The book is based on a course of lectures given at the Hammersmith Hospital to advanced students in immunology and consists of four major sections, each of several chapters, on immunochemistry, immunobiology, immunogenetics and immunopathology. Each chapter covers one aspect of the subject and offers a comprehensive résumé, including crucial experimental data, in such a way that the subject matter can be assimilated by the non-expert. The book's value to the toxicologist lies in the fact that each chapter can be taken out of context and used as a source of background information without recourse to other sections. The style is chatty and not overburdened with references, and consequently the uninitiated will find the whole book very readable.

Some sections are dealt with in more depth than others, the most detailed reflecting to some extent the research interests of the Hammersmith group of immunologists. Toxicologists would perhaps have welcomed more on immunopathology, but if they take the trouble to read the section on immunobiology they will find that it adequately augments the pathology section. This book is to be highly recommended and, at £5, should not be beyond the reach of any interested individual.

Immunological Tolerance. Edited by D. W. Dresser. British Medical Bulletin, Vol. 32, no. 2. Medical Department, The British Council, 65 Davies Street, London W1Y 2AA, 1976. pp. 192. £3.

Until comparatively recently, immunological tolerance was considered to be an alternative host response to allergization and this was reflected to some extent in the choice of model systems used to investigate the phenomenon; they tended to analyse the response in isolation.

The major contribution of this volume of the British Medical Bulletin is that it presents tolerance as an integral part of the immune response and emphasizes its importance as a regulator for the magnitude of immunological reactions. This is quite an achievement, as each of the 16 chapters is written by an expert specializing in a different aspect of tolerance. Some of these chapters accurately trace the authors' experimental progress in the field and are obviously more partisan than others. But this is not a criticism; when trying to assess complex immunological relationships, it is important to know which experiments the experimenter feels are crucial for demonstrating a particular point and how much weight is put on their interpretation.

Considerable thought has clearly been given to the design of this volume. The excellent choice of chapter headings ranges from the role of tolerance in transplantation, auto-immunity and tumour rejection to the effect of tolerized subpopulations of lymphocytes in the development of the allergic response. The reader is expected, however, to have a considerable background knowledge of immunology and to be familiar with the concept of tolerance. It is not a book that could be used as an introduction to the subject and it is doubtful, therefore, whether it would be of much value to the majority of toxicologists. Nevertheless, it is a volume to be recommended.

Human Histology. An Introduction to the Study of Histopathology. By D. L. Gardner and T. C. Dodds. 3rd Ed. Churchill Livingstone, Edinburgh, 1976. pp. xi + 432. £15.50.

Modern teaching of histology tends to fall into two distinct categories—that dispensed by the traditionalist with his anecdotes, limericks and collections of drawings and notes acquired over previous decades, and that favoured by the teacher *nouveau* with boxes of colour transparencies, other visual aids and little else. In between these extremes there is a need for "a concise but fully illustrated account" of the subject. The authors of this book set out with this in mind and have produced a text ideally suited to the medical student or medical laboratory scientist and to the biologist who requires a sound basic knowledge of histology.

To appreciate and understand histology, repeated reference is necessary to text, photograph and diagram, and the arrangement used in this book allows such a procedure to be followed effectively. The text is well written and concise (possibly too concise in places). The subjects are discussed by systems, with chapters, for example, on the respiratory, digestive and urinary systems, and a particularly good one on the central and peripheral nervous systems. The volume concludes with a brief but very useful chapter on common artefacts.

As one is entitled to expect from a modern histology book, the photographs are technically magnificent and most are in colour. Numerous low-power photomicrographs of the tissues are augmented with higher powers of the individual cells. There are a few black-and-white photomicrographs (including one of the tongue duplicated on pp. 110 and 366) and a series of clearly labelled drawings.

It is unfortunate that the book gets off to a poor start. One gets the impression that the authors were anxious to complete the first chapter (on cell structure and division) in order to get to Chapter 2 (on epithelial membranes and glands) and thus to the more familiar ground of the classical histologist. This first chapter makes no mention of peroxisomes, and the other organelles receive little attention. Moreover, some statements are oversimplified and ambiguous. Glycogen is said to be soluble in aqueous fixatives, but this statement is not amplified to explain how the majority of laboratories in the country demonstrate glycogen after formalin fixation. It is implied that osmium fixation is required if fat is to be demon-

strated, whereas in practically every laboratory the Sudans or Oil Red O are in use. In the section on pigment, there is no mention at all of lipofuscin, one of the more complex pigments found in man.

Despite these faults, the authors have achieved their main objective by presenting a comprehensive and yet clear and concise description of human histology. On this count alone their book can be recommended as a constant companion to the microscope and slide.

Meyler's Side Effects of Drugs. A Survey of Unwanted Effects of Drugs Reported in 1972–1975. Vol. VIII. Edited by M. N. G. Dukes. Excerpta Medica, Amsterdam, 1975. pp. xv + 1132. Dfl. 215.00.

This book is a survey of unintended effects of drugs, and covers reports that appeared between 1972 and 1975. Any book that attempts to give information on the undesirable effects of medicines is likely to be a tome and this eighth volume (the first in the series was published in 1957) is no exception. Running to well over 1000 pages, it is a massive but well-bound book, not unduly heavy but rather expensive. Fortunately, it is extremely well organized. Its 46 chapters, each concerned with a particular functional group and written by an expert in the field, deal with an extensive range of drugs prescribed throughout the world. The book has three indexes—for locating drugs or groups of drugs, for identifying synonyms and for tracing the various side effects, the latter naturally being the most extensive.

Each chapter commences with a review of well-established facts and then analyses the more recent literature, indicating where firm conclusions may be drawn or where the data provide only pointers. Closely related drugs with similar side effects are discussed as a group. Tables are relatively few, but most of those presented are useful, serving in some cases a greater purpose than several pages of text. Good as the book is, its impact could perhaps have been sharpened by the presentation of more results in tabular form. Numerous references (usually more than 100, often over 200 and in one case over 500) conclude each chapter, and many of these references indicate whether the reader will find substantially more information by consulting the original publication. Thus it is easy to follow up any specific point of interest.

Because they are written by different authors, individual chapters vary somewhat in the amount of detail given. Even allowing for differences in the importance and degree of use of various drugs or types of drugs, the variations in this respect are sometimes surprising. It seems slightly unreasonable, for example, for about twice as many pages to be devoted to aspirin alone as are occupied with all the antihistamine drugs. While appreciating the widespread occurrence of aspirin poisoning in very many countries, one may question the need for detailed case histories of so many individuals. This is an extreme example, however, and, in the main, a far more appropriate balance is maintained.

Because this book is concerned solely with the side effects of drugs, there is little discussion of therapeutic effects. It is important for this point to be borne in mind, since in any other context this approach could be considered to give an unfairly distorted appraisal of any particular drug. Nevertheless, short of a computerized retrieval system, no more comprehensive consideration of this subject can be available, and it must, therefore, prove a most valuable book to everyone concerned with drug therapy. It is intended that, in future, the book will be kept up to date by the annual publication of current developments in all relevant fields. This arrangement will enhance the already considerable value of this series.

The Pharmacological Basis of Therapeutics. 5th Ed. Edited by L. S. Goodman and A. Gilman. Macmillan Publishing Co., Inc., London, 1975. pp. xvi + 1704. £19.50.

The first edition of this book appeared in 1941 and its fluency and cohesion soon earned it world-wide recognition. As a result of the unstinting efforts of Drs L. S. Goodman and A. Gilman, each subsequent edition has augmented this reputation, and the fifth edition, following established tradition, presents with clarity and style the most authoritative and comprehensive text on pharmacology in the English language.

This latest edition has undergone extensive updating, involving the incorporation of recent advances and the condensation or elimination of less important and outmoded areas. Although attention is focussed mainly upon the major therapeutic agents, consideration is also given to the less important congeners and to materials of limited therapeutic value. Impressive advances in pharmacokinetics have prompted a more detailed consideration of basic principles at a general level and of their specific application to individual drugs. Where appropriate, the relationships between physico-chemical factors and the distribution and fate of administered compounds are summarized in tabular form.

Throughout the book repeated reference is made to the roles played by adenylate cyclase and cyclic adenosine 3′,5′-monophosphate in the mechanism of action of an apparently ever-increasing number of drugs. A prominent example is the inhibition of dopamine activation of adenylate cyclase by antipsychotic drugs, such as chlorpromazine and haloperidol. This inhibition has been demonstrated in parts of the basal ganglia and limbic system and it is tempting to speculate that both the antipsychotic and the extrapyramidal side effects of these drugs may be the result of a common basic mechanism.

One outstanding group of new drugs included in this edition is the group of antihistamines known as the H_2 receptor antagonists. These drugs, exemplified by two imidazolylthioureas, burimamide and metiamide, competitively inhibit the gastric acid secretion evoked by histamine or pentagastrin and are of immense potential therapeutic value in peptic ulceration and gastric hypersecretory states.

The prostaglandins, which currently command widespread interest in biology in view of the multiplicity of their actions, make their appearance in the sections on autacoids and on drugs affecting uterine motility. In the latter context, prostaglandins E and

F are discussed with reference to their potential use as abortifacients and oxytocic agents at term. Similarly the hypothalamic releasing factors are discussed in terms of both their potential therapeutic value and the mechanisms by which other drugs may act.

The above examples illustrate some of the many new topics discussed in this edition, which maintains the high standards previously established. Although the unity and objectives embodied in this volume must be credited to the Editors and the two new Associate Editors, Drs A. G. Gilman and G. B. Koelle, a large measure of the continuing success of this book stems from the erudite contributions of the 43 eminent authors.

Scientific Analysis on the Pocket Calculator. By J. M. Smith. John Wiley & Sons Ltd., London, 1975. pp. xii + 380. £8.30.

It has not been too easy keeping up with Jones during the solid-state revolution of the past few years. His pocket calculators have more and more keys and perform ever more sophisticated calculations. The pocket calculator you had last Christmas with its square, square root and reciprocal functions, which impressed everybody so much at the time, is already outclassed. Jones has just shown everybody his new one, which has in addition *e*, logs, trig functions and parentheses—and all for the price of your earlier model.

In direct confrontation with Jones one does not have much of a chance. There is, however, one simple act of oneupmanship which is open to anybody. It is to be able to use the keys one has—to know what they are for and to have a use for them. This most effective ploy is not difficult to develop and offers incidental advantages to the user.

The book named above explains it all in simple language. The author describes the use of the four-function ($+ \; - \; \times \; \div$) calculator as well as the more advanced types, and shows how the different models work through the same calculation. He explains the differences between fixed and floating decimal points and between algebraic ($A + B \div C$) and reverse polish ($A \uparrow B + C \div$) instructions, or language. He points out that the addition of functions to the pocket calculator keyboard reduces the number of key strokes associated with data inputs. Beyond four functions, the addition of square root, reciprocal and square instructions adds entirely new capabilities to the calculator instruction set. Storage capacity also reduces the number of key strokes, and programming reduces them still further.

This book is not likely to be outdated by increasingly sophisticated calculators as they stream into the shops, although in some ways it is perhaps already outdated. The author gives a method for computing roots on the simplest four-function calculator, by Newton's method. This is interesting, but somewhat academic, since anyone involved in practice with *n*th roots could easily acquire both a calculator with the instant function and a facility for using it.

There are useful sections on difference tables, useful for fitting curves and for the most part ignored by biologists, and on Fourier analysis, essential for assessing cyclical data. Quoted from Hamming is a 12-point formula for Fourier analysis for use on four-function calculators. This again is academic, however. In practice, it would be too complicated and open to error.

Chapter 10 on 'Statistics and Probability' is an excellent introduction to the subject, although the author says that he wishes only "to impart a working knowledge of statistical analysis on the pocket calculator, rather than a theoretical knowledge of the field of statistics and probability". Definitions, examples, significance tests and their interpretation are all clearly presented in simple, non-technical language, along with a discussion on the choice of 1- and 2-tailed tests and degrees of freedom, in a survey which takes less than 40 pages. Some people will probably find the book valuable for this chapter alone.

Professor Smith writes easily, as one well accustomed to discussing technical and mathematical niceties with people who know little of the background. He has produced an approachable book, which can help anyone who owns a pocket calculator to get the best out of it. And by way of light relief he invites the reader to contemplate $57738{\cdot}57734 \times 10^{40}$ upside down on the calculator display.

BOOKS RECEIVED FOR REVIEW

Health Hazards from New Environmental Pollutants. Report of a WHO Study Group. Technical Report Series no. 586. World Health Organization, Geneva, 1976. pp. 96. Sw.fr. 8.00 (available in UK from HMSO).

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

Manual on Urban Air Quality Management. WHO Regional Publications, European Series No. 1. Edited by M. J. Suess and S. R. Craxford. World Health Organization, Copenhagen, 1976. pp. 200. Sw.fr. 36.00 (available in UK from HMSO).

Residue Reviews. Residues of Pesticides and Other Contaminants in the Total Environment. Vol. 61. Edited by F. A. Gunther. Springer-Verlag, Berlin, 1976. pp. viii + 166. DM 41.00.

Use of Isolated Liver Cells and Kidney Tubules in Metabolic Studies. Edited by J. M. Tager, H. D. Soling and J. R. Williamson. North-Holland Publishing Company, Amsterdam, 1976. pp. xix + 476. $37.50.

Selected Methods of Measuring Air Pollutants. WHO Offset Publication No. 24. Published under the joint sponsorship of the United Nations Environment Programme and the World Health Organization, Geneva, 1976. pp. ix + 112. Sw.fr. 20.00 (available in UK from HMSO).

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

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

Fd Cosmet. Toxicol. Vol. 15, pp. 243–249. Pergamon Press 1977. Printed in Great Britain

Information Section

ARTICLES OF GENERAL INTEREST

CARBON DISULPHIDE BROUGHT UP TO DATE

Exposure to carbon disulphide (CS_2) vapour continues to present a hazard to workers in certain industrial processes and particularly to those involved in the production of viscose rayon. Since our last review (Cooper, *Fd Cosmet. Toxicol.* 1976, **14,** 57), several aspects of CS_2 toxicity have been studied further, and we now report the results of investigations on the metabolism of CS_2 by the mixed-function oxidase system, a follow-up to the cohort study on 343 viscose-rayon workers (*Cited in F.C.T.* 1971, **9,** 599) and a proposed method for the early diagnosis of CS_2 poisoning.

Effect on hepatic microsomal enzymes

In a systematic study of the effect of ^{35}S-labelled CS_2 and carbonyl sulphide (COS) on microsomal-enzyme preparations, Dalvi *et al.* (*Chemico-Biol. Interactions* 1975, **10,** 347) confirmed their earlier suggestion (*Cited in F.C.T.* 1976, **14,** 58) that CS_2 might be metabolized to COS with the release of a reactive form of sulphur which could bind to the microsomal membrane. They demonstrated a simultaneous CS_2-induced decrease, occurring only in the presence of NADPH, in both the rate of benzphetamine metabolism and the concentration of cytochrome *P*-450 detectable as its carbon monoxide complex. The degree of each inhibition correlated with the amount of COS formed and with the amount of sulphur bound, from which the authors deduced that COS or the reactive form of sulphur were the most likely inhibitors.

In an attempt to determine which was the active species, Dalvi *et al.* (*loc. cit.*) incubated COS with hepatic microsomes and observed qualitatively similar inhibitions to those with CS_2. Quantitatively, however, the inhibitions were less than those demonstrated with CS_2, despite COS concentrations up to 2000 times as great as those of CS_2. Thus, it appeared that the reactive sulphur, and not COS, was the direct cause of the inhibition. The sulphur observed to bind to the microsomes under these conditions probably originated from the metabolism of COS to CO_2, since CS_2 is known to be metabolized to CO_2 *in vivo*. Inhibition of the mixed-function oxidase system with SKF-525A gave some protection against the decrease in cytochrome *P*-450 *in vivo*, and it was shown that *in vitro* this compound reduced the amount of sulphur bound to microsomes, supporting the identification of sulphur as the inhibiting moiety.

The decrease in cytochrome *P*-450 concentration was not accompanied by a decrease in the level of protohaem associated with the microsomes, suggesting that the binding of sulphur interfered with the ability of the haem group to complex with carbon monoxide. It is possible that the sulphur either prevents the reduction of the haem iron of cytochrome *P*-450, so that carbon monoxide can no longer complex, or interferes (perhaps sterically) with the approach of carbon monoxide to the reduced haem iron. Either mechanism would also have to explain the observation that 9 mol sulphur inactivated only 1 mol cytochrome *P*-450 from complexing with carbon monoxide, but for an elucidation of this mechanism we must await the results of further studies.

In contrast to this report, Kromer & Freundt (*Arzneimittel-Forsch.* 1976, **26,** 189) suggested that CS_2 itself acts as the inhibitor. They studied the kinetics of CS_2 inhibition of oxidative aminopyrine *N*-demethylase by measuring the formaldehyde obtained in rat-liver microsomes in the presence of NADPH. The pattern of inhibition obtained *in vitro* was identical to that in rats exposed to 20–400 ppm CS_2 for 8 hours, indicating that the inhibitory process was based on the same molecular mechanism *in vivo* and *in vitro*. From their kinetic data, these authors determined that CS_2 attacks at two different sites of the enzyme molecule; binding at the first site was followed by inhibition until saturation of the site occurred, when a second site was occupied, resulting in reactivation and, overall, activation. CS_2 showed a high affinity for the first site, at which binding was reversible, and a low affinity for the second, but neither site was the active centre of the enzyme molecule. The CS_2 added to the microsomes could be expelled by helium gas, such treatment resulting in a return to normal enzyme activity, and it was concluded from this that CS_2 itself, and not a metabolite, was acting as the inhibitor.

The rat, although one of the commonest species of experimental animal, is not necessarily the ideal animal from which to extrapolate data to man (*Cited in F.C.T.* 1977, **15,** 70) and it is therefore of interest to compare results obtained in rats with those obtained in other animals. Gopinath & Ford (*J. comp. Path. Ther.* 1976, **86,** 251) studied the effect of CS_2 on the activity of hepatic microsomal aminopyrine *N*-demethylase in normal and phenobarbitone-pretreated calves and ponies. Pretreatment with phenobarbitone produced a marked increase in demethylase activity which, in the absence of other treatment, gradually fell to the predosing level over some 16 days after cessation of phenobarbitone administration. Without this pretreatment, no demethylase activity could be detected. On administration of approximately 0·1 ml CS_2/kg by stomach tube after phenobarbitone pretreatment, there was a sudden fall in the level of demethylase activity, and it was suggested that the reported protective effect of CS_2 against various halogenated hydrocarbons in sheep, rats and

horses is related to this inhibition, since the potentiation of the hepatotoxicity of such compounds by microsomal enzymes is well known.

No significant hepatotoxic effect was seen in calves or ponies given CS_2 alone, but in those pretreated with phenobarbitone, liver-specific enzymes were released into the plasma, and centrilobular liver-cell necrosis was observed, with ballooning of the central and midzonal cells, fatty changes and loss of glycogen and of intracellular enzyme activity. The pretreated calves also developed bilirubinaemia and clinical jaundice. Gopinath & Ford (*loc. cit.*) suggest that, although CS_2 inhibits demethylase activity, it does not completely prevent the stimulated microsomal enzymes from increasing the rate of the CS_2 metabolism and producing toxic metabolites.

A further aspect of CS_2 metabolism and enzyme inhibition arises from the work of Freundt *et al.* (*Int. Archs occup. environ. Hlth* 1976, **37,** 35) on the effect of CS_2 on alcohol metabolism. Rats subjected to a single 8-hour exposure to 20 or 400 ppm CS_2 or to twelve 8-hour exposures to 400 ppm at 40-hour intervals were given 2 g ethanol/kg ip and were then left in the CS_2 atmosphere for up to 4 hours. The concentration of ethanol in their blood decreased linearly and at the same rate as in controls not exposed to CS_2, whereas the concentration of acetaldehyde rose to 50% above the control values, thereafter falling only slightly. The half-life of acetaldehyde in the blood of rats injected with 1 mmol/kg after an 8-hour exposure to 400 ppm CS_2 was 144 seconds compared with 105 seconds in controls.

Humans exposed to 20 or 40 ppm CS_2, while ingesting ethanol to give a constant blood level of 75 mg/100 ml, had acetaldehyde levels in their blood up to 50% above those of controls not exposed to CS_2, and a slight additional increase was demonstrated in those exposed to 80 ppm CS_2. A similar elevation was also observed when alcohol was ingested during the last of five 8-hour exposures to 20 ppm CS_2 and 16 hours after an 8-hour exposure to 20 ppm CS_2. At no time did blood alcohol levels differ from those of controls.

Freundt *et al.* (*loc. cit.*) argue that CS_2 has a half-life of only some 40 minutes in the body, and thus it would not be expected to persist after 16–24 hours. CS_2 metabolites of the thiocarbamate type, however, would still be present and could cause the observed inhibition of aldehyde dehydrogenase, since many of their structurally related products are known to do so. The authors conclude that despite elevated acetaldehyde levels, subjects with up to 80 mg ethanol/100 ml blood are unlikely to suffer any CS_2–alcohol intolerance, provided exposure to CS_2 does not exceed the generally accepted maximum allowable concentrations of 10–20 ppm.

Epidemiological study continued

Some years ago (*Cited in F.C.T.* 1971, **9,** 599) we reported the results of an epidemiological survey of 343 workers in a viscose-rayon plant. This study, which supported earlier data relating the incidence of coronary heart disease to prolonged exposure to CS_2, involved workers exposed to CS_2 for at least 5 years between 1942 and 1967 and has now been extended over the years 1967–1972 by Tolonen *et al.* (*Br. J. ind. Med.* 1975, **32,** 1). During these 5 years, fourteen men had died from coronary heart disease in the exposed group as against three in the matched-control group, while other causes of death were evenly distributed. In addition, 11 non-fatal first infarctions had occurred in the exposed group compared with four in the controls. Nearly 25% of the exposed men, as against 13% of the controls, had a history of angina. The relative risks were 4·8 for fatal attacks, 3·7 for all infarctions, 2·8 for non-fatal infarctions, 2·2 for angina and 1·4 for electrocardiographic findings indicative of coronary heart disease, implying that the causal role of CS_2 in developing coronary heart diseases becomes more evident with increasing severity and specificity of the manifestations. Tolonen *et al.* (*loc. cit.*) consider that the cause–effect relationship between CS_2 and coronary heart disease is well established and that more knowledge of the exposure–response relationship is now urgently needed so that a no-effect level can be defined.

Early diagnosis of poisoning

Of obvious importance is the ability to detect exposure to CS_2 before the later, sometimes irreversible, lesions occur. Cavalleri (*Archs envir. Hlth* 1975, **30,** 85) showed that reductions in serum thyroxine values correlated statistically with duration of CS_2 exposure, a perceptible decline beginning after a few years of exposure. Values for exposed subjects who showed definite changes in the retinal vessels were significantly lower than those in subjects with electromyographic signs of peripheral neuropathy or with no changes in the retinal vessels, emphasizing that ophthalmoscopic examination, although a more specific test, will only show positive results after prolonged periods of exposure.

In contrast, measurements of serum lipids were found to be a poor diagnostic sign, although a correlation was noted between the decrease in thyroxine and the increase in cholesterol. Cavalleri (*loc. cit.*) concluded from this that the development of vasculopathy was independent of that of neuropathy, that the two effects occur through different pathogenic mechanisms and that hypothyroidism, even if subclinical, may be responsible for the metabolic alterations that lead to CS_2 vasculopathy.

[H.R. Potter—BIBRA]

CHLOROMETHYL METHYL ETHER IN THE AIR

The respiratory toxicity of chloromethyl methyl ether (CMME) and a clear relationship between levels of exposure to this compound and the development of chronic cough, excessive expectoration and lung cancer have been documented (*Cited in F.C.T.* 1976, **14,** 650). When it is considered that both CMME and its industrial contaminant bis-chloromethyl ether (BCME) break down in the presence of water to form

formaldehyde and hydrochloric acid, while CMME yields methanol as well, effects of this kind are perhaps hardly surprising. Nevertheless it would be difficult to relate all the observed tumorigenic effects of these compounds to the direct action of their breakdown products.

Epidemiology

An epidemiological survey carried out among workers in a Philadelphia chemical plant some years ago indicated that there was an increased overall mortality among workers aged 55 and older, with a significantly raised incidence of lung cancer (De Fonso & Kelton, *Archs envir. Hlth* 1976, **31,** 125). The most likely culprit was thought to be CMME, but the CMME used at the plant contained 0·5–4% BCME and it was found impossible to distinguish between the two as far as exposure was concerned. In groups of men exposed during 1948–72 there were 19 observed deaths from malignant neoplasms of the trachea, bronchus and lung, compared with an expected incidence of 5·6, giving a relative risk of 3·8. The interval between the first known exposure to CMME and death ranged from 8·3 to 25·2 years for men who had first been exposed while in their twenties.

Animal studies on CMME and BCME

In a study of the inhalation toxicity of CMME and BCME, rats and hamsters were exposed to varying concentrations of CMME for 7 hours (Drew *et al. ibid* 1975, **30,** 61). The 14-day LC_{50} was 55 ppm for rats and 65 ppm for hamsters. The 14-day LC_{50} for BCME was 7 ppm in both species. The results of exposure were similar for both ethers, with congested, oedematous and haemorrhagic lungs and acute necrotizing bronchitis. Rats exposed to 10 ppm CMME for 6 hours daily for up to 30 days showed a mortality rate of 88%, the first deaths occurring on day 3 of exposure. During week 1 of CMME exposure, body weight was markedly reduced, but there was some indication of recovery towards the end of the exposure period. All the animals that died showed a high ratio of lung weight to body weight. In ten of the 25 animals there was hyperplasia of the bronchial epithelium, and one of the group developed squamous metaplasia. Only two rats (8%) exposed to 1% CMME died, and lung changes were slight after termination of exposure. In 13 animals retained for observation until their natural death, only a few showed mucosal changes (regenerative hyperplasia in two and squamous metaplasia also in two, in one case in the bronchial epithelium and in the other in the trachea).

A single 7-hour exposure to 2·1 ppm BCME reduced the median life span of rats to 36 days and of hamsters to 68 days, and caused severe weight loss (Drew *et al. loc. cit.*). This degree of exposure raised the incidence of tracheal hyperplasia threefold in rats and four- or fivefold in hamsters, and increased the incidence of bronchial hyperplasia in both species. In rats it also more than doubled the incidence of squamous metaplasia of the bronchial epithelium. In one rat dying at 15 days, hyperplasia and advanced squamous metaplasia extended into the alveoli, while in another dying at 219 days, advanced squamous metaplasia was associated with hyperkeratosis. The median survival time during daily exposure to 0·7 ppm BCME was 420 days for rats (controls 462 days) and 657 days for hamsters (controls 675 days). Pathological changes of the respiratory tract in these chronically exposed animals were mainly non-specific, but tracheal epithelial hyperplasia in rats increased from 36 to 67% and pneumonitis in hamsters from 23 to 67%. A dose-dependent increase in mortality in both species followed exposure to 1 ppm BCME on three or more successive days. Tracheal changes in rats appeared to depend on the cumulative dose, with a rise in hyperplasia incidence from 27% for one exposure to 89% for 30 exposures. The peak incidence of tracheal hyperplasia in hamsters was reached after ten doses. Some animals of both species exposed to 10–30 doses of BCME developed extreme irritability, which was associated with the appearance of microscopic subarachnoid haemorrhage. One rat dying at 570 days developed a squamous-cell skin carcinoma after only three exposures to 1 ppm BCME, while one hamster dying at 1000 days developed a malignant nasal tumour after one exposure to the same concentration.

In a further study of CMME by the same investigators (Laskin *et al. ibid* 1975, **30,** 70), no significant effect on mortality or weight gain appeared in rats and hamsters exposed to 1 ppm CMME for 6 hours daily on '5 days/week for life. One of 74 rats so exposed developed a squamous-cell carcinoma of the lung at 700 days and another developed an aesthesioneuroepithelioma of the olfactory epithelium, invading the forebrain at 790 days. Of 90 hamsters, one killed at 134 days after 90 exposures had a lung adenocarcinoma and another died at 683 days with a tracheal squamous papilloma. A pituitary tumour found in a rat that died at 776 days was not thought to be connected with the experiment.

The same group (Kuschner *et al. ibid* 1975, **30,** 73) conducted studies in rats and hamsters exposed to 0·1 ppm BCME for 6 hours daily on 5 days/week either throughout life or for only 10–100 exposures in the case of some rats, which were then kept for observation without further treatment until they died. Two tumours, one nasal aesthesioneuroepithelioma appearing 383 days after the first exposure, the other a keratinizing squamous-cell carcinoma of the lung appearing at 578 days, occurred in 20 rats removed from the long-term study after 80 exposures. One undifferentiated lung carcinoma appeared in a hamster after 334 exposures. Among the 200 rats observed until death after 10–100 exposures to 0·1 ppm BCME in the second part of the study, there were 40 respiratory-tract cancers. These included 13 squamous-cell carcinomas which appeared to arise from metaplastic bronchial epithelium, extending later into the lungs, replacing pulmonary parenchyma and penetrating pulmonary veins. Tumours of the nasal cavities were usually composed of sheets and cords of cuboidal to columnar cells, replacing the normal olfactory epithelium, invading the nasal bones, and progressing into the brain. Nasal tumours were most numerous after 80 exposures, and pulmonary tumours after 100 exposures to BCME. The high incidence of aesthesioneuroepitheliomas was unexpected.

Other α-chloro ethers

Van Duuren *et al.* (*Cancer Res.* 1975, **35,** 2553) have extended the net further and studied the carcinogenic potential in mice of several other α-chloro ethers. Those studied were bis-1,2-(chloromethoxy)ethane (I), bis-1,4-(chloromethoxy)butane (II), bis-1,6-(chloromethoxy)hexane (III), bis-1,4-(chloromethoxy)-*p*-xylene (IV) and tris-1,2,3-(chloromethoxy)propane (V). In addition *trans*-1,4-dichlorobutene-2 (VI), used in the manufacture of copolymers and as an intermediate, was included in the study since it is capable of being metabolized to an epoxide with the structure of a β-chloro ether. These six compounds were tested by skin application and sc or ip injection for 502–569 days, depending on survival. Injections of 0·1 or 0·3 mg of compounds I–V, and of 0·05 mg of VI in tricaprylin were made once weekly. Skin applications of 0·3 mg or 1·0 mg of I–V in cyclohexane and of 1·0 mg of VI in acetone were made three times weekly.

I, IV and V produced significant numbers of sarcomas or squamous carcinomas at the injection sites or area of application, irrespective of the route of administration. VI failed to induce any skin tumours, and yielded only three sarcomas at subcutaneous sites and two at intraperitoneal sites among groups of 30 mice. No significant incidence of tumours arising at a distance from the application sites was observed with any of these compounds.

[P. Cooper—BIBRA]

OCHRATOXIN A AND THE KIDNEY

Ochratoxin A (OTA), a dihydro-isocoumarin derivative linked through its 7-carboxy group to L-β-phenylalanine, is a secondary metabolite of several species of Aspergillus and Penicillium. It is toxic mainly by reason of its effect on the proximal kidney tubules, and many experimental observations in animals have been reported (*Cited in F.C.T.* 1975, **13,** 282). Evidence has been presented to suggest that this nephrotoxicity results from the capacity of OTA to act as a competitive inhibitor of several mitochondrial transport processes (Meisner & Chan, *Biochemistry* 1974, **13,** 2795).

Occurrence in feedstuffs

Cereal products are liable to contamination with OTA. Making a survey of this problem, Krogh *et al.* (*Acta path. microbiol. scand. Sec. B* 1974, **82,** 301) collected samples of barley or oats from 84 farms in central and southern Sweden during the spring of 1972. Ochratoxin was extracted and estimated by thin-layer chromatography. Only seven of the 84 samples (five of barley and two of oats) contained detectable amounts of OTA, 16–410 ppb (μg/kg) in the barley samples and 29 and 77 ppb in the oats. No ochratoxin B or C or esters could be detected. There was no evident correlation beween the OTA concentration of the samples and their water content, nor between OTA concentration and impaired germination capacity.

The interaction of humidity and temperature in promoting the formation of OTA is a complex one (*Cited in F.C.T.* 1974, **12,** 797). Sansing *et al.* (*Can. J. Microbiol.* 1973, **19,** 1259) measured OTA production by *A. ochraceus* in stationary cultures containing 4% sucrose and 2% yeast extract at different temperatures and for different times. At 10, 40 or 45°C, no OTA was produced, while at 35° 0·1 mg OTA/25 ml medium resulted. Peak production occurred over 10–12 days at 25°, but the yields after these times differed little from those at 8 and 14 days (6 mg OTA compared with 4·9 mg/25 ml, respectively). At 30°, OTA production after 6–8 days was close to the peak production attained at 25° but it fell rapidly thereafter. Maximal OTA production was always associated with a rise in the pH of the medium. Maximal production of mycelium occurred by day 6 in cultures maintained at 20 or 25°, and growth was negligible at 40 or 45°.

Endemic nephropathy

There are striking similarities between the characteristics of endemic (Balkan) nephropathy in man and the porcine nephropathy induced by feeding OTA. Krogh & Elling (*Lancet* 1976, **II,** 40) have pointed out that most pigs and poultry exposed to OTA in mouldy feed carry the heat-stable toxin in their meat. In one Yugoslavian village where nephropathy in man is endemic, it was found that 10–20% of home-grown cereals and home-raised pork contained significant amounts of OTA. Moreover, in three Balkan countries, a highly significant correlation was apparent between the excess of rainfall over evaporation during harvest and the early period of storage and the number of deaths from endemic nephropathy during the following 2 years (Krogh & Elling *loc. cit.*). Clearly this possible connexion between endemic nephropathy and OTA deserves further study and a start has been made in Zagreb, where total-diet studies are being used to determine levels of total exposure of village populations in endemic areas, and in Copenhagen where immunofluorescence is being used in attempts to demonstrate OTA in the kidneys of patients with this form of nephropathy.

Pigs fed OTA in the feed at levels of 4, 1 or 0·2 ppm for up to 4 months developed only one observable lesion—a form of kidney damage identical with that seen in naturally occurring porcine nephropathy (Krogh *et al. Acta path. microbiol. scand. Sec. A* 1974, suppl. 246). A dose-related impairment of proximal tubular function was indicated by a decrease in maximal tubular excretion of *p*-aminohippurate and in the ratio of this to inulin clearance, a decrease in urine-concentrating ability and an increase in the excretion of glucose, leucine aminopeptidase and proteins. Microscopically, tubule degeneration, interstitial formation of connective tissue and, at later stages, the development of atrophied and sclerotized glomerular tufts were observed. In connexion with the

problem of endemic nephropathy mentioned above, it is of interest that the kidney, liver, fat and muscle of the animals contained OTA in concentrations that could constitute a public health problem.

Ochratoxicosis has been produced in beagle dogs by daily oral doses of 0·3 mg OTA/kg (Szczech *et al. Vet. Path.* 1974, **11,** 385). The animals were moribund by days 11–15 and were then killed for examination. Major morphological alterations were seen in the epithelial cells of the proximal convoluted tubules. These cells contained lipid droplets and cytoplasmic phospholipid accumulations. Large apical vacuoles were present below the brush border in some cells. Hypertrophy of the smooth endoplasmic reticulum was apparent. Tubules were separated and interstitial cells showed an increase in secretory activity. It was concluded that the primary effect of OTA was on the endomembrane system of the renal tubular cells.

In rats, oral administration of 5 or 15 mg OTA/kg/day for 3 days reduced the uptake of PAH by renal cortical slices prepared from animals killed 24 hr after receiving the final dose of OTA (Suzuki *et al. Toxic. appl. Pharmac.* 1975, **34,** 479). After treatment with the lower dose of OTA, intact rats showed a depression of PAH clearance and inulin clearance, particularly the former. Electron-microscopic examination showed that there was degeneration of the convoluted tubules. but the glomeruli retained a normal appearance. Tubular malfunction was possibly due, at least in part, to thickening of the basement membrane.

In broiler chicks, growth was inhibited by OTA fed at levels of 2–8 ppm in the feed from day 1 to wk 3 after hatching (Huff *et al. Appl. Microbiol.* 1975, **30,** 48). At dietary levels of 1 ppm or more, OTA caused kidney enlargement, while 4 and 8 ppm reduced renal function by 15 and 31% and increased urate excretion by 38 and 48%, respectively, compared with controls. Potassium was the only plasma electrolyte to be significantly decreased by these doses of OTA. Kidney sections from treated chicks showed swollen tubular epithelial cells. Changes in the proximal tubules were more marked than those in distal tubules and included some generalized necrosis. Renal function was also studied in hens given feed containing 0·3 or 1·0 ppm OTA for 49 weeks, or 1·0 ppm for only 2 weeks (Svendsen & Skadhauge, *Acta pharmac. tox.* 1976, **38,** 186). Plasma osmolality was not significantly affected, but plasma-protein concentrations were reduced by up to 17% in birds receiving the higher level. The glomerular filtration rates fell by 12 and 28% in the first two groups, respectively, and by 8% in the third. Renal concentrating ability after a lysine vasopressin injection was about 15% lower in the OTA-treated birds than in controls, irrespective of dose, and OTA treatment reduced tubular excretion of PAH at high plasma concentrations (50–90 mg/100 ml) but not at lower levels.

Elling *et al.* (*Acta path. microbiol. scand. Sec. A* 1975, **83,** 739) have reported that muscle taken from five of 14 birds condemned during a poultry-meat inspection because of enlarged pale kidneys was found to contain 4·3–29·2 ppb OTA. Four of these birds showed toxic nephropathy characterized by atrophy and degeneration of both proximal and distal tubules, together with interstitial fibrosis. It seems, therefore, that the chicken as well as the pig must be given serious consideration as a possible vehicle for introducing OTA into the human diet.

[P. Cooper—BIBRA]

ANTICONVULSANTS, VITAMIN D AND CALCIUM METABOLISM

Introduction

Anticonvulsant drugs, particularly phenytoin (diphenylhydantoin), have been suspected of inducing foetal malformations when taken during pregnancy (*Cited in F.C.T.* 1974, **12,** 151). Anticonvulsant therapy has been associated with disturbances of metabolism, especially folate metabolism and the intake and utilization of calcium, but observations are clouded by the tendency of patients with epilepsy to suffer from metabolic abnormalities that may not be related directly to the drug therapy.

Anticonvulsant-associated osteomalacia

Latham *et al.* (*J. clin. Pharmac.* 1973, **13,** 337) reported a study of 237 epileptic patients who were receiving long-term treatment with up to four anticonvulsant drugs for major seizures. The most frequently used drugs were phenytoin, phenobarbitone, primidone and pheneturide. Subnormal serum calcium levels were detected in 24% of those taking phenytoin, phenobarbitone or primidone and in 38% taking pheneturide. A broad metabolic disturbance was indicated by a decrease in both serum folate and serum calcium in these patients. Moreover, in 53 patients, the D-glucaric acid output in 24-hour urine samples was between 38 and 770 μmol whereas in non-epileptic controls taking no anticonvulsants the output was less than 25 μmol/day. The highest urinary excretion of D-glucaric acid occurred in patients treated with pheneturide. Since increased D-glucaric acid excretion is accepted as an indication of liver-enzyme induction, it is reasonable to assume that anticonvulsant-induced osteomalacia may be the result of disturbed calcium and vitamin D metabolism, secondary to increased liver-enzyme activity.

In discussing the case of a young woman who developed osteomalacia after treatment with 200–300 mg phenytoin/day for 8 years, Varkey *et al.* (*J. neurol. Sci.* 1973, **19,** 287) reported that the patient improved steadily after phenytoin was replaced by carbamazepine and oral supplements of calcium and vitamin D were administered. These investigators concluded that there were two possible mechanisms for the osteomalacia: liver enzyme induction by the phenytoin, or a specific calcium absorption defect affecting the intestinal mucosa. A study of intestinal absorption of calcium, using a ^{47}Ca label, was carried out in nine epileptic subjects and 12 healthy controls by

Caspary *et al.* (*Hormone metab. Res.* 1975, **7**, 271). Seven of the epileptics were taking phenytoin, six were taking phenobarbitone plus methylphenobarbitone, two were taking primidone, and all were recognized as having defective calcium absorption. Serum levels of ^{47}Ca were measured 30–240 minutes after a single oral dose of $^{47}CaCl_2$, and the measurements were repeated after daily administration of 50 μg 25-hydroxycholecalciferol (a vitamin D metabolite) for 3 weeks and again after 25 μg cholecalciferol (vitamin D_3) had been given daily for 3–4 months. In all the patients given anticonvulsants, there was a marked depression of calcium absorption. This was restored to normal in all patients treated with 25-hydroxycholecalciferol and in four of eight treated with cholecalciferol.

Klein & Deguia (*N.Y. St. J. Med.* 1975, **75**, 2545) examined a 12-year-old child taking phenytoin, phenobarbitone, primidone and methsuximide to control convulsions. His serum calcium level was slightly lower than normal (8·2 mg/100 ml) and his serum alkaline phosphatase greatly raised (more than 700 mIU/ml). Marked rachitic changes were seen in his wrist metaphyses. Treatment with calciferol (vitamin D_2) resolved these abnormalities without any interruption of anticonvulsant therapy. In a girl aged nearly 6 years, rickets occurred when phenobarbitone, phenytoin and primidone had been given for more than 5 years, and this condition was accompanied by decreased levels of serum calcium and phosphorus, elevated alkaline phosphatase and characteristic X-ray changes (Medlinsky, *Pediatrics, Springfield* 1974, **53**, 91). Vitamin D treatment reversed the condition, which was attributed to increased vitamin D catabolism brought about by the induction of liver microsomal-enzyme activity by the anticonvulsants.

In contrast to these findings, Livingston *et al.* (*J. Am. med. Ass.* 1973, **224**, 1634) maintained that, during a period of 36 years, some 15,000 ambulatory epileptic patients undergoing anticonvulsant treatment had shown no biochemical or X-ray abnormalities that indicated depressed absorption of calcium. They suggested, therefore, that the development of rickets in such patients was coincidental. Routine X-rays of the skull, but not of long bones, had been made. This finding was disputed by Aponte & Petrelli (*ibid* 1973, **225**, 1248), who had observed severe osteomalacia in a young woman in whom other causes (malabsorption, renal failure, aminoaciduria and hyperphosphaturia) had been rigorously excluded. The patient had maintained a normal vitamin D intake, but her serum level of 25-hydroxycholecalciferol was depressed. She had been taking methsuximide for 6 years when the osteomalacia became troublesome, and her condition improved when the drug was discontinued.

Role of vitamin D

A study of the mineral content of bone in 23 epileptic and 20 normal subjects, using photon absorptiometry of the forearms, showed a subnormal bone mass in the patients undergoing phenytoin therapy (Christiansen *et al. Br. med. J.* 1973, **2**, 208). This improved substantially after treatment with calcium lactate and vitamin D for 1 month. There was no response in healthy subjects given vitamin D or a placebo. The same investigators (*idem, ibid* 1973, **4**, 695) also studied 226 epileptic patients undergoing treatment with phenytoin, phenobarbitone or primidone or a combination of two of these, using the same technique. The mean bone mineral content of all the epileptics was initially 87% of normal, but increased by an average of 4% after 3 months of daily treatment with 2000 IU calciferol. Control patients treated with calciferol or a placebo showed no difference in the mineral content of bone. Hypocalcaemia was observed in 12% of the epileptic patients and raised serum alkaline phosphatase in 43%.

The interpretation of these observations was challenged by Rowe & Stamp (*ibid* 1974, **1**, 392), who maintained that a significant rise in total alkaline phosphatase in the plasma of adult epileptics depended on an increase in the hepatic level of the isoenzyme, and considered that, unless the mean level of alkaline phosphatase of bone origin could be shown to be elevated, the diagnosis was in doubt. Hypocalcaemia is common among epileptics without any other biochemical manifestations of oesteomalacia and may represent an effect of anticonvulsants on calcium metabolism. Rødbro & Christiansen (*ibid* 1974, **2**, 667) consider that the lack of observed histological abnormalities associated with hypocalcaemia in epileptics may reflect the insensitivity of the diagnosis of osteomalacia in bone biopsies, and comment that there is no way at present of establishing the value of vitamin D prophylaxis in those taking anticonvulsants. In a further investigation, Christiansen *et al.* (*ibid* 1974, **2**, 258) found that in 23 epileptic patients whose seizures were counted during anticonvulsant therapy before and after daily administration of 4000–16,000 IU calciferol or a placebo, the incidence of seizures was reduced in all groups taking the vitamin, although there were no significant alterations in serum levels of calcium or magnesium.

In a group of 56 children with epilepsy who were receiving phenytoin, phenobarbitone or both, Hahn *et al.* (*New Engl. J. Med.* 1975, **292**, 550) observed that the combined therapy produced the greatest fall in serum calcium, serum 25-hydroxycholecalciferol and bone mass. In all children, the level of calcium in serum was related directly to the level of 25-hydroxycholecalciferol and to vitamin D intake. At any given level of vitamin D in the diet, children taking an anticonvulsant had a lower serum 25-hydroxycholecalciferol level than controls. Henderson *et al.* (*Lancet* 1974, **1**, 379) suggested that the form in which vitamin D is given may affect the degree to which it improves calcium metabolism in epileptics. They showed in 11 patients with chronic renal failure that small doses of 1,25-hydroxycholecalciferol (0·35–2.7 μg) given for 4–8 days increased intestinal calcium absorption without producing large changes in plasma calcium or phosphate, or urinary hydroxyproline excretion. Resistance to treatment with vitamin D in chronic renal failure (or presumably in anticonvulsant-induced osteomalacia) may involve impaired metabolism of the vitamin to its active dihydroxy derivative.

Animal testing possibilities

The use of animal studies to test a drug for suspected vitamin D antagonism is an attractive pros-

pect. Villareale *et al.* (*Science, N.Y.* 1974, **183,** 671) have reported that chicks given 1·0 or 2·5 g phenytoin/kg of diet develop rickets. When given the test meal together with sc injections of cholecalciferol, the degree of severity of the rickets was inversely proportional to the size of the vitamin supplement and depended upon the dose of phenytoin taken. No direct effect of phenytoin could be observed on the intestinal absorption of calcium. From these findings it appears that chicks may be a useful animal model for the study of vitamin D antagonists, whether anticonvulsants or not.

[P. Cooper—BIBRA]

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TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS

FLAVOURINGS, SOLVENTS AND SWEETENERS

3137. Species difference in *d*-limonene metabolism

Kodama, R., Yano, T., Furukawa, K., Noda, K. & Ide, H. (1976). Studies on the metabolism of *d*-limonene (*p*-mentha-1,8-diene). IV Isolation and characterization of new metabolites and species differences in metabolism. *Xenobiotica* **6**, 377.

d-Limonene is a naturally occurring compound widely used in the food and cosmetic industries and of potential use as an agent for dissolving cholesterol gallstones. Various metabolites formed in the rat and rabbit have been identified and certain species differences have been noted (*Cited in F.C.T.* 1975, **13**, 474; *ibid* 1977, **15**, 77). The authors cited above not only isolated further metabolites but demonstrated an even wider variation between species.

In the rat and rabbit the major metabolite was 8,9-dihydroxyperillic acid, in the hamster it was perillyl-β-D-glucopyranosiduronic acid (the glucuronide of perillic acid) and in the dog *p*-menth-1-ene-8,9-diol (uroterpenol). In both man and the guinea-pig the major metabolite was 8-hydroxy-*p*-menth-1-en-9-yl-β-D-glucopyranosiduronic acid, but this does not indicate that the guinea-pig is necessarily the ideal experimental animal for limonene studies aimed at providing data for extrapolation to man. A second metabolic pathway in the guinea-pig, involving oxidation of the 1-methyl group rather than formation of 8,9-epoxide intermediates, gives perillylglycine, probably via perillic acid, in quantities almost equal to those of the major metabolite, whereas this compound is almost absent in man.

[Animal experiments are of value in the safety evaluation of food additives and cosmetics only if the results can be meaningfully extrapolated to man. Animal experiments on *d*-limonene have produced a long list of its possible metabolites in man, but, as yet, little more.]

EMULSIFIERS AND STABILIZERS

3138. Revision of a carrageenan hypothesis

Sawicki, J. E. & Catanzaro, P. J. (1975). Selective macrophage cytotoxicity of carrageenan *in vivo*. *Int. Archs Allergy appl. Immun.* **49**, 709.

Carrageenan, a sulphated polygalactose widely used as a stabilizer in food, has a well-documented immunosuppressive activity. Its ability, for example, to suppress the cutaneous expression of delayed hypersensitivity was thought to be a direct result of the cytotoxic effect of carrageenan on the macrophage (*Cited in F.C.T.* 1973, **11**, 1141). Macrophage cytotoxicity has been demonstrated *in vitro*, but the study cited here investigated this property *in vivo* by administering 100 or 300 mg carrageenan dissolved in saline to guinea-pigs by ip injection.

At the lower dose level, which was sufficient to suppress the subcutaneous expression of delayed hypersensitivity, histochemical methods clearly demonstrated the presence of carrageenan in the Kupffer cells of the liver and in the macrophages of the peritoneal cavity, but not in the lymph nodes, spleen or bone marrow at any time up to 6 days after its administration. Even at the higher dose level, only occasional macrophage cells of the splenic red pulp, bone marrow and lymph nodes contained carrageenan.

Cytotoxicity was restricted to peritoneal macrophages and Kupffer cells in the liver, and it was doubtful whether this selective macrophage toxicity would be crucial in delaying hypersensitization. As immunosuppressive behaviour would involve the lymph nodes, spleen and bone marrow, the authors suggest that the previously held ideas regarding the mechanism of immunosuppression by carrageenan, namely *in vivo* macrophage cytotoxicity, are untenable.

Because of this and the evidence of a link between changes in the clotting system and delayed hypersensitivity, the ability of carrageenan to interfere with the intrinsic pathway of the coagulation system is now being investigated.

PRESERVATIVES

3139. Competitive inhibition of nitrosamine metabolism...

Couch, D. B. & Friedman, M. A. (1976). Suppression of dimethylnitrosamine mutagenicity by nitrososarcosine and other nitrosamines. *Mutation Res.* **38**, 89.

Various *N*-nitrosamines are known to occur in nitrite-preserved foods and to be formed both during the cooking of such foods and by the reaction of nitrite with nitrosatable compounds during digestion. Although toxicity studies have been carried out on individual nitrosamines, the biological responses these compounds elicit are known to be susceptible to

modification by many other compounds, and this has prompted the authors cited above to investigate the effects of various nitrosamines on the mutagenicity of one specific member of the group, namely dimethylnitrosamine (DMN).

The effects of nitrososarcosine were studied as this compound is known to be produced along with DMN in the nitrosation of dimethylglycine, and diethylnitrosamine (DEN) and dibutylnitrosamine (DBN) were selected, despite their infrequent occurrence in the environment, because of their structural similarity to DMN. Nitrososarcosine, not mutagenic itself in the host-mediated assay using *Salmonella typhimurium* G46 as the indicator organism, lowered the mutant frequency produced by DMN. A 71% reduction was observed with 500 mg nitrososarcosine/kg administered by gavage prior to DMN injection, and a significant reduction was observed with 37·2 mg/kg, which is less than 0·01 of the LD_{50} of nitrososarcosine. The inhibitory effect was rapid, occurring within 30 min and lasting between 2 and 19 hr. Pretreatment with 165 mg DMN/kg 45 min before inoculation with bacteria and administration of a second dose of DMN resulted in a 50% decrease in mutant frequency compared to a single treatment with DMN just before inoculation, and similar responses were observed on pretreatment with a 500-mg/kg dose of DEN or DBN, neither of which were themselves mutagenic in the host-mediated assay.

The explanation for these effects may lie in the inhibition of DMN metabolism, which is required to produce the active mutagen. If so, this would appear to be a matter of competitive inhibition by the other nitroso compounds, because nitrososarcosine has been found to inhibit mouse-liver mixed-function oxidase activity only in doses much higher than those used in this study, and DBN had no effect on this enzyme system in doses up to 1000 mg/kg.

3140. .... But no inhibition of acute nitrosamine toxicity

Friedman, M. A. & Sanders, V. (1976). Acute toxicity of dimethylnitrosamine in the presence of inhibitors of DMN demethylase. *Experientia* **32,** 495.

From the laboratory responsible for the paper abstracted above has come a report of a further aspect of the modification of biological responses to dimethylnitrosamine (DMN).

Citing earlier literature, the authors state that the carcinogenic action of DMN is related to DMN-demethylase activation with consequent methylation of biological molecules. Inhibition of this enzyme activity generally results in a suppression of the acute toxicity. Nitrososarcosine, piperonyl butoxide, dibutylnitrosamine and diethylnitrosamine (DEN) have been shown to inhibit DMN-demethylase activity as well as to suppress the mutagenicity of DMN (see previous abstract), but in the study cited above the first three of these compounds had no effect on acute DMN toxicity while DEN had a synergistic effect, reducing the DMN LD_{50} when injected ip 45 min before the ip DMN injection. The toxicity of DMN was actually increased by some 20% by prior injection of a DEN dose which itself showed no toxic effect. It appears therefore, that, in contrast to mutagenicity, the acute toxicity of DMN does not depend on enzymatic activation.

3141. Doubts over propylene oxide

Rosenkranz, H. S., Wlodkowski, T. J. & Bodine, S. R. (1975). Chloropropanol, a mutagenic residue resulting from propylene oxide sterilization. *Mutation Res.* **30,** 303.

The alkylating agent propylene oxide is known to produce non-volatile chlorohydrins when used as a sterilant for foodstuffs containing chloride ions (*Cited in F.C.T.* 1966, **4,** 456), and in some foodstuffs the concentrations of 1-chloro-2-propanol may reach 47 ppm. Thus, 1 lb of fumigated food may contain up to 21 mg 1-chloro-2-propanol, and human intake may be considerable.

The authors cited above demonstrated the mutagenicity of 1-chloro-2-propanol containing 25% 2-chloro-1-propanol to *Salmonella typhimurium* strain TA1530 but not to strain TA1538, indicating that the mutations induced were of the base-substitution not the frameshift type. The degree of reversion to histidine independence in TA1530 was dose related within the experimental range of 1·1–22 mg test material/plate. In view of these results, the authors suggest that studies of the carcinogenicity of chloropropanols in animals would be desirable and that alternatives to the use of propylene oxide for food sterilization should be investigated.

AGRICULTURAL CHEMICALS

3142. Dieldrin and reproduction in the mouse

Virgo, B. B. & Bellward, G. D. (1975). Effects of dietary dieldrin on reproduction in the Swiss-Vancouver (SWV) mouse. *Envir. Physiol.* **5,** 440.

Dieldrin residues have been shown to build up in the tissues of some animal species fed the insecticide in the diet (*Cited in F.C.T.* 1975, **13,** 397), and its placental transfer has been demonstrated in mice (*ibid* 1965, **3,** 656) and rabbits (*ibid* 1969, **7,** 536), but no foetal deaths or abortions were seen in sows fed up to 15 mg dieldrin/kg (*ibid* 1973, **11,** 513).

In the experiment cited above, female mice that had raised one litter were fed 2·5–25 ppm dieldrin in the diet, starting 4 wk before the second mating and continuing until day 28 after birth. Deaths among the dams fed 20 or 25 ppm dieldrin accounted for 89 and 56%, respectively, all the deaths occurring before parturition. With 10 or 15 ppm dieldrin, 18% failed to

become pregnant, but all those on lower doses and the survivors from higher doses were fertile, with no variation in their gestation period. Litter size in mice fed 25 ppm dieldrin was reduced by 17%. Survival of pups to weaning did not differ significantly from controls in groups fed dieldrin at 2·5 or 5 ppm, but it showed a general downward trend and reached zero with feed levels above 10 ppm. Decreased litter size was associated with a lesion occurring between ovulation and implantation, but study of its biochemistry was impracticable. Liver enlargement in the dam was associated with an inability to rear the pups. An important cause of litter loss with dietary levels of dieldrin of 15 ppm or more was pup killing or neglect. This behavioural toxicity of dieldrin is tentatively explained as a suppression of normal adult behaviour towards offspring. The conclusion from these observations and those of other studies is that the death of offspring represents the most severe toxic effect of organochlorine insecticides on the mammalian reproductive pattern.

PROCESSING AND PACKAGING CONTAMINANTS

3143. Phthalate delivery

Gibson, T. P., Briggs, W. A. & Boone, B. J. (1976). Delivery of di-2-ethylhexyl phthalate to patients during hemodialysis. *J. Lab. clin. Med.* **87,** 519.

Di-2-ethylhexyl phthalate (DEHP) is commonly used as a plasticizer in PVC blood-storage bags and haemodialysis tubing, and its ability to migrate into blood in contact with such material is well documented (Cooper, *Fd Cosmet. Toxicol.* 1976, **14,** 501). The authors cited above determined quantitatively the amount of DEHP delivered to patients undergoing haemodialysis.

Of nine patients, five had no detectable DEHP in predialysis venous blood samples. The four with detectable DEHP had received previously either blood transfusions or haemodialysis treatment for long periods, but two others who had received long-term haemodialysis treatment had no DEHP in their blood. The amount of DEHP added to venous blood was estimated from the difference between the DEHP concentrations in venous and arterial blood and varied from 1·5 mg in a patient dialysed for 15 min to 150 mg in a patient dialysed for 5 hr.

The results of consecutive analyses were often highly variable, possibly because of such factors as unrecorded alterations in blood flow, subtle changes in perfusion of DEHP-metabolizing organs and erratic leaching of DEHP from the tubing. In addition, it is not known whether DEHP can be dialysed.

The authors state that the fate of DEHP delivered during haemodialysis is also unknown, as is the toxicity of the compound following repeated parenteral administration, but the toxicity of DEHP to cells of the embryonic chick heart (*Cited in F.C.T.* 1971, **9,** 909), the previously reported presence of DEHP in bovine heart mitochondria and the finding of DEHP in the hearts of infants exposed to PVC tubing suggest that DEHP may be an aetiological factor in the development of the accelerated atherosclerosis associated with prolonged haemodialysis treatment. Further elucidation of this question will depend on the acquisition of more data on the tissue levels and metabolism of DEHP.

3144. Phthalate metabolism in fish

Melancon, M. J., Jr. & Lech, J. J. (1976). Distribution and biliary excretion products of di-2-ethylhexyl phthalate in rainbow trout. *Drug Metab. Dispos.* **4,** 112.

The presence of the ubiquitous phthalates in aquatic species has been the cause of some concern, but more recent reports have suggested that accumulation in fish occurs only under conditions of constant exposure, such as near heavily industrialized areas, because of the ability of fish to metabolize and excrete these esters (*Cited in F.C.T.* 1975, **13,** 147). Di-2-ethylhexyl phthalate (DEHP) is one of the most commonly used phthalate plasticizers and the authors cited above have studied its metabolism in the rainbow trout.

After 24-hr exposure of rainbow trout to 0·5 ppm [^{14}C]DEHP, half of the radioactivity in the trout was present in the bile at a concentration over 200 times higher than that in the water. The biliary metabolites were isolated by selective solvent extraction and characterized by thin-layer chromatography and combined gas chromatography–mass spectrometry. The major metabolite, mono-2-ethylhexyl phthalate (MEHP) glucuronide accounted for 72% of the total biliary activity, the other metabolites being phthalic acid glucuronide (2%), MEHP (0·5%) and two partly characterized polar compounds. Only 1% of biliary radioactivity was accounted for by unchanged DEHP. Since glucuronide formation is generally considered to facilitate excretion, the authors considered it to be unlikely that the MEHP glucuronide was further metabolized by oxidation and suggested that the partly characterized polar glucuronide metabolite, which accounted for 9·5% of the biliary radioactivity, was probably formed by oxidation of MEHP followed by glucuronide formation.

THE CHEMICAL ENVIRONMENT

3145. The arsenical kidney

Brown, M. M., Rhyne, B. C., Goyer, R. A. & Fowler, B. A. (1976). Intracellular effects of chronic arsenic administration on renal proximal tubule cells. *J. Toxicol. envir. Hlth* **1,** 505.

It has been suggested that arsenic (As) may be making a come-back in the environment (*Cited in F.C.T.*

1975, **13**, 285), and since the major route of elimination of absorbed As from the body lies through the kidney, studies of the biochemical effects of As on the proximal tubules of this organ are important.

Rats were given drinking-water containing 40, 85 or 125 ppm As as sodium arsenate ($Na_2HAsO_4.7H_2O$) for 6 wk. Some animals reduced their water consumption while given As, and there was a slight reduction in weight gain in those given 125 ppm. At all levels of As intake there was an increase in the ratio of kidney to body weight. A study of the respiration of kidney mitochondrial preparations indicated that the main effect of As was upon the ADP-dependent (state 3) respiration rate, which was reduced by all three levels of As, but a reduction in ADP:O ratio was observed only in rats given 125 ppm. In As-treated animals, renal tissue contained some 5–10 times the control level of As, but no dose-response relationship could be seen above the 85 ppm As level. Ultrastructural kidney changes did not extend beyond the mitochondria of the proximal tubules. At all the dose levels tested, these mitochondria were swollen and there was an increase in dense autophagic lysosome-like bodies, the difference from controls being most evident at the higher levels of As intake.

3146. Bismuth and the brain

Krüger, G., Thomas, D. J., Weinhardt, F. & Hoyer, S. (1976). Disturbed oxidative metabolism in organic brain syndrome caused by bismuth in skin creams. *Lancet* **II**, 485.

Toxic effects due to soluble bismuth compounds have resulted almost exclusively from their use in therapeutics (Browning, *Toxicity of Industrial Metals*, 2nd ed., p. 87; Butterworth, London, 1969). It has been known for some time that these compounds may cause kidney damage varying from asymptomatic proteinuria to acute or chronic renal failure, and, more recently, a number of papers have appeared describing the neurological effects of bismuth toxicity (Lhermitte *et al. Nouv. Presse med.* 1975, **4**, 419; Loutre *et al. Revue neurol.* 1975, **131**, 883).

The paper cited above reports the case histories of two patients with an organic brain syndrome thought to be due to bismuth present in a proprietary skin cream. Both individuals exhibited intellectual impairment and memory loss punctuated by periods of confusion, tremulousness, clumsiness, difficulty in walking and myoclonic jerks. On examination, both were found to have an impaired cerebral blood flow and cerebral uptakes of glucose and oxygen were reduced. In one of the patients, who was still using the cream, increased cerebral lactate production was observed. Bismuth was found in significant amounts (160 and 100 μg/ml) in the cerebral venous blood of both patients and a level of 3 μg bismuth/ml was found in the cerebrospinal fluid of one patient. No bismuth was detectable in the blood or cerebrospinal fluid 3 wk after use of the cream was discontinued.

The authors suggest that bismuth, like mercury, binds with the thiol groups of enzymes concerned with oxidative metabolism in the brain. This hypothesis would explain the reduced utilization of oxygen and glucose. Increased lactate production in the patient still using the cream was consistent with a reduction in the oxidative decarboxylation of pyruvate. It is concluded that heavy metals such as bismuth may have a triple effect on the brain—by exerting a direct action on nervous tissue, by damaging the blood-brain barrier and thus increasing its permeability and by inducing indirect effects as a result of damaging other organs, such as the liver.

3147. A closer look at chromates

Mass, M. J. & Lane, B. P. (1976). Effect of chromates on ciliated cells of rat tracheal epithelium. *Archs envir. Hlth* **31**, 96.

The inhalation of chromates is known to cause irritation of the respiratory tract, bronchopneumonia, chronic bronchitis and tracheitis being common ailments among workers in the chromate industry. In addition, evidence has been accumulating to indicate that there is a specific hazard of broncho-pulmonary carcinoma for individuals handling chromium compounds in industry (*Cited in F.C.T.* 1976, **14**, 215). In an attempt to prevent these respiratory effects, a TLV of 0·1 mg/m^3 has been adopted for the chromates. The authors cited above investigated some specific subcellular effects that might form the basis for the pathological changes observed in connexion with chromate exposure.

Tracheal rings from Wistar-Lewis female rats were placed in culture media containing 0, 5, 10 or 20 μg calcium chromate/ml and examined by vital phase microscopy at regular intervals over 2 wk, so that the gross features of the exposed tissue could be observed and the persistence of ciliary activity ascertained. After examination, specimens from each group were prepared for light and electron microscopy. Freshly excised whole tracheas were immersed in media containing 0, 0·1, 1, 10 or 100 mg sodium chromate/ml in order to observe the effects of lethal doses. Specimens were fixed after exposure for 20 min.

In order to observe a threshold for ciliostasis and to determine a relationship between ciliostasis and cytotoxicity, tracheal slices were exposed to sodium chromate at concentrations of 1, 10 and 100 mg/ml. Exposure was continued until ciliostasis occurred or until 30 min had elapsed. Specimens were then either fixed for light and electron microscopy or returned to normal medium to determine whether the ciliary beat would resume.

The effect of chromate on protein and RNA synthesis in cultured tracheal epithelium was investigated by autoradiographic measurement of tritiated precursor uptake, while the activity of the mitochondrial enzyme succinic dehydrogenase in chromate-treated tissue was tested histochemically. Morphological studies were also undertaken on treated trachea to correlate structural changes in the mitochondria with histochemical data.

Vital phase microscopy indicated that both the control tissue and the tracheal rings exposed for 2 wk to 5 μg chromate/ml exhibited a smooth luminal surface with beating cilia. In the specimens exposed to 10 μg chromate/ml a jagged appearance of the epithelium was observed. The ciliary activity of tracheal rings exposed to 10 and 100 mg chromate/ml ceased

within 20 min and did not recover on return to a normal medium. Light microscopy suggested that epithelia exposed to more than 5 μg chromate/ml exhibited pathological alterations in direct proportion to the length of exposure and the chromate concentration. Ultrastructural changes in the mitochondria were observed in chromate-treated cells on examination by electron microscopy.

No gross inhibition of protein synthesis was shown in studies involving incorporation of a tritiated precursor, nor was there any modification of succinic dehydrogenase activity.

It is known that the removal of foreign substances from the respiratory tract depends largely on the ciliary movement of mucus carrying entrapped particles and it has been speculated that the effect of chromates might be primarily ciliostatic. These studies appear to contradict this idea, in that cytotoxic concentrations are far below the levels that induce ciliostasis. The preliminary experiments on specific cell functions likely to be affected, such as protein and RNA synthesis, failed to demonstrate any changes. It may be that a more selective effect on specific species of RNA or protein should be considered. An absence of effects on the enzyme succinic dehydrogenase suggests that mitochondria are not the primary site of injury. By exclusion, the authors suggest that the cell surface is the most likely site, as it has been shown that chromate rapidly traverses the cell membrane and is immediately reduced from Cr^{6+} to Cr^{3+} which cannot recross the membrane. A study of this process is presumably the next candidate for the clarification of chromate toxicity mechanisms.

3148. Epoxidation likely in bromobenzene metabolism

Ruzo, L. O., Safe, S. & Hutzinger, O. (1976). Metabolism of bromobenzenes in the rabbit. *J. agric. Fd Chem.* **24,** 291.

Bromobenzenes appear to be among those compounds that are metabolized to more reactive agents capable of producing biochemical and structural damage in animal tissues (*Cited in F.C.T.* 1972, **10,** 879). Like many other halogenated aromatic compounds, the bromobenzenes can undergo hydroxylation *in vivo*, raising the possibility of the formation of epoxide intermediates. Some support for this hypothesis has now been provided by a metabolic study of bromobenzenes in the rabbit.

A series of brominated benzenes (Br_1–Br_5) was administered to pairs of rabbits by ip injection of corn oil containing 50 mg bromobenzene/kg body weight. Urine and faeces were collected for 10 days after dosing and the metabolites were identified by mass-spectrometric analysis. Mono-, di- and tribromobenzenes yielded at least two major phenolic metabolites, typified by 3- and 4-bromophenol from bromobenzene itself. In the metabolism of 1,4-dibromo- and 1,3,5-tribromobenzene, hydroxylation was accompanied by a 1,2-migration of a bromine atom, suggesting an arene oxide intermediate. Tetra- and pentabromobenzenes yielded only trace amounts of metabolites in the urine and none was detected in the faeces. Thus, in the rabbit, benzenes substituted with one, two or three bromine atoms are metabolized principally to brominated phenols, the toxicological properties of which are largely unknown.

3149. Isocyanate hypersensitivity

Charles, J., Bernstein, A., Jones, B., Jones, D. J., Edwards, J. H., Seal, R. M. E. & Seaton, A. (1976). Hypersensitivity pneumonitis after exposure to isocyanates. *Thorax* **31,** 127.

The severe irritant properties of isocyanate vapour are well documented and problems associated with long-term exposure to isocyanate levels below the TLV of 0·02 ppm have been identified (*Cited in F.C.T.* 1971, **9,** 279). The authors cited above report the case histories of four workers who developed interstitial disease after being exposed to isocyanate, usually toluene diisocyanate (TDI), at relatively low levels.

A 50-yr-old man who had worked for 5 yr on a polyurethane-foam process developed dyspnoea, weight loss and fever. An alveolar filling lesion, visible in chest radiographs of both lungs, responded to antibiotic treatment, and after several weeks the subject returned to work. The symptoms recurred within 2 months, however, and pulmonary function tests showed a restrictive ventilatory defect and a reduced transfer factor. The pathology observed in an open-lung biopsy was consistent with a hypersensitivity response to an inhaled allergen, but the subject's reaction to challenge by an intradermal injection of TDI–albumin did not differ from that of a control individual. Similarly, challenge by a range of bacterial and miscellaneous allergens produced no adverse reactions.

Following daily treatment with corticosteroids, the overall condition of the subject improved sufficiently for him to return to work, although to a process not directly involved with TDI. Attempts to discontinue the steroid treatment were unsuccessful, rapid deterioration in lung function and dyspnoea being the result.

Three other subjects whose work involved contact with TDI showed similar signs of dyspnoea and reduced performance in pulmonary function tests. Two of these workers, maintenance fitters on a polyurethane-foam plant, had been exposed to high TDI concentrations only in "accident" situations, while the third, a painter, had been exposed to hexamethylene diisocyanate through the use of a polyurethane-paint spray over a period of 6 months.

Acute exposure to isocyanate at levels in excess of the TLV has been reported to produce airway obstruction, but in the cases reported here reduced gas transfer was also encountered. In two of the subjects, the adverse effects occurred after only "light" exposure and in all cases the effects persisted after the patient's removal from direct contact with isocyanate.

An attempt was made to reproduce the clinical signs in animal studies. Rabbits were sensitized to TDI–albumin by twice-weekly intramuscular injections for 3 wk, before being challenged by endotracheal deposition of the antigen. This produced, in the lungs of the treated animals, reactions that were classified as a hypersensitivity Arthus response—polymorphonuclear cellular exudation, peribronchial lymphoid hyperplasia, interstitial pneumonitis and pul-

monary oedema. On skin testing, a reddened wheal was produced at the site of a TDI–albumin injection with a maximum intensity at 4–8 hr, a finding also consistent with an Arthus reaction.

The results suggested that an inhalational allergic pneumonitis was being observed, but the picture was complicated because the one subject examined immunologically reacted "normally" to a TDI injection, and the other three presented normal radiographs.

3150. Methanol toxicity: the search for a model animal

McMartin, K. E., Makar, A. B., Martin, A. G., Palese, M. & Tephly, T. R. (1975). Methanol poisoning I. The role of formic acid in the development of metabolic acidosis in the monkey and the reversal by 4-methylpyrazole. *Biochem. Med.* **13,** 319.

Makar, A. B. & Tephly, T. R. (1976). Methanol poisoning in the folate-deficient rat. *Nature, Lond.* **261,** 715.

Methanol metabolism differs substantially in rats and monkeys (*Cited in F.C.T.* 1966, **4,** 625; *ibid* 1970, **8,** 109), with the situation in the latter species resembling more closely that in man. For this reason, the use of rats and, indeed, of other small laboratory animals for studies relating to human methanol poisoning is generally considered inappropriate.

In the first paper cited above, a single dose of ^{14}C-labelled methanol (3 g/kg) given by nasal tube to rhesus and pigtail monkeys was shown to produce effects similar to those seen in cases of methanol poisoning in man. Moderate central nervous depression, lasting 1–2 hr, was followed first by a latent period of 8–12 hr during which no obvious toxic signs appeared, and then by anorexia, photophobia, weakness, restlessness and profound hyperpnoea. The animals went into coma and died 12–33 hr after the dose. The characteristic biochemical finding was a gradually developing metabolic acidosis that became severe just before death. Plasma bicarbonate levels declined from 21 to about 8 mequiv/litre within 12 hr, and sometimes reached 3 mequiv/litre in the severest acidosis. Coincidental with this fall was a plasma accumulation of formate, but this was insufficient to account entirely for the loss of bicarbonate.

Administration of an alcohol-dehydrogenase inhibitor, 4-methylpyrazole (50 mg/kg), 15 min before the dose of methanol, reduced the rate of methanol metabolism to CO_2 by about 75% and averted early acidosis. A single dose was effective for at least 36 hr, after which metabolic acidosis started to develop.

The second paper cited reports profound acidosis in folate-deficient rats given methanol. The recovery of $^{14}CO_2$ derived from ^{14}C-labelled methanol in these animals was about 50% of that in the controls, which were not deficient in folate, and marked formic acidaemia was observed. The existence of folate deficiency therefore appears to eliminate some of the observed differences between rodents and primates in the toxic effects of methanol, and the authors suggest that the folate-deficient rat may be a useful model for studying certain aspects of methanol toxicity, obviating the need for some tests in monkeys, which previously have given a closer approximation to the effects observed in man. It is also suggested that the variability encountered in human sensitivity to methanol may be explained, at least in part, by differences in nutritional status, particularly as regards folate saturation or deficiency.

3151. MBK further indicted

Abdel-Rahman, M. S., Hetland, L. B. & Couri, D. (1976). Toxicity and metabolism of methyl *n*-butyl ketone. *Am. ind. Hyg. Ass. J.* **37,** 95.

Methyl *n*-butyl ketone (MBK) was recently implicated in an outbreak of peripheral neuropathy in workers at a printing plant. Preliminary complementary animal studies demonstrated that MBK was the likely aetiological factor but suggested methyl ethyl ketone (MEK), also used on the plant, as a significant potentiator of MBK toxicity (*Cited in F.C.T.* 1976, **14,** 218). The authors cited above examined the toxicity of MBK in more detail and investigated its interaction with MEK in the rat and guinea-pig.

Groups of rats, a species previously shown to be sensitive to MBK, were subjected to atmospheres of MBK (250 or 400 ppm) or MBK–MEK mixtures (225 and 750 ppm, respectively). Whereas blood levels of MBK up to 24 mg/100 ml were demonstrated in animals kept in the MBK–MEK environment for 23 days, MBK could not be detected in the blood of animals living in an MBK atmosphere for up to 2 months. After 23 days, all six animals kept in the MBK–MEK atmosphere had developed a severe neuropathy. The radical alteration of the pharmacodynamics of MBK by MEK provides a possible explanation for the observed synergistic interaction of the solvents in the factory.

Rats exposed to an atmosphere of MBK and given phenobarbitone in their drinking-water produced fewer signs of overt toxicity than did controls exposed to MBK alone. It is suggested that induction of liver microsomal-enzyme activity brought about this protective effect by increasing the efficiency of the conversion and excretion of MBK and any potentially toxic metabolites.

2-Hexanol, 2,5-hexanedione and unchanged MBK were identified as urinary metabolites in the rat after ip injection of MBK, and pretreatment with phenobarbitone enhanced the excretion of 2,5-hexanedione. Approximately 30% of the radioactivity from a tritium-labelled ip dose of MBK was excreted in the urine of the rat within 18 hr and a further 10% was eliminated in the next 72 hr, indicating that saturation of some biotransformation route was occurring. The metabolism of MBK in guinea-pigs followed a similar pattern, although 2-hexanol levels were higher in the unprimed animals and pretreatment with phenobarbitone produced a more marked increase in 2,5-hexanedione.

Previous workers have noted a similarity between the toxic effects of MBK and *n*-hexane. Metabolic studies of *n*-hexane in the guinea-pig showed that 5% of a given dose was excreted in the urine within 24 hr as 2-hexanol. Further studies are now in progress to compare the metabolism and toxicity of *n*-hexane and MBK.

[The occurrence of cases of peripheral neuropathy following industrial exposure to methyl *n*-butyl

ketone and the results of subsequent animal studies have indicated the need for a reduction in the TLV, currently standing at 100 ppm in the UK and USA (*Cited in F.C.T.* 1977, **15**, 159). The fact that several well-established TLVs have recently been called in question underlines the need for continual reassessment of these values in the light of new evidence, both epidemiological and experimental.]

3152. Weight-reducing methyl methacrylate

Tansy, M. F., Kendall, F. M., Benhayem, S., Hohenleitner, F. J., Landin, W. E. & Gold, M. (1976). Chronic biological effects of methyl methacrylate vapor. I. Body and tissue weights, blood chemistries, and intestinal transit in the rat. *Envir. Res.* **11**, 66.

The adverse effects observed in animal inhalation studies on methyl methacrylate (MMA) have occurred typically at levels unlikely to be present in industrial environments. Several acute studies have shown that at these high levels, the monomer can cause reversible impairment of the working of the gastro-intestinal tract by a neurotoxic mechanism. The study cited here demonstrated similar subacute effects in rats exposed to MMA at levels close to the TLV.

Groups of 25 rats were exposed to MMA vapour (116 ppm) for 8 hr/day on 5 days/wk for 3 or 6 months. The regime produced no deaths, and the only external sign of toxicity was the shaggy appearance of the animals. The rats killed at month 3 all exhibited a marked absence of visceral and subcutaneous fat deposits. The body, lung and spleen weights were significantly lower than those of the controls and the serum level of alkaline phosphatase was increased. There were no significant differences in visceral fat levels between experimental and control animals at month 6, but subcutaneous and popliteal fat weights in the exposed group were markedly lower than those in the control animals.

At month 6, although all the organ weights were normal, the haematology of the treated rats differed from that of the controls. Levels of serum protein, cholesterol, blood urea nitrogen and serum glutamic–oxalacetic transaminase and the calcium/phosphate ratio were all significantly lower in the treated rats and increased levels of serum alkaline phosphatase and inorganic phosphate were observed.

A number of the animals exposed for 6 months exhibited, 3 days after the final MMA exposure, a lower rate of intestinal transit than was demonstrated in the unexposed controls. As previously reported changes in intestinal performance following MMA exposure were reversible within minutes of cessation of exposure, this more persistent change was noteworthy. Further experiments to determine the time taken for the intestinal transit times to return to control values are being undertaken.

3153. Teratology of PBB in rodents

Corbett, T. H., Beaudoin, A. R., Cornell, R. G., Anver, M. R., Schumacher, R., Endres, J. & Szwabowska, M. (1975). Toxicity of polybrominated biphenyls (Firemaster BP-6) in rodents. *Envir. Res.* **10**, 390.

While the environmental impact of polychlorinated biphenyls (PCBs) needs no introduction, remarkably little is known about the toxicity of the structurally similar polybrominated biphenyls (PBBs). Ironically, our familiarity with both chemical groups is derived more from environmental disaster than from experimental design. Undoubtedly the most serious disaster was an outbreak of PCB poisoning in Japan which affected at least 1000 people (*Food Chemical News* 1971, **13** (35), 41), and more recently an error involving the addition of hexabromobiphenyl to animal feed resulted in the need for the widespread destruction of cattle and other livestock in Michigan (*ibid* 1976 **18** (4), 33). The latter occurrence, combined with the demonstration of a weak teratogenic response to PCB in chickens (Fishbein, *Ann. Rev. Pharmac.* 1974, **14**, 139), has prompted the authors cited above to examine the effects of a PBB mixture (Firemaster BP-6) on pregnant rodents.

Groups of female Swiss/ICR mice and Sprague–Dawley rats were exposed to 50, 100 or 1000 ppm Firemaster BP-6 in a chow diet on days 7–18 (mice) or 7–20 (rats) of pregnancy. A further group of non-pregnant female mice was exposed to 1000 ppm Firemaster BP-6 in the diet for 11 days.

There was a dose-related decrease in the mean foetal weight in rats and mice, and a significant incidence of exencephaly in the foetuses of mice given 100 or 1000 ppm PBB and of cleft palate and hydronephrosis in those given 1000 ppm. Non-pregnant mice fed 1000 ppm PBB exhibited a marked increase in liver size and weight compared with control values, and an increase in *P*-450 microsomal protein. Histological examination of the livers from mice treated in this way revealed a swelling of the hepatocytes with focal areas of coagulative necrosis. Whole-tissue foetal and liver concentrations of PBB were in the range 0·17–0·86 and 1·52–3·57 ppm, respectively, compared with levels of $<0{\cdot}1$ ppm PBB detected in human fat specimens taken at autopsy from three individuals from the PBB-contaminated area of Michigan.

The authors concluded that the demonstration of the weak teratogenicity of this commercial PBB in rodents justifies continued surveillance of the exposed human population in Michigan, with emphasis on teratogenic, mutagenic and carcinogenic parameters.

[Whether brominated dibenzofuran impurities are responsible for any of the teratogenic activity of commercial polybrominated biphenyls, as seems likely to be the case with the chlorinated dibenzofurans known to be present in commercial polychlorinated biphenyls, has yet to be established.]

3154. Flame retardants under fire

St. John, L. E., Jr., Eldefrawi, M. E. & Lisk, D. J. (1976). Studies of possible absorption of a flame retardant from treated fabrics worn by rats and humans. *Bull. env. contam. & Toxicol. (U.S.)* **15**, 192.

Several organophosphorus compounds, including tris-(2,3-dibromopropyl) phosphate (TDBP), are used extensively as flame retardants on fabrics. Recent studies have shown that appreciable amounts (up to 10 μg/sq in. of fabric) of this and other organophosphorus flame retardants are released from fabrics dur-

ing a simulated laundering step, this rate of release being maintained during several subsequent launderings (*Cited in F.C.T.* 1976, **14,** 512). In the investigation cited above, the possible diffusion of TDBP from fabrics to skin, its absorption and its toxicological significance were examined.

Exposure studies were undertaken in rats and man. TDBP monomer (100 mg) was spread over the surface of the gauze pad of a 1-in. bandage and applied tightly against the skin of a rat for 7 days. The skin of a second rat was exposed for 9 days to fabric treated with TDBP. Similarly, two male volunteers were exposed to treated pyjama fabric for a period of 7 nights. Since in mammals organophosphorus insecticides typically undergo enzymatic or chemical hydrolysis to the corresponding acids and alcohols, analytical methods were devised to reveal the presence in urine of free or conjugated 2,3-dibromopropanol (DBP) derived from TDBP hydrolysis.

No detectable residues of free or conjugated DBP were found in urine samples from the rat exposed to TDBP fabric or in the urine of the two human subjects, but small amounts appeared in the rat urine when the animal was allowed to chew the fabric. In the rat to which the pure liquid TDBP was applied, the appearance in the urine of free or conjugated DBP was slow, with maximum concentrations of 12·8 and 10·7 ppm, respectively, being recorded on day 5. *In vitro* studies showed that the 10,000 g supernatant fraction of fresh rat liver produced about a 5% conversion of TDBP to DBP during incubation for 30 min.

The migration rate and absorption of flame retardants will depend very much on their chemical structure, their lipid solubility, the nature of the fabric, the operating conditions during commercial production of the fabric, the thickness of the flame retardant coating, the closeness of fit of the fabric and differences among individuals, such as oiliness of or breaks in the skin. Migration may also be enhanced under conditions of profuse sweating, and there is the likelihood of extraction of the retardant and its ingestion by children chewing on the garment. The synthesis and use of isotopically labelled flame retardants would greatly facilitate further studies on the safety of these chemical finishes on various fabrics.

3155. TOCP not the sole cause of shoemakers' neuropathy

Abbritti, G., Siracusa, A., Cianchetti, C., Coli, C. A., Curradi, F., Perticoni, G. F. & De Rosa, F. (1976). Shoe-makers' polyneuropathy in Italy: the aetiological problem. *Br. J. ind. Med.* **33,** 92.

The first cases of so-called shoemakers' polyneuropathy were reported in 1957. Since that time nearly 400 cases have been described in the Italian literature, the substance implicated being tri-*o*-cresyl phosphate (TOCP), which is present in glues, artificial leathers and some types of paint. The clinical picture and the histopathological changes found among workers affected by this disease were similar to those found in patients with polyneuropathy caused by non-occupational exposure to TOCP, and to those found in experimental animals given TOCP. However, chemical analyses of glues and leathers taken from factories where the disease occurred showed that in almost all instances little or no TOCP was present. It was also found that the disease manifests itself more frequently during winter and spring. This is not consistent with the absorption of the causative agent through the skin and/or gastro-intestinal tract.

In an attempt to clarify the aetiology of the disease, 122 affected workers were studied during the period 1971–74, and samples of glue and solvents were taken from workplaces where cases of polyneuropathy had occurred. The disease was found mainly to affect workers engaged in the glueing and cleaning process, but it also affected individuals who did not have direct contact with the glues or solvents. Men (32%) were shown to be less susceptible than women (68%), but no direct relationship was found between the severity of the disease and the number of years of work in the shoe industry. Workers in small shoe factories, where standards of hygiene were low, appeared to be most susceptible to the disease.

The authors suggest that the disease is caused by volatile substances which accumulate in the working environment under conditions of poor hygiene. A likely hypothesis is that the primary causative agents are paraffin hydrocarbons of low boiling point (pentane, hexane, heptane and isomers). Analysis of glues and cleaning fluids collected from different factories revealed the presence of pentane, 2- and 3-methylpentane and *n*-hexane in concentrations of more than 80% by weight. However, it is not clear whether only one of these substances or the combined action of several hydrocarbons of the same group is responsible.

3156. Unravelling the neurotoxicity of triethyltin

Graham, D. I., de Jesus, P. V., Pleasure, D. E. & Gonatas, N. K. (1976). Triethyltin sulfate-induced neuropathy in rats. Electrophysiologic, morphologic, and biochemical studies. *Archs Neurol.* **33,** 40.

Lock, E. A. & Aldridge, W. N. (1975). The binding of triethyltin to rat brain myelin. *J. Neurochem.* **25,** 871.

The alkyltins induce widespread oedema in the white matter of the central and peripheral nervous systems. Morphologically, these lesions are characterized by intramyelinic vacuolation and in the case of the peripheral nerves by an apparent increase in the number of neurofilaments in the axons of the vacuolated fibres (*Cited in F.C.T.* 1971, **9,** 893; Graham & Gonatas, *Lab. Invest.* 1973, **29,** 628). The papers cited above report two further investigations on the neurotoxicity of triethyltin (TET) in the rat.

The first of these was an extensive investigation into the morphological, electrophysiological and biochemical aspects of the neuropathy induced when 20 mg TET sulphate/litre was added to the drinking-water of rats. Three groups of animals underwent continuous treatment for 5, 10–12 or 20 days. In the last two groups, some of the animals were allowed to recover on a control regime before being tested. A further group of rats was maintained for alternate 5-day periods on TET-treated and normal water over a period of 50–65 days.

None of the animals given TET for 5 days exhibited neurological signs of intoxication, but they weighed less than the controls. Animals treated for 10 days or longer showed symmetrical weakness of the hind legs, accompanied by weight loss and muscle wasting by day 20. Rapid improvement occurred when the rats were convalesced on the control diet. Interestingly, only one of seven rats on the alternating regime developed similar clinical signs of neuropathy.

These clinical signs of neuropathy were not accompanied by changes in the biochemistry of the brain tissue. There was no evidence of demyelination, nor were there any significant differences in the floating fractions of the experimental and control animals. Normal collagen values were found in the treated groups, which suggested that scar tissue was not being formed.

The only significant changes in the electrophysical properties of the sciatic nerve were in the motor nerve conduction velocity (MNCV), which decreased with increasing length of TET treatment, a 33% decrease being observed after 20 days. A significant reduction in MNCV was observed also in rats given the alternating regime, even when no clinical signs of poisoning were apparent. On day 50 of this regime the rats exhibited a reduction in MNCV equivalent to that in rats treated continuously for 20 days. Again a convalescence period after TET poisoning produced a normalization in MNCV, control values being regained after 20 days without TET treatment.

The main morphological indication of neuropathy in the rats was the already well documented formation of intramyelinic oedema. Microscopic examination of the sciatic nerve indicated that the vacuoles were formed by separation of the myelin lamellae at the intraperiod line. Although the electrophysical and morphological effects of the TET treatment paralleled each other during intoxication, MNCV values returned to normal at a time when excess fluid was still present in the sciatic nerve. As a consequence, the authors suggested that the neurological effects of the alkyltin could be attributed only in part to structural changes in the central and peripheral nervous systems and that, in addition, some metabolic factor must be involved. An increase in the number of neurofilaments and neurotubules was demonstrated within the axons of both vacuolated and nonvacuolated nerve fibres of intoxicated animals. Although the exact significance of this increase was not known it was thought not to be a direct cause of the neuropathy.

The second investigation concerned the binding of ^{113}Sn-labelled TET chloride to myelin isolated from the rat brain. After a 15-min incubation, the myelin and free alkyltin were separated by centrifugation and the radioactivity associated with the myelin was measured. Analysis of the results indicated the presence of more than one class of binding site on the myelin, the major site having a high affinity for TET. Hexachlorophene and 3,5-diiodo-4′-chlorosalicylanilide, both of which produce oedematous lesions of the central nervous system similar to those produced by TET, had no observable effect on the binding of TET to myelin. However, trimethyltin and trimethyllead, which do not produce oedematous lesions in the central nervous system, both competed with TET for the high-affinity binding site. Trimethyltin had a 30-fold lower affinity than TET, whereas the affinities of trimethyllead and TET were similar. These data suggest that the binding of TET to myelin is not an important factor in the production of the observed oedema.

NATURAL PRODUCTS

3157. Is ascorbic acid an abortifacient?

Alleva, F. R., Alleva, J. J. & Balazs, T. (1976). Effect of large daily doses of ascorbic acid on pregnancy in guinea pigs, rats, and hamsters. *Toxic. appl. Pharmac.* **35,** 393.

No fewer than five studies have suggested that ascorbic acid may increase abortion at high dose levels in rats and guinea-pigs, a finding with potentially alarming implications for pregnant women taking this vitamin in relatively large quantities to combat minor ailments such as the common cold. The authors cited above have sought, therefore, to clarify the situation in rats, guinea-pigs and hamsters, using dose levels of the same order as were reported to be effective in previous studies.

L-Ascorbic acid was given orally at dose levels of 50, 150 or 450 mg/kg/day to rats and hamsters on days 1–19 and 1–15, respectively, of pregnancy, and at a daily dose level of 400 mg/kg to guinea-pigs from about day 23 until days 45–53 of pregnancy. Other guinea-pigs were given twice-daily sc injections of 200 mg/kg (400 mg/kg/day) on days 6–10 of pregnancy before the start of oral treatment on day 11. No increase in abortion or in deaths of offspring was observed in any species, and the only effect noted was a slight increase in pup weight in one group of guinea-pigs (treated on days 23–50) and in hamsters given the highest dose level. The study thus confirmed a recent finding that daily oral doses of 150–1000 mg/kg in rats and 250–1000 mg/kg in mice had no effects on pregnancy (Frohberg *et al. Arzneimittel-Forsch.* 1973, **23,** 1081). The conflicting results of earlier workers could not be explained.

3158. Nutrition and the alcoholic liver

Jordö, L. & Olsson, R. (1975). The effect on the rat liver of long-term administration of different alcoholic beverages together with inadequate diets. *Acta path. microbiol. scand. Sec. A* **83,** 717.

The effects of nutritional factors on the relation between ethanol consumption and adverse liver effects are highly complicated (*Cited in F.C.T.* 1969, **7,** 71). The possibility that the cirrhotic effect of alcoholic beverages may be due to ingredients other than ethanol has been investigated with little success and, in view of the generally poor food intake of human

alcoholics, the possible role of malnutrition in cirrhogenesis has now been examined.

Ethanol was given to rats, as 36% of the total calorie intake of an otherwise standard diet, in the form of gin, brandy, whisky, red wine or pure ethanol, the intake of ethanol being about 4 g/kg/day for 12 wk in each case. Liver biopsies carried out in each group indicated only slight histological changes, and the number of deaths was about 25% in all groups. A second experiment was performed in which the dietary protein given to the rats was reduced to 10·9% of total calorie intake, thus increasing the calorie intake from ethanol to 39·6%, from fat to 43·7% and from glucose to 5·8%. Each group consisted originally of 20 rats and the numbers of survivors were eight controls, three on ethanol, four on red wine, two on brandy, two on gin and none on whisky. Apart from one rat in the gin group which showed pronounced liver steatosis, none showed steatosis, liver necrosis, inflammation or fibrosis. In a third experiment carried out for 15 wk, vitamin allowances of the standard diet were halved. Again no steatosis, necrosis, inflammation or fibrosis of the liver appeared, except in one rat which was on pure ethanol and which showed a few discrete liver necroses with lymphatic infiltration.

These results support the previous findings that congeners present as flavours and bouquets in different alcoholic beverages lack any important hepatotoxic potential (*ibid* 1975, **13,** 676), but do not provide any evidence that an inadequate diet is an important factor in the development of cirrhosis as a result of a high ethanol intake.

3159. Pass the rye bread

Reiss, J. (1976). Mycotoxins in foodstuffs. VI. Formation of sterigmatocystin in bread by Aspergillus versicolor. *Z. Lebensmittelunters. u.-Forsch.* **160,** 313.

Sterigmatocystin has been reported to be a possible contaminant of country-cured ham infected with *Aspergillus versicolor* (*Cited in F.C.T.* 1974, **12,** 797). Evidence is now presented that this toxin may occur also in bread under certain conditions.

Two potent sterigmatocystin-producing strains of *A. versicolor* grew well on types of bread of low acidity (whole wheat bread, whole wheat bread with wheat germ and whole wheat bread with linseed) and formed moderate amounts of sterigmatocystin, the highest yields of the toxin being in the range 0·1–0·4 μg/g bread. The optimal temperature for mould growth was 20–30°C and maximal mycotoxin production occurred at 20°C, most of the toxin being formed within 10 days. Neither strain grew well on whole rye bread, with a high acid content, but when the total acid content of this bread was reduced, there was a marked increase in mycelial growth. The production of toxin, however, remained insignificant on rye bread even when the total acid content was low. Clearly some factor in the composition of the bread other than its acidity affects the course of fungal metabolism.

3160. Offshoot of the potato blight controversy

Keeler, R. F., Young, S. & Brown, D. (1976). Spina bifida, exencephaly, and cranial bleb produced in hamsters by the solanum alkaloid solasodine. *Res. Commun. chem. Path. Pharmac.* **13,** 723.

Keeler, R. F., Brown, D., Douglas, D. E., Stallknecht, G. F. & Young, S. (1976). Teratogenicity of the solanum alkaloid solasodine and of 'Kennebec' potato sprouts in hamsters. *Bull. env. contam. & Toxicol. (N.Y.)* **15,** 522.

The debate about the possible teratogenic effects of blighted potatoes (*Cited in F.C.T.* 1973, **11,** 311; *ibid* 1974, **12,** 772) led to a suggestion (Keeler, *Lancet* 1973, **I,** 1187) that the spirosolane solanum alkaloids might be teratogens. Despite studies on various solanum alkaloids, no data are available on the teratogenicity of those with a configuration of terminal furan and piperidine rings like that found in solasodine, although the veratrum alkaloids, such as cyclopamine, terminating in a fused furanopiperidine group are well-known teratogens (*Cited in F.C.T.* 1973, **11,** 705). The authors cited above studied, therefore, the teratogenicity of solasodine, which occurs in the egg plant but has not, as yet, been demonstrated in potatoes.

In the first study cited, hamsters were gavaged with a high single dose of 1184–1628 mg solasodine/kg on day 7 or 8 of gestation, and one third of all the litters from surviving dams had one or more deformed offspring. Of the 727 offspring from 84 dams, 50 had malformations, including nine classed as spina bifida, 13 as major exencephaly and 25 as cranial bleb, as well as two cases of hare lip and one of anophthalmia. In comparison, only five of the 1948 offspring from 192 control dams were abnormal. Administration of solasodine also resulted in a large increase in resorptions. The solasodine analogues tomatidine and diosgenin, each given in a dose about twice as high as that shown to be teratogenic in the case of solasodine, caused no malformations or increase in resorption rate. These differences were tentatively associated with the presence or absence of a piperidine ring and the positioning of the nitrogen on the α or β side of the molecule.

In the second study cited, a similar dose of solasodine given to hamsters on day 7 or 8 of gestation produced 48 deformed offspring in 23 of 89 litters from survivors and these figures were compared with those from hamsters gavaged with potato-sprout preparations from a cultivar ('Kennebec') known to produce high concentrations of spirosolane-type alkaloids. Administration of the dry sprouts in a single 500-mg dose on day 7 or 8 resulted in 64 abnormal offspring in 26 of 113 litters from survivors. The deformities showed a gross resemblance to those induced by dosing with solasodine, and in addition the number of resorptions was increased.

However, at the present time, these findings must be seen as coincidental, since the teratogen present in potato sprouts has not yet been characterized as a spirosolane alkaloid, and solasodine has not been isolated from potatoes.

COSMETICS, TOILETRIES AND HOUSEHOLD PRODUCTS

3161. Metabolites of 2,4-toluenediamine

Waring, R. H. & Pheasant, A. E. (1976). Some phenolic metabolites of 2,4-diaminotoluene in the rabbit, rat and guinea-pig. *Xenobiotica* **6,** 257.

2,4-Toluenediamine (*m*-toluenediamine; MTD) produced liver damage and hepatomas in rats when fed at levels of 0·06 or 0·1% in a semisynthetic diet for 36 wk (*Cited in F.C.T.* 1969, **7,** 700). However, when an oxidation hair-dye formulation containing MTD was applied to mouse skin once weekly or fortnightly for 18 months, there was no evidence of systemic toxicity or carcinogenicity (*ibid* 1975, **13,** 353). Recent work has shown that the 4-amino group of MTD is acetylated in the rat (Glinsukon *et al. Xenobiotica* 1975, **5,** 475), and the present investigation was undertaken to determine whether orally administered MTD gives rise also to hydroxylamines, aminobenzoic acids or aminophenols, which might account for its high toxicity.

Female rats, rabbits and guinea-pigs were given 50 mg MTD/kg by stomach tube, and urine was collected for the following 48 hr. All three species were found to excrete trace amounts of unchanged MTD (0·1–1·3% of the administered dose), with small amounts (0·7–2·3%) of the 4-acetyl derivative. Rats also produced traces of both the diacetyl and 2-acetyl derivatives of MTD. However, the predominant metabolites were phenolic in nature, with 5-hydroxyMTD accounting for as much as 22% of the dose in rabbits, 12% in rats and 9% in guinea-pigs. Rats and rabbits also converted appreciable amounts (8–10%) to the 3-hydroxy derivative, and another metabolite, identified as 6-hydroxyMTD, accounted for a further 5% of the dose in the rat. Also identified were the 3- and 5-hydroxy derivatives of 4-acetylMTD, with guinea-pigs excreting 17% of the dose as the latter and only traces as the former, while the corresponding excretion figures for rabbits were 6 and 10% and for rats 14 and 18%.

Large quantities of three different glucuronides were eliminated by all three species, accounting in total for 65% of the administered MTD in guinea-pigs, 44% in rabbits and 34% in rats, but their precise identity was not established. No hydroxylamines or aminobenzoic acids could be detected in the urine of any species, even after pretreatment with phenobarbitone (which increased the amounts of free phenols and glucuronides but decreased excretion of acetylated phenols).

The amount of methaemoglobin in the blood reached a maximum 6–8 hr after dosing, with levels of 3·7% in the guinea-pig, 6·0% in the rat and 20·4% in the rabbit. The authors state that this toxic effect correlates well with the total excretion of aminophenols (tabulated as some 26, 57 and 48% in the guinea-pig, rat and rabbit, respectively), but the rat data seem somewhat at variance with this statement. Animals that had received a previous dose of MTD, even as long as 6 months before, showed a much lower excretion of glucuronides, an effect which again was most pronounced in rabbits. The authors suggest, therefore, that metabolism of MTD to aminophenols may cause tissue damage, perhaps to the kidney as one might expect the liver to have regenerated during this time interval.

3162. A whiff of enzyme

Richards, D. E., Scheel, L. D. & Groth, D. H. (1975). An evaluation of the inhalation toxicity of one commercial proteolytic enzyme preparation. *Am. ind. Hyg. Ass. J.* **36,** 266.

Tolos, W. P., Richards, D. E. & Scheel, L. D. (1975). Histamine induction and release following proteolytic enzyme exposure. *Am. ind. Hyg. Ass. J.* **36,** 272.

Milne, J. & Brand, S. (1975). Occupational asthma after inhalation of dust of the proteolytic enzyme, papain. *Br. J. ind. Med.* **32,** 302.

Respiratory disturbances as well as skin irritation have been reported in people exposed to detergent powders containing proteolytic enzymes (Charlesworth, *Fd Cosmet. Toxicol.* 1976, **14,** 61).

The first paper cited here describes the exposure of guinea-pigs, rats and rabbits to different concentrations of the dust of a proteolytic enzyme preparation containing 12% subtilisin, inactive proteins, non-protein organic material and inorganic salts for periods of 6 hr. Only the guinea-pig showed a toxic response to exposure. No abnormal effects occurred with a concentration of 1 mg/m^3, but at concentrations above 4·2 mg/m^3 mild hyperactivity appeared from the start of exposure, followed by sneezing, salivation and laboured spasmodic breathing. Deaths occurred only in guinea-pigs that had been sensitized by intradermal injections of subtilisin (3 doses/wk for 3 wk, then a rest period of 2 wk prior to the experiment). The 6-hr LC_{50} for pretreated guinea-pigs was 24·7 mg/m^3. Treatment with diphenhydramine, an antihistamine, before exposure to the enzyme preparation protected these animals. In contrast, normal rats and normal or sensitized rabbits survived exposure to the powder in concentrations up to 36·8 mg/m^3. Lung changes seen at autopsy in sensitized guinea-pigs included severe diffuse congestion and oedema, with patchy haemorrhages covering more than half of the lung. In normal guinea-pigs, rabbits and rats, any initial lung lesions resolved apparently spontaneously.

In the study discussed in the second paper, groups of guinea-pigs were pretreated with a 12% subtilisin preparation, an alkaline protease prepared from *Aspergillus oryzae* or a non-proteolytic lipase mixture from cotton seed, given by intradermal injection in saline solution. Such pretreatment increased histamine concentrations in lung, liver and ear tissue. When given an intratracheal challenge with subtilisin, guinea-pigs pretreated with subtilisin showed a marked reduction in liver histamine concentration. Inhalation of subtilisin by any of the animals pretreated with any of the three intradermally injected preparations resulted in a fall in the histamine concentration in the lung and liver. These observations are presented in support of the view that histamine

release by a proteolytic enzyme is the result of metabolic changes in the mast cells.

The third paper reports that of four food technologists who were occupationally exposed to high concentrations of papain dust in air, two developed an acute asthmatic reaction followed by signs of obstructive airways disease that persisted as long as they remained exposed to papain. When all the subjects were tested 17 months after exposure, their ventilatory capacity was within normal limits. No evidence of emphysema was sought. Two of the subjects, one with and one without a history of allergy, showed a heightened bronchial reactivity when challenged with methacholine. In view of the emphysema that has been noted in experimental animals exposed to papain by inhalation, the minimal effects on respiratory function hitherto observed in people encountering high concentrations of papain dust seem remarkable.

3163. Negative Ames test for optical brighteners

McGregor, D. B. & Ainsworth, L. (1976). Lack of mutagenic activity in *Salmonella typhimurium* of four optical brighteners. *Mutation Res.* **40,** 169.

The reported induction of mutations in yeast by optical brighteners (Gillberg & Aman, *Mutation Res.* 1971, **13,** 149) was not confirmed in some later experiments (Kilbey & Zetterberg, *ibid* 1973, **21,** 73). Further support for the negative findings has now been obtained using the Ames test. The strains of *Salmonella typhimurium* used were TA1535 and TA1538, the former for detecting base-pair substitutions and the latter for frameshift mutagens.

Tests for the reversion of histidine-dependence in these two strains were carried out in the presence and absence of a rat-liver postmitochondrial supernatant as metabolic activator, and the optical brighteners tested were disodium 4,4′-bis-([4-anilino-6-morpholino-1,3,5-triazin-2-yl]amino)stilbene-2,2′-disulphonate, sodium 2-(4-styryl-2-sulphophenyl)-2*H*-naphtho-[1,2-*d*]triazole, disodium 4,4′-bis-([4-anilino-6-(*N*-methyl-*N*-2-hydroxyethylamino)-1,3,5-triazin-2-yl]-amino)stilbene-2,2′-disulphonate and disodium 4,4′-bis-(2-sulphostyryl)biphenyl.

Neither before nor after metabolic activation did any of the test substances show mutagenic activity to either bacterial strain, even at a concentration of 2 mg/plate. The possibility that penetration of the test materials into the bacteria was low is considered unlikely, since the cell-wall permeability in these strains is relatively high; moreover, penetration of these compounds into yeast cells has been demonstrated although the cell walls of yeasts are generally less permeable to foreign compounds than are those of bacteria.

TOXICOLOGY

3164. The role of the gut flora in toxicity

Reddy, B. G., Pohl, L. R. & Krishna, G. (1976). The requirement of the gut flora in nitrobenzene-induced methemoglobinemia in rats. *Biochem. Pharmac.* **25,** 1119.

The gut flora plays a major role in the reduction of *p*-nitrobenzoic acid in the rat, even when the compound is given parenterally (*Cited in F.C.T.* 1975, **13,** 485), as was demonstrated in a study in which the nitro-reductase activity of the gut contents was virtually eliminated by antibiotic pretreatment. Many other nitro compounds are also reduced by the gut flora, often to metabolites that are more toxic than the parent compound (*ibid* 1970, **8,** 120). The relationship between the reduction of nitrobenzene by the gut flora and methaemoglobin formation *in vivo* has now been investigated.

When nitrobenzene (200 mg/kg body weight in sesame oil) was injected ip into normal (control) rats, about 30–40% of the blood haemoglobin was converted to methaemoglobin within 1–2 hr. The same dose given to germ-free or antibiotic-pretreated rats produced no measurable methaemoglobinaemia even after 7 hr, but in initially germ-free animals acclimatized in a normal animal room for 7 days it induced methaemoglobinaemia to the same extent as in control rats. In liver, kidney and gut-wall homogenates, the rate of aniline formation from nitrobenzene did not differ significantly whether the tissues were taken from normal germ-free or acclimatized germ-free rats, being low in all cases (about 2–3 nmol aniline/mg protein/hr in the liver and gut wall and 0·5–0·8 nmol/mg protein/hr in the kidney). However, the gut contents from control and acclimatized germ-free rats produced aniline at a much faster rate (11–15 nmol/mg protein/hr), whereas in actual germ-free rats the nitro-reductase activity of the gut contents was virtually zero. It appears, therefore, that the gut flora present in normal and acclimatized animals was responsible for nitrobenzene reduction and subsequent methaemoglobin formation.

CANCER RESEARCH

3165. Azoxymethane and colon carcinogenesis

Narisawa, T., Wong, C.-Q. & Weisburger, J. H. (1976). Azoxymethane-induced liver hemangiosarcomas in inbred strain-2 guinea pigs. *J. natn. Cancer Inst.* **56,** 653.

Azoxymethane (AOM), on intrarectal instillation, produced a higher incidence of colonic tumours in germ-free than in conventional rats, indicating that metabolism by the gut flora was not a necessary prerequisite for carcinogenesis at this site (*Cited in F.C.T.* 1976, **14,** 79). Mice and hamsters, but not guinea-pigs,

were also shown to be susceptible to AOM-induced cancer of the colon (Weisburger, *Dis. Colon Rectum* 1973, **16,** 431). Tumours at this site have, however, developed in guinea-pigs after intrarectal instillation of *N*-methyl-*N*-nitrosourea (Narisawa *et al. J. natn. Cancer Inst.* 1975, **54,** 785), and a further attempt to induce colon cancer in this species with AOM has now been made.

Female guinea-pigs were instilled intrarectally with 1 mg AOM as a 0·2% aqueous solution twice weekly for 33 wk, and were subsequently maintained until death which occurred between wk 32 and 54. Treatment was temporarily discontinued in some animals which showed loss of weight and appetite and general weakness after 16 wk, and the total dose administered thus ranged from 52 to 64 mg. Autopsy revealed that 15 of the 16 guinea-pigs had developed multiple liver haemangiosarcomas, which had invaded and destroyed the surrounding liver tissue and which, in three animals, had metastasized to the lungs, spleen, adrenal glands and kidneys. In addition there was usually evidence of hepatocellular injury, fatty degeneration of the liver cells, post-necrotic fibrosis and bile-duct hyperplasia. No neoplasms were found in other organs, and preneoplastic changes were not observed microscopically in random sections of the large intestine.

As AOM requires metabolic activation to be carcinogenic (Weisburger, *loc. cit.*), the findings suggest that the colonic mucosa of the guinea-pig, unlike that of the rat, does not contain sufficient amounts of the required enzymes. On the other hand, AOM must be metabolized to an active form by specific cells in the guinea-pig liver. The high incidence of haemangiosarcomas in this study was unexpected, because such tumours have been reported previously only in rats given AOM together with an immunosuppressive treatment, while rats treated (sc) with AOM alone developed only colon cancer (Kroes *et al. Cancer Res.* 1975, **35,** 2651).

Fd Cosmet. Toxicol. Vol. 15, p. 265. Pergamon Press 1977. Printed in Great Britain

LETTER TO THE EDITOR

PHOTOTOXICITY OF FRAGRANCE MATERIALS

Sir,—In our report on phototoxicity testing (Forbes *et al. Fd Cosmet. Toxicol.* 1977, **15,** 55), we indicated that all but one of the listed phototoxic fragrance materials were derived from the botanical families Rutaceae or Umbelliferae. The exception was a sample of verbena oil (no. 75–141) from the family Verbenaceae. While the paper was in press we tested two other samples of verbena oil (no. 75-141NUR and no. 76-313); these samples were not phototoxic. At the same time, a phototoxicity test of an opopnax tincture (no. 72-260) was negative. Thus, verbena and opopnax belong to the category of generic names (along with lemon, lime, orange and bergamot) associated with both phototoxic and non-phototoxic samples.

The purpose of this letter is to emphasize that the phototoxic response to a given sample may be influenced by a number of factors, including the sources of the material and the method of preparation. Thus, the generic name is a helpful though not definitive indication of the phototoxic potential of a given sample. Additional information on specific batches of test materials (identified by code number) is available from the Research Institute for Fragrance Materials, Inc., 375 Sylvan Avenue, Englewood Cliffs, New Jersey 07632, USA.

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Fd Cosmet. Toxicol. Vol. 15, p. 267. Pergamon Press 1977. Printed in Great Britain

FORTHCOMING PAPERS

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

Feeding tests in rats on mixtures of nitrite with secondary and tertiary amines of environmental importance. By W. Lijinsky and H. W. Taylor.

Primary and secondary amines in the human environment. By G. B. Neurath, M. Dünger, F. G. Pein, D. Ambrosius and O. Schreiber.

Toxic synergism of methylmercury with sodium nitrite and ethylurea on reproduction and survival of progeny in rats. By J. E. Nixon.

A 2-year feeding study of instant coffees in rats. II. Incidence and types of neoplasms. By H.-P. Würzner, E. Lindström, L. Vuataz and H. Luginbühl.

Evaluation of acute and short-term administration of 2,4,5-trichlorophenoxyacetate with respect to renal proximal tubular transport. By F. J. Koschier and W. O. Berndt.

Mycotoxins in foodstuffs. X. Production of citrinin by *Penicillium chrysogenum* in bread. By J. Reiss.

Safety testing of alkyl polyethoxylate nonionic surfactants. I. Acute effects. By G. M. Benke, N. M. Brown, the late M. J. Walsh and R. B. Drotman.

Safety testing of alkyl polyethoxylate nonionic surfactants. II. Subchronic studies. By N. M. Brown and G. M. Benke.

Primary sensitization potentials of some halogenated salicylanilides and their cross-sensitivity in guinea-pigs. By C. W. Chung and A. L. Giles, Jr.

Ability of adult and foetal rat tissues to metabolize chlorinated fatty acids. By H. M. Cunningham and G. A. Lawrence. (Short paper)

Excretion and metabolism of 2,4,5,2′,5′-pentachlorobiphenyl in the squirrel monkey. By S. Holm. (Short paper)

Teratogenic evaluation of piperonyl butoxide in the rat. By G. L. Kennedy, Jr., S. H. Smith, F. K. Kinoshita, M. L. Keplinger and J. C. Calandra. (Short paper)

[*Contents continued*]

Aims and Scope

The Journal provides a wide-ranging service of general articles and abstracts on food and cosmetics toxicology. The Journal is primarily intended for the use of food scientists and technologists, manufacturers and administrators who neither read nor have access to the medical, pharmacological or toxicological literature. In addition, the journal will constitute a medium for the publication of reviews and original articles relating to the fields of interest covered by the British Industrial Biological Research Association.

Instructions to Authors

General. Authors from the United Kingdom should send *Original Papers and Reviews* to the Assistant Editor. All other papers and reviews should be sent to the appropriate Regional Editor, All 'Letters to the Editor' should be sent to the Editor and must be signed before they can be considered for publication.

Submission of a paper to the Editor will be held to imply that it reports unpublished original research, that it is not under consideration for publication elsewhere and that if accepted for the Journal, *Food and Cosmetics Toxicology*, it will not be published again, either in English or in any other language, without the consent of the Editor.

Forms of Papers Submitted for Publication. Papers should be headed with the title of the paper, the surnames and initials of the authors and the names and addresses of the institutions where the work was done. A shortened version of the title not exceeding 45 letters and spaces, to serve as a running title, should be supplied.

In general the text should be subdivided as follows: (a) Summary: brief and self-contained, not exceeding 3% of length of paper (b) Introductory paragraphs (c) Experimental (d) Results, presented as concisely as possible (e) Discussion (if any) and general conclusions (f) Acknowledgements and (g) References. Each subdivision should commence on a separate sheet. Manuscripts should be typewritten on *one side of the paper* and *double spaced*. At least *two copies* should be supplied (one original and one, or preferably two, carbon copies). Papers will be accepted in English, French and German.

References. These should be listed on a separate page, in alphabetical order and arranged as follows: author's name and initials, year, title of the journal [abbreviated according to the rules adopted in the *World List of Scientific Periodicals 1963*, 4th Ed., Butterworth & Co. (Publishers) Ltd. London], volume, first page number:

e.g. Hickman, J. R., McLean, D. L. A. & Ley, F. J. (1964). Rat feeding studies on wheat treated with gamma-radiation. **1.** Reproduction. *Fd Cosmet. Toxicol.* **2**, 15.

References to books should include the author's name followed by initials, year, title of book, edition, appropriate page number, publisher and place of publication:

e.g. Dow, E. & Moruzzi, G. (1958). *The Physiology and Pathology of the Cerebellum*. 1st Ed., p. 422. The University of Minnesota Press, Minneapolis.

The names of all the authors of papers to be cited should be given when reference is first made in the text. In cases where there are more than two authors subsequent citations should give the first-named author followed by the words *et al.*:

e.g. (McLaughlin, Bidstrup & Konstam, 1963); (McLaughlin *et al.* 1963).

Where more than one paper by the same author(s) has appeared in any one year, the references should be distinguished in the text and the bibliography by the letters, a, b etc. following the citation of the year:

e.g. 1943a, 1943b or (1943a,b).

Footnotes. These as distinct from literature references should be avoided as far as possible. Where they are essential, reference is made by the symbols * † ‡ § ‖ ¶ in that order.

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