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Research Section

FEEDING TESTS IN RATS ON MIXTURES OF NITRITE WITH SECONDARY AND TERTIARY AMINES OF ENVIRONMENTAL IMPORTANCE*

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(*Received 15 January 1977*)

Abstract—The possible formation of *N*-nitroso compounds *in vivo* from ingested secondary or tertiary amines and nitrite was tested with 13 amino compounds selected on the basis of their widespread availability to man. The amino compounds were administered to rats in the drinking-water with or without sodium nitrite. Survival rates differed little between the various groups and only a few of the amines, either alone or in combination with nitrite, induced a significant incidence of malignant tumours. Lucanthone gave rise to a 30% incidence of liver tumours when given alone but not when given in combination with nitrite. Chlordiazepoxide plus nitrite induced nervous system tumours in 10% of the animals, methapyrilene plus nitrite induced liver tumours in 30%, and dimethyldodecylamine plus nitrite induced tumours of the urinary bladder in 10% of the treated rats. These results provide further evidence that ingestion of secondary and tertiary amines together with nitrite can lead to the formation of significant amounts of carcinogenic *N*-nitroso compounds in the stomach.

INTRODUCTION

It has been amply demonstrated both *in vivo* and *in vitro* that under appropriate conditions, such as those existing in the stomach, secondary and tertiary amines can interact with nitrite to produce *N*-nitroso compounds (Eisenbrand, Ungerer & Preussmann, 1974; Lijinsky & Greenblatt, 1972; Mysliwy, Wick, Archer, Shank & Newberne, 1974; Sander, Schweinsberg & Menz, 1968; Sen, Smith & Schwinghamer, 1969). Several examples of tumour induction following simultaneous feeding of amines and nitrite have been reported (Greenblatt, Mirvish & So, 1971; Ivankovic & Preussmann, 1970; Sander, 1970; Sander & Bürkle, 1969; Taylor & Lijinsky, 1975a,b).

To amplify and extend these findings several amines have been studied. All the compounds selected either occur naturally or are widely used as drugs, food additives or agricultural chemicals, and all have been shown to react chemically to form carcinogenic *N*-nitroso compounds. Only a few of these amines have been tested before in combination with nitrite. The tests were by no means exhaustive; the number of animals was small for reasons of economy, and the concentrations used were limited by the tolerance of the rats for nitrite and also by the solubility of the compounds in water.

The compounds studied were arginine (an amino-acid), chlorpromazine and chlordiazepoxide (tranquillizers), cyclizine (an anti-motion-sickness drug), dimethyldodecylamine (an anti-suckering agent used on tobacco plants), dimethylphenylurea (or fenuron, a herbicide), hexamethylenetetramine (formerly used, together with its salts, as a urinary antiseptic), lucanthone (an antischistosomiasis agent), methapyrilene (an antihistaminic agent and a component of many over-the-counter cold remedies), methylguanidine (present in the body and a constituent of meat, particularly of partially putrified meat), piperidine (a cyclic secondary amine, produced in the body, occurring in foods and also used as a food additive), tolazamide (a hypoglycaemic drug taken instead of insulin by some diabetics) and trimethylamine oxide (a constituent of fish).

EXPERIMENTAL

Chemicals. The following compounds were generous gifts from the manufacturer: chlordiazepoxide (Hoffman-LaRoche, Nutley, N.J.), chlorpromazine hydrochloride (Smith, Kline & French Research Laboratories, Philadelphia, Pa), cyclizine hydrochloride (Burroughs-Wellcome, Research Triangle Park, N.C.), lucanthone hydrochloride (Sterling-Winthrop Pharmaceutical Co., Rensselaer, N.Y.), and tolazamide (Upjohn Company, Kalamazoo, Mich.).

Arginine, hexamethylenetetramine and piperidine hydrochloride were obtained from Aldrich Chemical Co., Milwaukee, Wisc. Dimethyldodecylamine, methylguanidine sulphate and trimethylamine oxide dihydrate were from Eastman Organic Chemicals, Rochester, N.Y. Methapyrilene was from Sigma Chemical Co., St. Louis, Mo., and sodium nitrite was from Fisher Chemical Co., Pittsburgh, Pa.

Dimethyldodecylamine was purified by fractional distillation (bp 138–140°C) and for conversion to the hydrochloride it was mixed with a slight excess of

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10 N-HCl and the semi-solid was allowed to dry in air for several days. Dimethylphenylurea was prepared as previously described (Elespuru & Lijinsky, 1973).

Preparation of test materials. Each of the amines was dissolved in distilled water (pH 6·8–7·0) at the concentration shown in Table 1 and, where appropriate, the requisite amount of sodium nitrite was added to the solution. In the case of lucanthone an immediate reaction occurred even at pH 7. and a precipitate appeared which was suspected of being the nitrite salt of the amine. A precipitate also appeared in the solution of chlordiazepoxide and nitrite: in view of the report that chlordiazepoxide reacts with nitrite in neutral solution to form the nitroso derivative of the drug (Walser, Fryer, Sternbach & Archer, 1974), we suspect that this was largely nitrosochlordiazepoxide. However, this was not pursued and the material was not analysed. For administration to the animals, the suspension was simply shaken before being put in the drinking-water bottles.

Animals and treatment. Each solution was given to a group of 15 male and 15 female Sprague–Dawley rats. The rats were 8–10 wk old at the beginning of the experiment, were housed three to a plastics cage and were fed Rockland rat diet *ad lib.* On the five working days of the week each cage of rats was given 60 ml of the solution to drink and on the remaining 2 days tap-water was given. Each of the amines was administered with nitrite and most were also administered alone, i.e. without nitrite, to similar groups of rats. The result of feeding 0·2% sodium nitrite solution for 2 yr to the group of 26 males and 30 females which formed the concurrent control, has been reported previously (Taylor & Lijinsky, 1975a). From the end of the treatment, which lasted in most cases for 50 wk but in some for as long as 90 wk, the animals were kept until they died spontaneously or until

Table 1. *Survival patterns of rats given amines and nitrite in the drinking-water*

Amine	Amine concn in water (%)	Concn of added sodium nitrite (%)	Length of treatment (wk)	Total amine dose/rat (g)	Total nitrite dose/rat (g)	Sex	No. of survivors at wk						
							0	30	50	70	90	110	130
Arginine	0·1	0·2	50	5	10	F	15	15	15	15	9	5	1
						M	15	15	15	14	11	8	3
Chlordiazepoxide	0·2	0·2	50	10	10	F	15	15	15	13	11	2	0
						M	15	15	13	13	13	7	0
Chlorpromazine	0·2	0	50	5	0	F	15	14	14	13	11	5	3
						M	15	15	15	15	15	12	3
	0·1	0·2	50	5	10	F	15	15	15	15	14	10	6
						M	15	15	15	15	14	9	3
Cyclizine	0·1	0·2	80	8	16	F	15	14	12	12	11	10	0
						M	15	15	15	14	13	9	0
Dimethyldodecylamine	0·18	0·2	80	14	16	F	15	15	15	15	8	4	0
						M	15	14	13	13	12	10	0
Dimethylphenylurea	0·1	0	50	5	0	F	15	15	15	15	12	9	3
						M	15	14	14	14	14	7	1
	0·1	0·2	50	5	10	F	15	15	14	13	10	3	0
						M	15	15	14	14	11	7	4
Hexamethylenetetramine	0·1	0	50	5	0	F	15	14	14	13	11	6	0
						M	15	15	13	11	9	5	0
	0·1	0·2	50	5	10	F	15	15	15	15	11	3	0
						M	15	14	14	13	10	3	0
Lucanthone	0·14	0	50	7	0	F	15	15	14	12	10	5	2
						M	15	10	6	5	4	2	0
	0·14	0·2	50	7	10	F	15	15	15	13	12	2	0
						M	15	14	14	14	12	4	1
Methapyrilene	0·1	0·2	90	9	18	F	15	15	15	15	14	11	5
						M	15	15	15	15	15	11	7
Methylguanidine	0·1	0·1	50	5	5	F	15	15	15	14	11	6	1
						M	15	15	15	14	13	5	1
Piperidine	0·09	0	50	4·5	0	F	15	15	15	15	11	7	1
						M	15	15	15	15	10	4	0
	0·09	0·2	50	4·5	10	F	15	15	15	15	10	3	1
						M	15	15	15	15	11	2	0
Tolazamide	0·1	0	50	5	0	F	15	13	13	12	11	9	3
						M	15	15	15	14	14	9	3
	0·1	0·2	50	5	10	F	15	14	14	14	13	7	1
						M	15	14	14	14	13	9	2
Trimethylamine oxide	0·08	0	50	4	0	F	15	15	15	14	11	2	0
						M	15	15	15	13	9	5	0
	0·08	0·2	50	4	10	F	15	15	15	15	11	3	0
						M	15	15	15	15	12	9	0

they became moribund and were killed. Each animal was subjected to a complete autopsy and the tissues were fixed for full histopathological examination.

RESULTS

A complete listing of all of the tumours found, together with their pathological description, has been given elsewhere (Lijinsky & Taylor, 1976) and will not be repeated here. We shall, instead, present a summary of the major findings.

There was no great difference between the survival in any of the groups treated with the amines and nitrite and the survival in those treated with sodium nitrite solution alone or with the amines themselves. In each case, many animals survived beyond 2 yr. The only exception was the group given lucanthone, which proved to be somewhat toxic and distasteful to the

Table 2. *Tumors induced in rats by feeding amines plus nitrite*

Treatment*	Sex	No. of rats autopsied	No. of animals with named tumours
Chlordiazepoxide + nitrite	F	15	Spinal cord tumor (1) Hepatoma (1) Liver cholangiocarcinoma (1) Pancreatic adenocarcinoma (1)
	M	15	Brain glioma (1) Malignant neurinoma of spinal nerves (1) Heart neurofibrosarcoma (1) Osteosarcoma of vertebrae (1) Liver cholangiocarcinoma (1) Lymphangiosarcoma of mandible (1) Skin keratoacanthoma (1)
Dimethyldodecylamine + nitrite	F	9†	Bladder papillomas (1) Kidney adenocarcinoma (1) Kidney haemangiosarcoma (1) Forestomach carcinoma (1) Pancreatic adenocarcinoma (1)
	M	15	Bladder transitional-cell carcinoma (1) Bladder leiomyosarcoma (1) Forestomach papilloma (2) Forestomach carcinoma (1) Hepatoma (1) Brain astrocytoma (1)
Lucanthone alone	F	15	Hepatoma (3) Hepatocellular carcinoma (1) Liver cholangiocarcinoma (1) Thyroid adenocarcinoma (1) Salivary gland adenocarcinoma (1)
	M	6‡	Hepatocellular carcinoma (1) Pancreatic islet-cell adenoma (2)
Methapyrilene + nitrite	F	14	Liver cholangiocarcinoma (4) Hepatocellular carcinoma (1) Liver haemangioendothelial sarcoma (1)
	M	15	Liver cholangiocarcinoma (1) Hepatocellular carcinoma (2) Spinal cord neurofibrosarcoma (1)
Sodium nitrite (0·2%; control group)	F	30	Thyroid adenoma (3) Thyroid adenocarcinoma (1) Multiple myeloma (1) Pancreatic islet-cell adenoma (1) Thymic lymphosarcoma (1) Squamous-cell carcinoma of ear (1)
	M	26	Thyroid adenoma (2) Thyroid adenocarcinoma (2) Brain ependymoma (1) Pancreatic islet-cell adenoma (2) Pancreatic islet-cell adenocarcinoma (2) Hepatocellular carcinoma (1) Parotid-gland squamous-cell carcinoma (1) Forestomach carcinoma (1) Adenocarcinoma of gut (2)

*For dose levels of amines and nitrite see Table 1.
†Two cages of three animals died from lack of water and were accidentally discarded without autopsy.
‡Nine animals died of dehydration during the first 6 months and were not autopsied.

animals. They tended to refuse to drink the solution and it was forced on them, the supply of plain water being withheld at the weekends; nevertheless, nine of the males died of dehydration rather than drink the solution. In all other cases the animals drank virtually all of the solution offered to them and thus the dose could be estimated reasonably well.

The great bulk of the tumours found in these animals, particularly those living beyond 2 yr, were tumours of endocrine organs, mammary tumours, adrenal tumours, pituitary tumours and tumours of the gonads, which are commonly found in old animals of this strain. The incidences varied somewhat but no significant differences were seen. However, in several of the groups there were scattered tumours rarely seen spontaneously in these rats, most notably liver tumours and kidney tumours. In few cases were the numbers of these tumours very large, but in three of the combined treatments, namely nitrite with methapyrilene, with dimethyldodecylamine and with chlordiazepoxide, the numbers of some tumours suggested an induction of tumours by a nitrosamine formed *in vivo* from the amine and nitrite (Table 2). These findings complement the previously reported results with aminopyrine, oxytetracycline and heptamethyleneimine hydrochloride combined with nitrite. These showed a significant incidence of tumours with heptamethyleneimine and aminopyrine (Taylor & Lijinsky, 1975a,b) and a marginally significant number of liver tumours with oxytetracycline hydrochloride (Taylor & Lijinsky, 1975b). The previous report of the test on oxytetracycline showed the presence of four liver tumours in a group of 30 animals. The three tumours of the central nervous system in rats treated with chlordiazepoxide and nitrite represent more such tumours than would be expected in a group of rats even larger than 30. Similarly the three bladder tumours seen in the animals treated with dimethyldodecylamine and nitrite are tumours that have never been observed spontaneously in our Sprague–Dawley rats. Six of the females in this group were discarded accidentally, having died of lack of water. The incidence of tumours induced in the liver by methapyrilene and nitrite is great enough to be highly significant. A large number of animals in this group, almost 50%, showed necrotic and other degenerative changes in the liver which often accompany, or precede, the development of frank neoplasia in animals treated with such well-known liver carcinogens as dimethylnitrosamine and diethylnitrosamine. In addition, there were nine liver tumours, five of which were cholangiocarcinomas.

One surprising finding was the high incidence of liver tumours (six in all) in the group treated with lucanthone hydrochloride alone (Table 2), although all but six of the males died of dehydration early in the experiment. This again is an incidence of tumours far in excess of the number to be expected in untreated animals. Indeed, in 56 control animals treated with sodium nitrite, only one hepatocellular carcinoma was observed, and incidences of 1% have been the usual experience with this strain of rat.

DISCUSSION

Although it is known that several of the tertiary amines administered with nitrite to rats do react to form *N*-nitroso compounds in weakly acid solution (Lijinsky, 1974; Lijinsky, Keefer, Conrad & Van de Bogart, 1972), it appears that in most cases insufficient nitroso compound was formed in the rats' stomachs during the long-term feeding described here to give rise to tumours. On the other hand, the results with three of the compounds are sufficiently positive to add them to the list of amines already known to form *N*-nitroso compounds in tumorigenic quantity when fed for a prolonged period with nitrite to rodents. These include methylbenzylamine (Sander & Bürkle, 1969), morpholine (Sander & Bürkle, 1969), piperazine (Mirvish, 1971), heptamethyleneimine (Taylor & Lijinsky, 1975a), aminopyrine (Taylor & Lijinsky, 1975b), oxytetracycline (Taylor & Lijinsky, 1975b) and a variety of alkylureas (Ivankovic & Preussmann, 1970; Sander, 1970). Of these, morpholine (Singer & Lijinsky, 1976), piperazine, aminopyrine and oxytetracycline are certainly of environmental significance, the latter two being drugs widely used by man.

Although there were only three tumours of the nervous system in the 30 rats given the chlordiazepoxide plus nitrite solution, which contained, as mentioned above, some nitroso derivative of the drug formed *in vitro*, these tumours are sufficiently rare to support the assumption of a carcinogenic effect of the combination. It is known from previous chemical studies that chlordiazepoxide reacts readily with nitrous acid to give a variety of products (Walser *et al.* 1974). A firm conclusion should not be drawn from the results of this small test, however: instead the experiment should be repeated in a larger group of animals, and should possibly involve the feeding of a combination of the drug with nitrite mixed in dry food.

The three bladder tumours in rats given dimethyldodecylamine plus nitrite must be ascribed to the treatment, since urinary bladder tumours have never been seen in any rats of our colony other than those treated with nitrosomethyldodecylamine (Lijinsky & Taylor, 1975a). Earlier chemical studies showed that formation of nitrosomethyldodecylamine with nitrous acid occurred readily (Lijinsky *et al.* 1972) and this reaction can reasonably be inferred to have occurred in the rats' stomachs in the test described here. Of all the dimethylamine derivatives examined, one of the most reactive with nitrous acid was methapyrilene (Lijinsky & Singer, 1974). This chemical finding was confirmed by the results of the tests reported here, in which feeding of methapyrilene and nitrite gave rise to a significant incidence of liver tumours and other liver lesions, due, presumably, to the dimethylnitrosamine formed in the stomach. This result confirms an earlier opinion that taking this over-the-counter drug when interaction with nitrite in the stomach is possible could expose people to significant amounts of the strongly carcinogenic dimethylnitrosamine.

On the other hand, several of the amines administered with nitrite did not lead to significant incidences of induced tumours in rats. This means only that, in these tests, insufficient nitroso compound was formed to induce tumours, but it is not proof that no such reaction took place *in vivo*.

Some of the negative results are surprising. For example, the failure of a combination of piperidine

hydrochloride plus nitrite to induce the typical tumours found in rats after treatment with nitrosopiperidine (Lijinsky & Taylor, 1975b) contrasts with the high incidence of tumours of the lung and oesophagus following feeding of rats with a mixture of the homologue heptamethyleneimine hydrochloride plus nitrite (Taylor & Lijinsky, 1975a). However, these results do confirm an earlier experiment in which piperidine as the free base plus nitrite also failed to induce tumours when administered to rats in water (Garcia & Lijinsky, 1973). It is probable that the relatively strong basicity of piperidine reduced the rate of reaction with nitrite to such an extent that an ineffective amount of nitrosopiperidine was formed. The same conclusion can be drawn from the results of feeding trimethylamine oxide plus nitrite, although formation of dimethylnitrosamine from trimethylamine oxide and nitrous acid occurs readily at higher concentrations (Lijinsky *et al.* 1972; Lijinsky & Singer, 1974).

Although arginine reacts with nitrous acid, no stable products have been isolated and, in particular, no nitroso derivative has been identified (Lijinsky & Epstein, 1970). Therefore, the lack of tumour response in rats fed arginine plus nitrite suggests that no carcinogenic product is formed under the conditions of our test, a finding that supports a previous observation (Garcia & Lijinsky, 1973).

Studies of the chemical reaction between methylguanidine and nitrous acid have led to the conclusion that two potent carcinogens, nitrosomethylurea and nitrosomethylcyanamide (Mirvish, 1971) are the products. Feeding methylurea and nitrite, or nitrosomethylurea, to rats at quite low concentrations has led to a high incidence of tumours (Ivankovic & Preussmann, 1970). The lack of tumour induction by the very much higher concentrations of methylguanidine plus nitrite used in our present tests indicates either that the chemical calculations reported previously (Mirvish, 1971) are incorrect in their prediction of the amount of nitroso compounds formed, or that the reaction demonstrated in a chemical system does not take place *in vivo*. That this is a possibility was demonstrated by previously unreported experiments in which it was not possible to isolate the products of reaction of methylguanidine with nitrous acid, but the absorption spectrum of the product was that of a nitrosoalkylguanidine (and was identical with that of the stable methylnitrosonitroguanidine) rather than of a nitrosoalkylurea. The product in solution was potently mutagenic to bacteria after partial neutralization (to pH 6), its activity being similar to that of methylnitrosonitroguanidine rather than to that of methylnitrosourea, which was much lower (R. K. Elespuru, unpublished results 1975).

The herbicide, dimethylphenylurea, given with nitrite did not induce tumours in rats, when administered either alone or with nitrite, although formation of dimethynitrosamine by its interaction with nitrite took place readily in acid solution (Elespuru & Lijinsky, 1973). Similarly, hexamethylenetetramine did not induce tumours by itself in rats, confirming an earlier report of its non-tumorigenicity (Della Porta, Colnaghi & Parmiani, 1968), or when given with nitrite, although it interacts with nitrite in acid solution to form at least two nitrosamines (Bachmann & Deno, 1951).

Neither tolazamide nor lucanthone induced significant incidences of tumours when administered to rats together with nitrite, although the formation of nitrosamines by their reaction with nitrite in acid solution has been demonstrated (Lijinsky, 1974). Tolazamide alone did not give rise to any tumours under these conditions. However, lucanthone given to rats alone in drinking-water gave rise to a significant incidence of liver tumours and must be assumed to be a liver carcinogen in the Sprague–Dawley rat. It was noticed that the drug was distasteful to the animals, which suffered considerable dehydration for a time at the beginning of the treatment through reluctance to drink the solution given to them. A small number of the animals of this group died early because of this, but the remainder accommodated later and drank sufficient solution to survive.

Our conclusion is that nitroso derivatives are formed *in vivo* by reaction of ingested secondary and tertiary amines with nitrite. In some cases, as with aminopyrine, oxytetracycline, chlordiazepoxide, dimethyldodecylamine and methapyrilene, sufficient carcinogenic nitroso derivative is formed to give rise to a significant incidence of malignant tumours in the small groups of rats used in our experiments. In other cases, the amount of nitroso compound formed was too small to give rise to a significant tumour incidence within the animals' lifetime. These tests, however, provide no assurance of safety for people ingesting these compounds when nitrite, from food or in saliva, is present in the stomach. In particular, we have not considered the possible augmentation of tumorigenic effect by interaction with nitrite of several such amines present at one time, or by interaction of one carcinogenic nitroso compound with another. A further concern is that other drugs, possibly as freely available to the general public as is methapyrilene, might react as readily, or perhaps more readily, with nitrite in the stomach to form carcinogenic nitroso compounds. This emphasizes the necessity to reduce exposure to nitrite and to nitrosatable amines to the minimum consistent with safety and health, and the need to test all widely used nitrosatable amines, by feeding with nitrite to animals, before certifying their safety.

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PRIMARY AND SECONDARY AMINES IN THE HUMAN ENVIRONMENT

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Abstract—Altogether 40 primary and secondary amines with different gas-chromatographic properties have been detected in samples of fresh vegetables, preserves, mixed pickles, fish and fish products, bread, cheese, stimulants, animal feedstuffs and surface waters, and 21 of these have been identified by mass spectrometry. Secondary amines, the precursors for the carcinogenic *N*-nitrosamines were generally found in concentrations below 10 ppm, although higher concentrations occurred in herring preparations, some cheese and samples of large radish and red radish. Besides dimethylamine and diethylamine, the most prevalent secondary amines were found to be pyrrolidine, piperidine, *N*-methylbenzylamine, *N*-methylaniline and *N*-methylphenethylamine, the latter apparently being the most widespread in foods of plant origin. The highest content of secondary amines found so far was in red radishes (38 ppm pyrrolidine, 20 ppm pyrroline 5·4 ppm *N*-methylphenethylamine and 1·1 ppm dimethylamine). Concentrations of secondary amines found in surface waters have generally been below 15 ppb (15 μg/kg).

INTRODUCTION

Although evidence for the carcinogenic activity of *N*-nitroso compounds in man is lacking, it is generally assumed that this class of compounds is active in man, since some 80% of all the *N*-nitroso compounds tested in many animal species have been shown to induce tumours.

Therefore, following demonstrations of nitrosamines in various environmental media, epidemiological research has focussed on the search for correlations between the incidence of various types of cancer and the presence of *N*-nitroso compounds in foodstuffs, stimulants and other components of the environment, and there is some evidence for the occurrence of these compounds as endemic factors in particular localities (Bogovski, 1972). Nitrosamines, however, can be formed only in the presence of secondary or tertiary amines on the one hand and nitrite or nitrate ions or nitrogen oxides on the other.

Since it was shown by Sander (1971) that nitrosamines can be formed from their precursors in the animal organism, it has been clear that a thorough knowledge of the occurrence of amines in the human environment is desirable. The probable extent of nitrosamine formation could then be assessed from an evaluation of the amine content of the various materials and this would be a useful tool in cancer epidemiology.

Only a small number of investigations on the occurrence of amines in foods and stimulants have been published and there have been fewer still on the occurrence in air and water.

Amines in plants were first discussed by Steiner & Stein von Kamienski (1953) and Stein von Kamienski (1957), while Drawert (1965) reported on the amines in wine. The results of analyses of several foods and stimulants were cited by Möhler, Mayrhofer & Hallermayer (1972), and sablefish, salmon and salmon roe have been investigated by Gruger (1972), but in both of these cases the data were restricted to dimethylamine and diethylamine. Several monoalkylamines have been reported to be present in Emmentaler cheese (Ney & Wirotama, 1972). Singer & Lijinsky (1976a) found morpholine and dimethylamine to be ubiquitous and reported finding piperidine and pyrrolidine, as well as smaller amounts of other amines, in plant-derived material.

Tobacco and its smoke have been thoroughly analysed for amines liable to promote nitrosamine formation (Neurath, 1969; Neurath, Dünger, Gewe, Lüttich & Wichern, 1966; Neurath, Krull, Pirmann & Wandrey, 1966; Pailer, Hübsch & Kuhn, 1967; Pailer, Völlmin, Karnincic & Kuhn, 1969; Singer & Lijinsky, 1976b) and the occurrence of pyrrolidines and piperidines, as well as of methylalkylamines, has been noted. According to Alliston, Cox & Kirk (1972), *N*-nitrosopyrrolidine occurs in many foodstuffs, a fact that underlined the necessity for more research on amines in the human environment.

EXPERIMENTAL

Test samples. Food products were generally purchased from retail outlets, some fruits and vegetables were freshly harvested, animal feeds were obtained from the manufacturers and samples of surface waters were freshly collected.

Amine determinations

The analytical procedure used was based on the formation of the trifluoroacetamides of the primary and secondary amines, followed by gas-chromatographic separation, preliminary identification and quantitative analysis using an integrator. Identifications were confirmed by mass spectrometric comparison with authentic samples. This procedure was first reported by Pailer & Hübsch (1966) and was adapted to meet the needs of our study.

Table 1. *Primary and secondary amines found in samples of fresh vegetables*

Amine	Levels (mg/kg) found in samples of											
	Spinach	Red cabbage	Cabbage*	Cauliflower	Kale	White beet	Swede†	Carrots	Red beet	Large radish	Red radish	Celery
Ammonia	18280	11060	3800	6376	15260	15400	2490	3970	8800	7865	8450	19600
Methylamine	12	22·7	3·4	65	16·6	17·6	—	3·8	30	42	—	6·4
Ethylamine	8·4	1·3	—	—	—	4·3	—	1	—	10	40	—
Dimethylamine	—	2·8	2	14	5·5	—	—	—	—	—	1·1	5·1
Methylethylamine	—	0·9	—	—	—	7·6	—	7	—	—	—	—
n-Propylamine	—	—	—	—	—	—	5	—	—	—	—	—
Diethylamine	15	—	—	—	—	—	—	—	—	—	—	—
n-Butylamine	—	—	—	—	7	—	—	—	—	—	—	—
Pyrroline	—	—	—	—	—	—	—	—	—	—	20	—
n-Pentylamine	0·3	0·6	1·4	3·3	0·4	—	1	—	—	—	6·9	0·8
Pyrrolidine	2·5	—	—	—	—	—	—	—	—	—	38	0·4
Isopentylamine	3·8	—	—	—	0·5	7·6	5	—	—	—	—	—
Aniline	—	1·0	4	22	0·7	1·2	—	30·9	0·6	2·8	4·6	0·7
N-Methylaniline	3·4	0·3	—	—	—	—	—	0·8	—	—	—	0·5
Toluidine	—	—	—	—	1·1	—	—	7·2	—	—	—	1·1
Benzylamine	6·1	3·3	2·8	1·4	3·8	5·3	—	2·8	0·1	1·8	4·8	3·4
Phenethylamine	1·1	8·6	2·1	1·8	3	1·3	40	2	0·3	1·2	0·2	—
N-Methylbenzylamine	—	—	—	—	—	—	—	16·5	—	—	—	—
N-Methylphenethylamine	2·4	3·7	0·5	1·6	2	1·6	2	2	0·4	6·6	5·4	0·5
NO_3 content	3100	600	80	150	200	4200	525	500	5300	5300	1900	400

*Methylmercaptopropylamine (30 mg/kg) and ethylmercaptopropylamine (10 mg/kg) identified by mass spectrometry on OV 11 columns.
†Four further primary amines.
Amines are listed in the sequence of the retention times of their trifluoroacetamides on SE 30 columns.

Table 2. *Primary and secondary amines found in samples of maize, salad and fruit*

Amine	Levels (mg/kg) found in samples of				
				Apple	
	Maize, grains	Green salad	Rhubarb	Flesh	Peel
Ammonia	10030	10260	6340	235	1029
Methylamine	26·8	37·5	—	5·6	4·5
Ethylamine	2·4	3·3	—	3	—
Dimethylamine	3·5	7·2	—	—	—
Methylethylamine + diethylamine	20*	7·5	—	—	—
Isopropylamine	2·3	—	—	—	—
Diethylamine	—	—	—	3	—
Isopentylamine	—	—	3·9	—	—
n-Pentylamine	—	3	—	0·3	—
Pyrrolidine	3·5	—	—	—	1·5
Aniline	0·6	0·6	5	1·5	1·7
Benzylamine	3·4	11·5	2·9	0·3	0·6
Phenethylamine	—	—	3·2	—	—
N-Methylbenzylamine	—	10	—	—	—
N-Methylphenethylamine	1·1	0·4	2·6	1·2	1·3
NO_3 content	20	1600	—	40	40

*Not resolved.
Amines are listed in the sequence of the retention times of their trifluoroacetamides on SE 30 columns.

Preparation of the trifluoroacetamides. The amines were isolated by steam distillation of 50–500 g of the material under investigation after addition of sufficient water and 100 ml 50% aqueous potassium hydroxide. The distillate (500 ml) was trapped in 100 ml 10% hydrochloric acid. Neutral materials were washed out twice with 50 ml dichloromethane, and the water phase was evaporated to dryness under vacuum. Trifluoroacetic acid anhydride (3–20 ml) was then added dropwise to the residue. The trifluoroacetamides of the primary and secondary amines were then separated from any tertiary amines on an ion-

Table 3. *Primary and secondary amines found in samples of preserved vegetables*

Amine	Levels (mg/kg) found in samples of									
	Broken beans	Broken butter-beans	Shelled peas	Bean salad	Mush-rooms	Kale	Red cabbage	Pickled cabbage	Paprika red	Corni-chons
Ammonia	142	327	173	296	—	129	386	214	104	144
Methylamine	—	—	—	0·1	—	—	—	9	—	—
Methylamine + *n*-propylamine	—	1·2	0·4	—	—	—	0·5	—	—	—
Methylamine + *n*-propylamine + piperidine	1·2	—	—	—	—	2·7	—	—	0·4	0·6
Ethylamine	<0·1	0·8	0·1	<0·1	—	0·3	0·1	—	0·1	0·1
Dimethylamine	0·6	<0·1	2·2	0·2	*	4·5	0·1	—	1	0·5
Diethylamine	<0·1	2·4	<0·1	1·5	*	—	2·4	—	0·5	0·1
Isopentylamine	—	—	1	—	*	—	0·1	—	—	—
n-Pentylamine	—	—	0·1	—	*	—	0·2	—	—	—
Pyrrolidine	0·2	—	—	<0·1	*	1·6	—	—	0·6	0·1
Piperidine + pyrrolidine	—	—	0·1	—	—	—	0·5	—	—	—
Piperidine	—	0·1	—	—	—	—	—	—	—	—
Di-*n*-propylamine	1	—	0·1	—	*	—	0·1	—	0·1	—
Aniline	<0·1	—	—	0·1	—	—	—	—	—	—
Toluidine	—	—	0·4	—	—	—	0·2	—	—	—
o-Toluidine	<0·1	—	—	—	—	—	—	—	—	—
Phenethylamine	—	—	—	—	—	—	0·2	1	—	—
Benzylamine	—	—	—	—	—	—	0·1	—	—	—
N-Methylbenzylamine	0·1	—	—	—	—	—	—	—	—	—
N-Methylphenethylamine	—	—	<0·1	—	—	—	0·1	—	—	0·1

*Quantity not determined; nine further components have been recognized as amines but not identified so far.
Amines are listed in the sequence of the retention times of their trifluoroacetamides on SE 30 columns.

Table 4. *Primary and secondary amines found in samples of pickles*

Amine	Levels (mg/kg) found in samples of											
	Paprika	Paprika brine (mg/litre)	Cucumber in aromatic vinegar	Cucumber in aromatic vinegar brine (mg/litre)	Peperoni	Peperoni brine (mg/litre)	Cucumber pickled with mustard	Cucumber pickled with mustard brine (mg/litre)	Pickled onions	Pickled onions brine (mg/litre)	Celery	Celery brine (mg/litre)
Ammonia	382	10	13050	37200	2660	212·2	8980	610·3	7130	401·5	15480	680
Dimethylamine	—	—	15·4	0·01	—	—	—	—	1	—	—	—
n-Propylamine	2·3	10·6	7·5	0·1	1·4	—	—	—	1·8	0·05	2·7	0·3
Diethylamine	—	—	1·4	0·01	—	0·1	—	—	3·2	0·05	—	—
n-Butylamine	—	—	0·6	0·06	—	—	5·3	0·2	—	0·4	—	—
n-Pentylamine	3	1·6	1·7	0·02	—	—	2·8	0·1	0·5	0·05	—	—
Pyrrolidine	1·4	8·4	5·6	0·05	1·8	0·2	1·5	0·5	8·4	0·5	2·6	0·6
Di-*n*-propylamine	0·3	2	1·4	0·01	—	—	1·2	—	1·1	0·2	0·9	0·1
Piperidine	5·2	5·6	—	—	3·4	0·4	—	—	—	—	1	0·1
N-Methylaniline	13·1	25·6	13·8	0·78	—	—	6·1	1·1	6·8	0·2	7	0·7
Di-*n*-butylamine	—	—	—	—	3·4	0·7	—	—	—	—	—	—
N-Methyl-phenethylamine	—	—	2·2	—	—	0·3	7·3	1·1	6·5	—	—	—
n-Dodecylamine	—	—	2·7	1·9	6·4	0·7	—	—	3·6	0·1	—	—
NO_3 content	1297	258	2688	509	2295	694	1520	416	2039	509	1150	247

Amines are listed in the sequence of the retention times of their trifluoroacetamides on SE 30 columns.

Table 5. *Primary and secondary amines found in samples of fish, cheese and bread*

Amine	Levels (mg/kg) found in samples of								
	Herring				Cod roe	Cheese			Brown bread
	Bismarck	Salted	Smoked	In oil		Tilsiter	Camembert*	Limburger†	
Ammonia	1570	2928	270	—	270	164400	—	—	13880
Methylamine	2	3·4	—	7	10·3	—	12	3	—
Methylamine + *n*-propylamine	—	—	10·3	—	—	—	—	—	—
Ethylamine	—	0·1	0·4	—	0·4	—	4	1	—
Dimethylamine	3·4	7·8	6·3	45	6·3	—	—	—	3·1
Methylethylamine	—	—	—	1	—	—	—	—	—
n-Propylamine	—	—	—	—	—	8·7	2	2	1·6
Diethylamine	0·1	1·9	5·2	—	5·2	—	—	—	—
Isobutylamine	—	—	0·3	—	0·3	—	0·2	0·2	—
n-Butylamine	—	—	—	—	—	3·7	—	—	1·1
Isopentylamine	—	—	—	—	—	—	0·2	trace	—
n-Pentylamine	—	—	—	17	—	1·2	—	—	0·2
Pyrrolidine	—	—	—	—	—	19·9	1	0·1	0·3
Di-*n*-propylamine	—	—	—	—	—	8·4	—	—	0·4
Piperidine	0·3	0·7	0·2	—	0·2	—	—	trace	—
N-Methylaniline	—	—	—	—	—	37·9	—	—	0·7
N-Methyl-benzylamine	—	—	—	2	—	—	—	—	—
N-Methyl-phenethylamine	—	—	—	0·1	—	2·6	—	—	—
n-Dodecylamine	—	—	—	—	—	—	—	—	0·9
NO_3 content	—	—	—	—	—	444	—	—	651

*Four other amines, not identified.
†Among the five further amines, which are not yet identified, there are *C*-alkylpiperidines and *C*-alkylpyrrolidines.
Amines are listed in the sequence of the retention times of their trifluoroacetamides on SE 30 columns.

exchange column (100 ml Amberlyst 15, acidic form; ID 3 cm; length 25 cm) by elution of the trifluoroacetamides with ether (300 ml) and subsequent elution of the tertiary amines with 200 ml 10% hydrochloric acid and 50 ml water. The solution of the trifluoroacetamides was evaporated to less than 10 ml and made up to 10 ml with ether. Trifluoroacetamides of 40 amines were prepared from authentic samples.

Gas chromatography of the trifluoroacetamides. The operating conditions were as follows: Varian Aerograph 1445 equipped with Hewlett-Packard Integrator 3380; columns—6 m packed glass column, 2 mm ID, 10% SE 30 on Gaschrom Q, 80–100 mesh; temperature—8 min at 90°C, then programmed at 8 degrees/min from 90 to 260°C and finally 20 min at 260°C; carrier gas—15 ml nitrogen/min, injector 180–280°C, detector—flame-ionization, 300°C, 40 ml hydrogen/min, 500 ml air/min; injected solution—2 μl. The limits of detection for the different amines varied slightly, being, for example, 5 ng for methylamine, 4 ng for *n*-octylamine and 1–15 ng for ammonia. Two runs were generally performed. In one run, 105·5 μg *n*-heptylamine as the trifluoroacetamide was added to one tenth of the amine fraction as an internal standard, since it had not been found in the environment; this permitted the quantitative evaluation of the fractograms as well as the determination of retention times relative to that for *n*-heptylamine (t_R = 1000).

Evaluation of the fractograms. The primary and secondary amines used as standards were chosen on the grounds that they occurred in the environment or were very likely to be formed, e.g. by decarboxylation of the respective amino acids or methylation of a precursor, that they were separable by gas chromatography to facilitate the evaluation, and that they covered a broad spectrum of chemical properties. On these bases, ammonia, methylamine, dimethylamine, diethylamine, *n*-propylamine, isopropylamine, di-*n*-propylamine, 2-aminobutane, isobutylamine, *tert*-butylamine, diisobutylamine, 3-methylbutylamine, *n*-pentylamine, (*n*-heptylamine), *n*-octylamine, *N*-methylaniline, *N*-methylbenzylamine, *N*-methylphenethylamine, benzylamine, phenethylamine, piperidine and pyrrolidine were selected as standards.

Nitrate determinations

Levels of nitrate were determined by means of an ion-specific electrode (Orion) in selected samples of fresh and preserved fruit and vegetables and in some samples of cheese and bread.

RESULTS AND DISCUSSION

The amines identified in the various test samples and the concentrations found are summarized in Tables 1–8.

Forty primary and secondary amines with different gas-chromatographic properties have been detected in samples of fresh vegetables, preserves, mixed pickles, fish and fish products, bread, cheese, stimulants, animal feedstuffs and surface waters, and 21 of these have been identified by mass spectrometry. Secondary amines, the precursors for the carcinogenic *N*-nitrosa-

Table 6. *Primary and secondary amines found in samples of stimulants*

Amine	Levels (mg/kg) found in samples of									
	Coffee extract	Coffee I freeze-dried*	Coffee II freeze-dried*	Cocoa defatted†	Black tea‡	Wine white I§	Wine white II‖	Barley	Hops	Malt
Ammonia	820	760	560	1150	400	56	69	8130	10660	1192
Methylamine	27	80	16	60	50	—	—	4·5	3·7	<0·1
Ethylamine	2	2	1·5	—	—	—	—	3·4	5·2	0·3
Dimethylamine	4	6	3	—	—	—	—	1·6	1·4	0·5
Methylethylamine	1	2	0·5	—	—	—	—	—	3·7	<0·1
n-Propylamine	0·5	trace	0·2	—	—	—	—	—	—	—
Diethylamine	—	—	—	—	—	—	—	5·7	3·1	0·6
Isobytylamine	1	1	1	6	—	—	—	—	—	—
Isopentylamine	1	1	1	10	—	2	3	—	0·4	—
n-Pentylamine	2	15	10	—	—	—	—	—	—	—
Pyrrolidine	10	11	7	0·5	—	—	—	0·9	1	1·5
Piperidine	2	2	1	9	—	—	—	1	2·5	<0·1
Phenethylamine	—	—	—	—	—	2	2	—	—	—
N-Methylbenzylamine	—	—	0·2	—	—	—	—	—	—	—

*And three other amines.
†And four other amines in concentrations between 4 and 50 mg/kg, probably including *C*-alkylpyrrolidines.
‡And five other amines in measurable concentrations.
§And 13 other amines between 0·06 and 0·6 mg/kg.
‖And 15 other amines between 0·06 and 0·6 mg/kg.
Amines are listed in the sequence of the retention times of their trifluoroacetamides on SE 30 columns.

Table 7. *Primary and secondary amines found in samples of animal feeding stuffs*

Amine	Levels (mg/kg) found in samples of			
	Soyabean, residual oilseed*	Rapeseed cake†	Linseed cake‡	Cocosexpeller§
Ammonia	8600	3100	3000	390
Methylamine	50	550	—	7
Ethylamine	0·5	—	—	—
Dimethylamine	8	—	—	1·2
Isopropylamine	trace	—	—	—
n-Butylamine	1	—	—	—
Isopentylamine	—	140	—	—
Aniline	—	120	—	—
Phenethylamine	—	90	—	—
N-Methylphenethylamine	50	—	—	—

*And one other amine.
†And six other amines in concentrations between 5 and 20 mg/kg.
‡Six amines that can be identified only by mass spectrometry.
§And four other amines in concentrations between 0·2 and 5 mg/kg.
Amines are listed in the sequence of the retention times of their trifluoroacetamides on SE 30 columns.

mines, were generally found in concentrations below 10 ppm, although higher concentrations occurred in herring preparations, some cheese and samples of large radish and red radish. Besides dimethylamine and diethylamine, the most prevalent secondary amines were found to be pyrrolidine, piperidine, *N*-methylbenzylamine, *N*-methylaniline, and *N*-methylphenethylamine, the latter apparently being the most widespread in foods of plant origin. The highest content of secondary amines found so far was in red radishes (38 ppm pyrrolidine, 20 ppm pyrroline, 5·4 ppm *N*-methylphenethylamine and 1·1 ppm dimethyl-

Table 8. *Primary and secondary amines found in samples of surface waters*

Amine	Levels (μg/kg) found in samples of								
	River Elbe I	River Elbe II	River Alster*	River Stör†	River Au near Hetlingen‡	River Krückau§	River Pinnau‖	River Ammersbek	Timmermoor Swamp
Ammonia	—	30	1210	525	600	2680	2610	7090	339
Methylamine	—	—	20·6	3·7	~1	1	9·3	2·3	6·2
Methylamine + propylamine	—	30·5	—	—	—	—	—	—	—
Ethylamine	—	16·2	2	6	~1	5	5	37·1	0·6
Dimethylamine	—	9·2	1·1	—	2–3	1	0·1	11·9	—
n-Propylamine	—	2·9	—	—	—	—	—	—	—
Diethylamine	—	9	1·6	8·2	14	0·5	1·8	7·1	—
n-Butylamine	—	1·5	—	—	—	—	—	—	—
Isopentylamine	—	4·2	—	—	—	—	—	0·9	—
n-Pentylamine	—	1·5	—	—	—	—	—	1·1	—
Pyrrolidine	—	—	1·5	—	2·5	0·2	0·9	—	—
Di-*n*-propylamine	—	1·7	0·3	0·3	0·3	2–3	—	—	—
Piperidine	—	—	3	1·4	0·5	1	—	9	0·9
Aniline	—	3	3·7	—	—	—	—	0·5	—
2-Methylpiperidine	—	—	0·5	—	1	—	—	—	—
N-Methylaniline	—	0·8	—	—	—	—	—	1·1	—
o-Toluidine	—	—	~1	—	0·5	—	0·3	—	—
Benzylamine	—	—	1	—	0·3	—	—	—	—
Phenethylamine	—	—	2	—	0·1	0·2	—	0·5	—
N-Methylbenzylamine	—	—	~1	0·3	0·3	—	—	—	—
N-Methylphenethylamine	—	1	4	—	0·5	—	—	—	—
Laurylamine	trace	—	—	—	—	—	—	—	—

*And propylamine, dimethylpyrrolidine or methylpiperidine.
†And dimethylamine, one methylpentylamine, one dimethylpyrrolodine or methylpiperidine.
‡And one dimethylpyrrolidine or methylpiperidine, *n*-propylamine.
§And one dimethylpyrrolidine or methylpiperidine, *n*-propylamine.
‖And di-*n*-propylamine, *n*-propylamine.
Amines are listed in the sequence of the retention times of their trifluoroacetamides on SE 30 columns.

amine). Concentrations of secondary amines found in surface waters have generally been below 15 ppb (15 μg/kg).

The simultaneous occurrence of relatively high levels of secondary amines and high concentrations of nitrate was confirmed in some vegetables, particularly in red radishes, spinach and green salad.

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TOXIC SYNERGISM OF METHYLMERCURY WITH SODIUM NITRITE AND ETHYLUREA ON REPRODUCTION AND SURVIVAL OF PROGENY IN RATS

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Abstract—Sodium nitrite and ethylurea were administered to pregnant rats that had been fed 10 ppm dietary methylmercury from weaning, to determine the effect of this combination of treatments on reproduction and the survival of progeny. Prolonged consumption of dietary methylmercury plus ingestion of sodium nitrite (2 g/litre in the drinking-water) and ethylurea (0·636% in the diet) from day 14 of the breeding period decreased litter rate, litter size, birth weight and weanling weight to about 30, 60, 60 and 30% of control values, respectively. The percentage of stillborn pups was 5–10 times higher than in the controls and only four pups survived to weanling from the six litters derived from 18 possible matings. In methylmercury-treated rats administration of sodium nitrite and ethylurea (50 and 100 mg/kg body weight/day, respectively) by stomach tube on days 17, 18 and 19 of gestation did not reduce litter rate and litter size markedly, but only six pups from eight litters survived to weaning. Reduction of the nitrite–ethylurea exposure improved most breeding parameters and survival. The combination of methylmercury and nitrite–ethylurea increased the number of deaths at the foetal and the pre- and post-weanling stages of development.

INTRODUCTION

In the environment an organism is rarely exposed to a single toxicant but rather to several potential toxicants in varying concentrations. Unfortunately little is known about the interaction of toxic compounds in biological systems because most investigations attempt to establish the effects of one toxicant while minimizing the effects of other toxicants and variables. This report describes the combined effects of methylmercury (MeHg) and ethylnitrosourea (ENU) in the transplacental system of the rat.

Druckrey, Ivanković & Preussmann (1966) reported that a single dose of ENU on day 15 of gestation in the rat produced neurogenic tumours in the offspring. As little as 2% of the adult LD_{50} resulted in a 63% incidence of neuroectodermal tumours. Swenberg, Koestner, Wechsler & Denlinger (1972) administered doses of 1, 5, 20 and 50 mg ENU/kg body weight into the tail vein of rats on day 20 of gestation. The 20-mg level produced a 100% incidence of neurogenic tumours in the progeny in a mean time of 288 days. Several types of tumour developed, but neural tumours predominated. Two groups reported transplacental induction of neural tumours by the feeding of nitrite and ethylurea (EU), the precursors of ENU. Ivankovic & Preussmann (1970) reported this effect in BD IX rats given, from day 13 of gestation, a daily oral dose of 100 mg EU/kg body weight followed by 50 mg sodium nitrite/kg. Osske, Warzok & Schneider (1972) reported a high incidence of neural tumours following administration of 0·5% EU in the diet together with sodium nitrite (0·5% in the diet or 0·3% in the drinking-water) to pregnant inbred strain E rats. Recently, Rustia & Shubik (1974) observed neurogenic tumours in 69% of female and 12·5% of male progeny following simultaneous oral administration of EU and sodium nitrite to hamsters on days 12–15 of gestation. Ivankovic, Zeller & Schmähl (1972) found that two heavy metals, cobalt and copper, increased the carcinogenic activity of ENU, but they did not test mercury.

Mercurial compounds, especially MeHg, cause marked physiological effects. A prominent effect is a degeneration of the central nervous system, which causes numbness, ataxia, disturbances of speech and vision, impairment of hearing and emotional disturbances (Borg, Wanntorp, Erne & Hanko 1969; Clarkson, Amin-Zaki & Al-Tikriti, 1976). Methylmercury has a tendency to accumulate in the body and in the brain. Suzuki, Miyama & Katsunuma (1971) found an accumulation in the human placenta. It readily passes the placental barrier (Buhler, 1973) and can lead to poisoning in the foetus with damage to the central nervous system (Matsumoto, Koya & Takeuchi, 1965).

Sodium nitrite and EU fed to rats can result in the *in vivo* formation of ENU (Mirvish & Chu, 1973) and the subsequent development of transplacental tumours. Ethylnitrosourea promotes tumours primarily in the central nervous system, the tissue that is attacked by MeHg. Since both compounds are deleterious to the foetus, it was not surprising to observe a toxic synergistic effect on reproduction and survival of progeny from rats fed MeHg in combination with nitrite and EU during the gestation period.

EXPERIMENTAL

Animals. Female Wistar rats from a closed Oregon State University colony were reared and bred to provide data on reproduction and survival of progeny. The rats were housed in clear plastics box-type cages with lids holding air filters. The cages measured 13 × 10 × 8 in. and held three or four rats each.

When males reached maturity, two were kept in each cage. During the breeding period, two females and one male were housed together for 19 days, after which the females were put in individual cages. Males were rotated every 3 days. Vaginal smears were taken to determine the day of conception.

Diets and test compounds. Semipurified powdered diets and water were available *ad lib.* The basal diet contained 23·3% casein, 65·2% glucose, 4·3% salt mixture (Draper no. 4164, U.S. Biochemicals, Cleveland, Ohio), 2·2% vitamin fortification mixture (U.S. Biochemicals) and 5% corn oil (Best Foods, San Francisco, Cal.). For the MeHg diet, methylmercury chloride (Ventron Corp., San Leandro, Cal.) was dissolved in the corn oil to give a final concentration of 10 ppm mercury in the complete diet.

Sodium nitrite and EU were administered either in the drinking-water and diet or by stomach tube. When added to the drinking-water, nitrite solution (reagent grade, C. P. Baker & Co., Philadelphia, Pa) was freshly made each week in the desired concentration and stored at 4°C. The nitrite solution given as drinking-water was held in brown-glass bottles and changed daily. Ethylurea (reagent grade, Aldrich Chemical Co., Milwaukee, Wisc.) was mixed into the dry diet. For administration by stomach tube, aqueous solutions of nitrite and of EU were freshly made each day and mixed together immediately before intubation. The concentrations were adjusted to give a dosage volume of 10 ml/kg body weight.

Experimental procedure. Female rats were fed the basal diet or diet containing 10 ppm MeHg from weaning until they delivered pups. Sodium nitrite and EU were administered to some females in each group during pregnancy. All dams were switched to the basal diet at parturition and the progeny were fed the basal diet during their lifespan. Table 1 shows the diet composition and regimen for dams on the various treatments. Treatments 1, 2a–e and 3 were controls and treatments 4a–e provided combinations of MeHg and nitrite–EU.

In the first experiment, 20 females were fed the basal diet (treatment 1) and 20 females were fed the MeHg diet (treatment 3) from weaning to day 14 of the breeding period. Then ten of the females on treatment 1 were switched to treatment 2a and ten females on treatment 3 were switched to treatment 4a. All females were continued on their respective regimens until parturition, when they were switched to the basal diet.

Experiment 2 was designed to improve the number and survival of progeny from treatment 4 and involved a repeat of treatments 2a and 4a as well as additional treatments with lower concentrations of nitrite–EU. Groups of 24 females were fed either the basal diet or the MeHg diet from weaning to day 14 of the breeding period. The females were then divided into groups of eight, those originally fed the basal diet being switched to treatments 2a, 2b and 2c (nitrite–EU) and those on the MeHg diet being switched to treatments 4a, 4b and 4c (MeHg + nitrite–EU). All females were fed their respective diets until parturition, when they were switched to the basal diet.

Experiment 3 was designed to compare the effect of nitrite–EU treatment by intubation with that of administration in the diet. Two groups of 16 females were fed the basal or the MeHg diet until they delivered pups, after which all were fed the basal diet. On each of days 17, 18 and 19 of gestation, eight rats from each group were given 50 mg nitrite and 100 mg EU/kg body weight by stomach tube (treatments 2d and 4d, Table 1) and the other eight from each group were given 25 mg nitrite and 50 mg EU/kg body weight (treatments 2e and 4e).

Data collection. Reproduction parameters, teratogenic effects, mercury accumulation and tissue pathology were recorded in these experiments. Litter rate, litter size, birth weight, weanling weight, number of stillborn pups and survival were included in the reproduction data. Total mercury was determined (Magos, 1971) in kidney, liver and muscle from the

Table 1. *Summary of treatments given to female rats from weaning*

Treatment		MeHg	EU		Sodium nitrite	
Function	No.	in diet* (ppm)	In diet† (%)	Dosage by gavage‡ (mg/kg)	In drinking-water† (g/litre)	Dosage by gavage‡ (mg/kg)
Negative control	1	0	0	—	0	—
Nitrite–EU	2a	0	0·636	—	2·0	—
positive control	b	0	0·318	—	1·0	—
	c	0	0·159	—	0·5	—
	d	0	0	100	0	50
	e	0	0	50	0	25
MeHg positive control	3	10	0	—	0	—
Experimental group	4a	10	0·636	—	2·0	—
	b	10	0·318	—	1·0	—
	c	10	0·159	—	0·5	—
	d	10	0	100	0	50
	e	10	0	50	0	25

MeHg = Methylmercury EU = Ethylurea

*As methylmercury chloride; fed to the dams from weaning until parturition.

†Given from day 14 of breeding period until parturition.

‡Daily, on days 17, 18 and 19 of gestation.

dams, in whole newborn pups and in the complete diet. Detailed tissue pathology will be discussed elsewhere. Gross effects such as ataxia, loss of balance, paralysis and hydrocephalus were used to assess neurological damage. When rats became incapacitated, autopsies were performed and tissues were fixed in 10% buffered formalin for histopathology.

Generally 25 progeny (13 males and 12 females) from control treatments 1, 2 and 3 were reared from weaning and observed for signs of neurological damage. All progeny from treatments 4a–e (nitrite–EU + MeHg) were saved for observation. Progeny surviving after 11 months were autopsied at the end of the experiment.

RESULTS AND DISCUSSION

Experiment 1

Female rats fed the MeHg diet for 10 wk weighed 217 ± 14 g (mean ± SD) compared with 239 ± 9 g for rats fed the basal diet. This 9% difference in body weight at the start of the breeding period appeared to be the effect of a difference in food consumption.

The data for experiment 1 shown in Table 2 illustrate a dramatic synergistic effect of nitrite–EU and MeHg on reproduction. Most reproduction parameters for experimental treatment 4a (nitrite–EU + MeHg) were reduced sharply to values below those for the control treatments 1, 2a (nitrite–EU) and 3 (MeHg). Birth weights and weanling weights were also significantly lower in pups from treatment 2a than in those from treatments 1 and 3, but they were still significantly higher than those from treatment 4a. Thus, the nitrite–EU treatment reduced birth and weanling weight, but not to the degree caused by nitrite–EU + MeHg. Females drinking nitrite solution consumed about 50% less water than the females on treatment 1 or 3, and this probably contributed to the low birth weights and subsequent low weanling weights.

The percentage of stillborn pups was nearly five times greater in the group on treatment 4a than in the control groups and the number of pups weaned per litter was less than 15% of the control figures. The pups in the experimental group were small and weak at birth and all the pups in two of the three litters died within 3 days of birth. Only four pups survived to 4 wk, compared with nearly 100 pups for each of the control treatments 1, 2 and 3. The four surviving pups matured slowly and were uneven in size. They displayed various degrees of slow hair growth, late eye opening and incoordination. One had a short tail and another developed hydrocephalus. Pups from the other treatments appeared normal except for some on treatment 2, which showed delayed eye opening and ocular hypoplasia; in some the eyes did not develop and remained closed.

The survival of the progeny and the incidence of neurological damage are shown in Table 3. All 25 treatment-1 control rats and all but two of the treatment-3 (MeHg control) rats survived to the end of the 11-month experimental period. No rats in these two groups displayed signs of neurological problems. However, no treatment-2 or treatment-4 progeny survived to the end of the test period. Twenty of 24 rats in the treatment-2a group exhibited signs of nerve damage and six had carcinomas not associated with nerve tissue. The mean survival time for rats from the nitrite–EU group was about 50% of that following control treatment 1, but was still about 50% longer than that of the nitrite–EU + MeHg progeny. The main effects displayed by the treatment-2 rats were paralysis of the hind legs, poor sense of balance and incoordination. Tumours in the central nervous system and in the meninges around the brain were common.

The mean survival time for the treatment-4 progeny was only 102 days. One pup died from an undetermined cause while the other three were afflicted with hydrocephalus, an ovarian tumour and a kidney tumour, respectively.

Experiment 2

The objective of determining the long-term effect of nitrite–EU + MeHg on tumorigenesis and nerve damage was not achieved in experiment 1 because the pups from treatment 4 did not survive. Experiment 2 was initiated to verify the synergistic effect

Table 2. *Reproductive performance in experiment 1*

Parameter	Reproductive performance following treatment* no.			
	1 (control)	2a (Nitrite–EU)	3 (MeHg)	4a (Nitrite–EU/MeHg)
Litter rate (%)†	100	90	90	30
Litter size (no.)‡	9·7 ± 2·8	$11·1 \pm 2·1^{1}$	9·2 ± 3·1	$6·3 \pm 3·8^{1}$
Stillborn pups (%)	2·0	1·0	0	9·5
Birth weight (g)	$5·4 \pm 0·4^{1,2}$	$4·3 \pm 0·6^{1,3,4}$	$5·4 \pm 0·2^{3,5}$	$3·2 \pm 0·5^{2,4,5}$
Weanling weight (g)§	$58·7 \pm 6·7^{1,2}$	$49·2 \pm 7·2^{1,3,4}$	$59·3 \pm 4·4^{3,5}$	$20·0^{2,4,5}$
No. of pups weaned‖	96	97	81	4
Survival at weaning (%)¶	98	94	98	21

MeHg = Methylmercury EU = Ethylurea

*See Table 1 for details of concentrations in diet and drinking-water.

†Percentage of total possible matings that resulted in litters.

‡Average no. of live pups/litter.

§Average weight of 4-wk-old pups.

‖The number assumes that pups taken for mercury analysis and histology would have survived.

¶Percentage of live pups that survived to weaning, assuming the killed pups would have survived.

Values for litter size, and birth and weanling weights are means ± SD. The same superscripts in a given parameter indicate a significant difference ($P < 0·05$) according to Student's t test.

Table 3. *Survival and signs of neurological damage (Experiment 1)*

Treatment*	Initial no. (at weaning)	Survival time† (days)	Effective no.‡	Signs of nerve damage§
1 (Control)	25	336	25	0
2a (Nitrite–EU)	25	183	24	20
3 (MeHg)	25	321	25	0
4a (Nitrite–EU/MeHg)	4	102	3	2

EU = Ethylurea MeHg = Methylmercury
*See Table 1 for details of concentrations in diet and drinking-water.
†Mean age at which the rats were autopsied.
‡Number of rats that were examined and on which a prognosis was made.
§Incoordination and paralysis.

of nitrite–EU and MeHg on reproduction, to improve survival of treatment-4 progeny and to determine a dose-response effect. The 10-ppm level of MeHg used in experiment 1 was satisfactory because reproduction and survival of progeny from the positive MeHg control group (treatment 3) were adequate. Reproduction in the nitrite–EU control group (treatment 2) was also satisfactory, but the incidence of nerve damage or tumours was nearly 100%. It was reasoned that a reduction in the concentration of nitrite–EU would improve survival of pups in the treatment-4 group and still promote an adequate level of nerve damage for comparative purposes.

The results of experiment 2, shown in Table 4, confirm that nitrite–EU in combination with MeHg is detrimental to reproduction. In fact no pups survived to weaning after treatment 4a. When the level of nitrite–EU was reduced to 50% (4b) and 25% (4c) of the concentration used in treatment 4a, there was a marked improvement in all parameters. However, except for litter rate and litter size, the improvement was not dose-dependent. In other respects, treatments 4b and 4c produced nearly the same effect. The significant difference was that 69 and 59% of the pups survived to weaning with treatments 4b and 4c compared with no survivors with treatment 4a. The positive-control nitrite–EU treatments, 2a, 2b and 2c, all produced comparable results, but for some unexplained reason the lowest level of nitrite–EU produced the lowest litter rate and litter size.

Experiment 3

Experiment 3 was run simultaneously with experiment 2. The nitrite–EU was given by stomach tube in three doses in an effort to increase the number and survival of the progeny of rats given treatment 4. Administration of nitrite–EU by stomach tube did improve the litter rate and litter size and the percentage of stillborn pups was also lower following stomach-tube treatments than after diet treatments (Table 5). However, birth weight was still lower with the higher nitrite–EU dose than in the controls (2d versus 4d), although reduction of the nitrite–EU concentration by 50% (4e) resulted in a mean birth weight comparable to that of the controls (2d and 2e).

Although most parameters of reproduction were improved when treatment was given by stomach tube, the improvement of survival to weaning was questionable (Table 5). Only 8% survived at the higher dose (4d) and all of those were in one litter. However, 54% survived when the nitrite–EU concentration was halved (4e). Although survival improved, the weanling weight of pups from 4e was about 20% below the control (2e) value and there was a large variation in the size of the pups. In contrast, the pups in the one

Table 4. *Reproductive performance in experiment 2*

	Reproductive performance following treatment* no.					
	2 (Nitrite–EU)			4 (Nitrite–EU/MeHg)		
Parameter†	a	b	c	a	b	c
Litter rate (%)	100	100	75	38	88	100
Litter size (no.)	10·1 ± 2·1	10·9 ± 2·7	6·2 ± 3·3	5·7 ± 5·1	7·9 ± 2·3	8·5 ± 3·1
Stillborn pups (%)	3	0	0	29	7	7
Birth weight (g)	5·2 ± 0·6[1]	5·6 ± 0·5	5·4 ± 0·3	3·3 ± 0·4[1,2,3]	5·0 ± 0·6[2]	5·0 ± 0·4[3]
Weanling weight (g)	58·6 ± 4·8[1]	61·0 ± 10·4	70·7 ± 6·7[1,2]	—	51·3 ± 9·6	46·7 ± 6·4[2]
No. of pups weaned	66	60	37	0	38	40
Survival at weaning (%)	93	81	100	0	69	59

EU = Ethylurea MeHg = Methylmercury
*See Table 1 for details of concentrations in diet and drinking-water.
†See Table 2 for definitions of reproduction parameters.
Values for litter size and birth and weanling weights are means ± SD. The same superscripts in a given parameter indicate a significant difference ($P < 0.05$) according to Student's t test.

Table 5. *Reproductive performance in experiment 3 (treatment by stomach tube)*

Parameter†	Reproductive performance following treatment* no.			
	2 (Nitrite–EU)		4 (Nitrite–EU/MeHg)	
	d	e	d	e
Litter rate (%)	100	75	100	88
Litter size (no.)	10·5 ± 3·3[c]	10·2 ± 1·5	9·0 ± 1·7	10·6 ± 2·6
Stillborn pups (%)	0	2	4	3
Birth weight (g)	5·2 ± 0·4[1]	5·0 ± 0·2	4·5 ± 0·5[1,2]	5·1 ± 0·4[2]
Weanling weight (g)	56·8 ± 7·9	56·3 ± 4·7[1]	55	44·8 ± 5·6[1]
No. of pups weaned	78	61	6	• 40
Survival at weaning (%)	93	100	8	54

EU = Ethylurea MeHg = Methylmercury

*See Table 1 for details of dietary concentration and dosage.

†See Table 2 for definitions of reproduction parameters.

Values for litter size and birth and weanling weights are means ± SD. The same superscripts in a given parameter indicate a significant difference ($P < 0·05$) according to Student's t test.

surviving litter from treatment 4d appeared as healthy as control pups.

When MeHg was present in the diet, exposure to nitrite–EU in the diet was more detrimental to litter rate and litter size than exposure by intubation. Examination of the uterus from some of the rats for sites of foetal implantation revealed that conception and the number of implantations was comparable for the two different nitrite–EU regimens. The difference in effect between the two treatments lay in the survival of the foetuses not in their conception. Evidently continuous exposure to lower levels of nitrite–EU in the diet, starting at approximately wk 2 of gestation was more detrimental to foetal survival than were larger single doses given on three consecutive days late in gestation.

The growth of pups from dams subjected to MeHg and the lower levels of nitrite–EU (treatments 4b, 4c and 4e) was very erratic. Some of the pups died within 2–3 days of birth but many appeared to grow normally to about 2 wk of age and then became weak and died. Surviving pups varied considerably in size. Some pups were as large as pups from control groups while others were 20–30 g runts. The range in body weight of litters from treatments 4b, 4c and 4e was from a minimum of 36 ± 14 g (mean ± SD) to a maximum of 56 ± 6 g, compared with a minimum of 53 ± 10 g to a maximum of 66 ± 11 g for control treatments 2b, 2c and 2e.

An attempt to foster pups was carried out to determine the effect of nursing capability on survival. Some pups from dams from treatments 2a and 2d were switched with pups from 4a and 4d, respectively. Most of the pups from 2a and 2d were raised by the dams from the 4a and 4d treatments, but none of the pups from treatment 4 were successfully fostered by control dams. The MeHg-treated dams could foster healthy pups but the pups from MeHg-treated dams could not compete with healthy pups in the control litters.

The results of total-mercury analyses of diet and rat tissue are shown in Table 6. Diets for treatments 3 and 4 were prepared to contain 10 ppm mercury as methylmercury chloride but assayed at 7·4–7·9 ppm. The mercury concentrations in tissues from the dams paralleled the level of mercury fed in the diet. Of the individual tissues, the kidney had the highest levels followed by the liver and then muscle. The amount of mercury in the female kidney from treatments 3 and 4, listed in Table 6, is 5–6 times higher than the level fed in the diet. The mercury concentration in whole pups from treatment 4 was

Table 6. *Mercury analyses of diet and rat tissue*

Treatment	Levels of mercury in			
	Diet (ppm)	Maternal kidney (ppm)	Pups*	
			ppm†	Total μg/pup
1 (Control)	<0·005 (2)	3·21 ± 3·66 (6)	0·012 ± 0·006 (9)	0·072 ± 0·038 (9)
2 (Nitrite–EU)	—	0·8 ± 1·8 (14)	0·025 ± 0·019 (6)	0·098 ± 0·068 (6)
3 (MeHg‡)	7·9 ± 3·3 (8)	50·25 ± 20·48 (4)	11·25 ± 2·40 (13)	61·36 ± 14·94 (13)
4 (Nitrite–EU/MeHg‡)	7·4 ± 1·2 (4)	63·3 ± 23·0 (20)	19·80 ± 4·12 (7)	55·7 ± 11·0 (7)

EU = Ethylurea MeHg = Methylmercury

*Pups from treatments 1, 2 and 3 were newborn; those from treatment 4 were 1–7 days old at sampling.

†Expressed as Hg concentration in whole pup on wet-weight basis.

‡10 ppm Hg added to diet as methylmercury chloride.

Values are means ± SD for the numbers of samples indicated in parentheses.

about 75% higher than the concentration in pups from the mercury control (treatment 3). However, the total mercury per pup was similar for the two treatments. Some of the treatment-4 analyses were not done on newborn pups but on pups that had become progressively dehydrated and starved with each day of survival. Since the mercury concentration was calculated from total body weight, a reduced body weight due to dehydration might explain the higher concentration in treatment-4 pups. In general the foetuses in treatments 3 and 4 were exposed to high levels of MeHg during gestation and the mercury accumulated in the pup tissue.

The data presented here clearly demonstrate that reproduction and survival of progeny are dramatically reduced in rats fed MeHg in combination with nitrite and EU administration during gestation. The combined effect of the MeHg and the ENU, formed from the nitrite and EU, was much more pronounced than the effect of either the MeHg or the ENU alone. The cause of death in the progeny is not known but was probably related to damage to the nervous system since both MeHg and ENU affect the central nervous system.

Premature deaths among the rats of the treatment-4 group occurred at all stages of development. There were more resorbed and stillborn pups, more deaths between birth and weaning and a lower survival of pups to maturity among the progeny from dams fed MeHg and nitrite–EU than among those from the control dams. There appear to be a shorter latent period and higher incidence of the neuroectodermal tumours induced by ENU when MeHg is in the diet. These points will be determined when the current experiments are completed.

A toxic synergistic effect of MeHg and nitrosourea analogues on the foetus could constitute a potential danger to man. Nitrosourea analogues can be formed in the gut from nitrite and ureides. Nitrite is used extensively as a preservative in the food industry, and urea derivatives are found in nature and some are used as drugs (Lijinsky, 1972). Methylmercury is found occasionally in some marine and fresh-water food products. Thus humans could be exposed to all of these compounds simultaneously. Unfortunately, little is known about the interaction of these compounds in biological systems or the impact of that interaction on human health. This study is a step towards the understanding of those processes and risks.

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A 2-YEAR FEEDING STUDY OF INSTANT COFFEES IN RATS. II. INCIDENCE AND TYPES OF NEOPLASMS

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Abstract—Regular and decaffeinated instant coffees were incorporated at the maximum tolerated level of 6% in a standard commercial diet and fed *ad lib.* to male and female rats for 2 yr. The average daily intake of coffee, not considering the first 4 wk, was 2·9 g/kg for males and 3·5 g/kg for females, corresponding to about 80 cups for a 70-kg man and 70 cups for a 50-kg woman. With the exception of caffeine, the other technological variables, such as freeze- and spray-drying, decaffeination and extraction rates, did not influence the incidence and types of neoplasms in a significant manner. With three of the treatments involving high levels of caffeine, the incidence of neoplasms was significantly lowered. In all treatment groups, the total number of neoplasms was either similar to or lower than the total in the control group.

INTRODUCTION

In the human populations of many highly industrialized countries, cancer ranks second only to cardiovascular diseases as a cause of death. In view of circumstantial evidence indicating that more than 80% of cancers may be dependent on the environment and therefore, at least theoretically, preventable, large screening programmes on potential carcinogenic chemicals have been started by national and international institutions. In general these are of two types, involving either the experimental approach, which uses short-term predictive screening tests or long-term experiments, or the epidemiological approach, which is based on the comparison of exposed and unexposed populations.

Particular difficulties in cancer epidemiology arise from the very long induction time of many human cancers, the difficulty of identifying slight increases in incidence of common cancer and the many interfering social and cultural habits of man (Higginson, 1976; Muir, McLennan, Waterhouse & Magnus, 1976). Available studies on coffee as a possible carcinogenic hazard are all retrospective and controversial. Stocks (1970) correlated age-specific death rates due to carcinomas in ovaries, prostate and pancreas, as well as to leukaemia, with the yearly coffee consumption in 20 countries, and concluded that a positive correlation existed between coffee consumption and the incidence of neoplasms in these organs. Heyden (1972), however, refuted these conclusions on the basis of inadequate statistical analysis and lack of biological evidence. Cole (1971) and Schmauz & Cole (1974) considered bladder cancer to be positively correlated with coffee consumption, but in a later publication (Simon, Yen & Cole, 1975) the observed association was judged to be unsubstantiated.

Earlier, another group (Dunham, Rabson, Stewart, Frank & Young, 1968) found that bladder cancer was not associated with coffee consumption, except among black females, in whom a weak correlation ($P < 0{\cdot}025$) was demonstrated. No increase in the incidence of bladder cancer was found in coffee drinkers by Morgan & Jain (1974). Attention was drawn by Shennan (1973) to a correlation between coffee consumption and mortality rates for renal cancer. On the other hand, Wynder, Mabuchi & Whitemore (1974) and Armstrong, Garrod & Doll (1976) concluded that there was no evidence of a positive association between renal cancer and coffee consumption.

Experimental data on the incidence of tumours in laboratory animals ingesting coffee are very scarce (Jans, 1972; Zeitlin, 1972). The study discussed here investigated the effects of long-term administration of various instant coffee samples to rats. The main objectives were to investigate the possible effects of extraction rates and decaffeination, as well as drying techniques using industrial installations. The experiment was not designed as a carcinogenicity bioassay, for which larger control and experimental groups as well as several dose levels for each treatment would have been required. However, observations on the incidence and types of neoplasms in a fairly large number of animals treated with maximum tolerated dose levels are of considerable interest. This study consisted of one control and thirteen experimental groups, including seven groups given coffees from which caffeine had been removed by extraction with methylene chloride. Effects of instant coffees treated with methylene chloride are of particular interest, since no data from chronic toxicological or carcinogenicity studies exist for this solvent.

EXPERIMENTAL

The experimental design, treatments, animal husbandry and other experimental details have been described by Würzner, Lindström, Vuataz & Luginbühl (1977). Details of the treatments and caffeine intakes of the various experimental groups are given in Table 1. The average daily intake of coffee during the experiments, excluding wk 1–4, was 2·9 g/kg in the case of the males and 3·5 g/kg for the females. At months 3 and 12, ten male and ten female Sprague–Dawley rats were randomly taken for autopsy from each experimental group and at 24 months all surviving animals were killed. The rats were killed by ether anaesthesia and bleeding from the opened aorta. Moribund rats were killed for examination and animals found dead were generally autopsied immediately; over the weekend, however, dead animals were refrigerated and autopsy was performed on the next working day. Organs from severely autolysed animals were discarded. Autopsy findings were recorded on separate sheets for each animal and important or representative lesions were photographed.

The organs fixed and processed for microscopic examination included the heart, aorta and major arteries, liver, kidney, lung, trachea, lachrymal gland, oesophagus, stomach, small intestine (proximal and distal parts), large intestine, salivary glands, mammary glands (only if grossly abnormal), thyroids, parathyroids, pancreas, several lymph nodes, spleen, thymus, adrenals, brain, eye, peripheral nerves, muscle, testes, epididymis, prostate, seminal vesicles, ovaries, uterus, cervix and vagina and urinary bladder. Neoplasms and any possibly abnormal tissues were recorded and processed for further examination. From all organs and tissues, comparable standard slices were fixed in Bouin's solution and Baker's Formol Calcium Solution and embedded in paraffin (Paraplast®, Sherwood Medical Industries, St. Louis, USA). Sections, approximately 5–6 μm thick, were cut and stained with haematoxylin and eosin. Heart, kidney and arteries were also stained by the Van Gieson–Elastica method. In addition, selected organs were stained with periodic acid–Schiff reagent.

RESULTS

Tumour incidence

All neoplasms observed at autopsy or only on microscopic examination were classified according to generally accepted morphological criteria. The prevalence and types of tumours in the different organs and tissues are listed for each experimental group and separately for males and females (Tables 2–6).

The most common types of neoplasm were embryonal nephromas, lymphosarcomas, mammary fibroadenomas and adrenal adenomas. These accounted for about 85% of all the tumours observed in the entire assay. Some 20 other types of tumours, found in between one and four animals in all groups combined, accounted for the remaining 15% of neoplasms. No urinary bladder tumours or microscopic foci of epithelial-cell anaplasia were detected.

The embryonal nephromas (nephroblastomas) varied in size from tumours only detectable microscopically to neoplasms several centimetres in diameter. They were ill-defined in the kidney but did not metastasize. Embryonal nephromas occurred in two controls and in one, two or three animals of each treated group except group G. For the sake of clarity, this tumour, which shows neoplastic growth of both epithelial and mesenchymal elements, has been listed as epithelial and malignant (Tables 2 & 3).

Lymphoid neoplasms, most frequently histioblastic and histiocytic lymphosarcomas, were multicentric or confined to liver or spleen and exceptionally to lymph nodes. Lymphosarcomas were found in four controls and in from one to five animals of each treated group except group S, in which this type of tumour was not found.

Fibroadenomas of the mammary glands were observed in nine controls and in from two to nine

Table 1. *Experimental groups and data on caffeine consumption*

Treatment group	Types of instant coffee administered	Extraction rate (%)	Drying method	Average intake* of caffeine (mg/kg/day) By males in 4-wk period following month				By females in 4-wk period following month			
				0†	3	6	12	0†	3	6	12
A	None (control)	—	—	0	0	0	0	0	0	0	0
B	Regular	23·0	Spray	347	194	165	169	336	219	219	198
C	Regular	37·4	Spray	252	130	117	114	241	150	149	144
D	Regular	50·2	Spray	193	88	79	76	168	104	104	97
E	Regular	50·2	Freeze	205	96	92	86	186	110	108	105
G	Decaffeinated	20·9	Spray	25	12	10	10	24	14	14	13
H	Decaffeinated	34·6	Spray	19	9	8	7	17	10	10	9
J	Decaffeinated	47·4	Spray	12	6	5	5	12	7	6	6
K	Decaffeinated	47·4	Freeze	14	6	5	5	13	7	7	6
L	Decaffeinated + caffeine	37·4	Spray	359	188	178	172	347	207	202	197
M	Decaffeinated + caffeine	50·2	Spray	355	194	173	172	353	218	213	202
O	Decaffeinated + caffeine	50·2	Freeze	370	207	170	177	356	227	209	198
P	Regular	47·0	Spray	304	151	128	134	277	168	148	154
S	Regular	47·0	Spray	278	135	125	118	268	150	134	135

*Based on the weekly recorded individual body weights and intake of food of all the animals alive at each period under consideration.
†First day of assay.

Table 2. *Prevalence of tumours in all animals of control and treated groups*

Group†	No. of rats	Neoplasms*					Average days in the assay‡
		Total	Malignant	Benign	Epithelial	Mesenchymal	
A	67	26	6	20	21	5	397
B	73	10	3	7	7	3	410
C	73	19	6	13	17	2	372
D	68	16	7	9	14	2	388
E	69	18	6	12	13	5	379
G	74	24	6	18	19	5	410
H	70	29	9	20	24	5	400
J	65	17	6	11	12	5	397
K	70	18	7	11	12	6	408
L	72	14	6	8	9	5	392
M	69	13	7	6	9	4	369
O	73	11	6	5	7	4	395
P	66	16	9	7	10	6	355
S	71	15	1	14	15	0	392

*No. of rats affected.
†For identification of groups see Table 1.
‡Average time at which the rats in the group died or were killed. Those killed at months 3 and 12 of the trial are included in the calculation.

females of each treated group. All of these benign epithelial tumours were evident macroscopically and had diameters of up to 2–3 cm.

Adrenal adenomas, located as proliferative lesions in the medulla or at the cortico-medullary junction, were in a majority of cases detected only microscopically, but single well-defined spheroid tumours were several times the size of a normal adrenal. For this reason all proliferative lesions have been listed as benign epithelial neoplasms, but the distinction from

Table 3. *Prevalence of tumours in males and in females of control and treated groups*

Group†	No. of rats	Neoplasms*					Average days in the assay‡
		Total	Malignant	Benign	Epithelial	Mesenchymal	
Males							
A	31	12	5	7	8	4	369
B	34	3	1	2	2	1	361
C	36	4	1	3	4	0	347
D	34	5	3	2	4	1	355
E	32	9	4	5	6	3	339
G	35	8	3	5	6	2	388
H	32	8	3	5	5	3	383
J	30	7	3	4	4	3	373
K	34	8	3	5	4	4	400
L	34	4	2	2	2	2	363
M	33	6	5	1	4	2	335
O	36	3	2	1	1	2	373
P	28	3	1	2	2	1	322
S	33	4	1	3	4	0	354
Females							
A	36	14	1	13	13	1	425
B	39	7	2	5	5	2	459
C	37	15	5	10	13	2	397
D	34	11	4	7	10	1	420
E	37	9	2	7	7	2	418
G	39	16	3	13	13	3	431
H	38	21	6	15	19	2	416
J	35	10	3	7	8	2	421
K	36	10	4	6	8	2	416
L	38	10	4	6	7	3	420
M	36	7	2	5	5	2	402
O	37	8	4	4	6	2	417
P	38	13	8	5	8	5	388
S	38	11	0	11	11	0	429

*No. of rats affected.
†For identification of groups see Table 1.
‡Average time at which the rats in the group died or were killed. Those killed at months 3 and 12 of the trial are included in the calculation.

Table 4. *Incidence and types of tumours in different organs of all animals in treated and control groups*

Organ and type of tumour	No. of rats...	No. of rats with named tumour in group*													
		A	B	C	D	E	G	H	J	K	L	M	O	P	S
		67	73	73	68	69	74	70	65	70	72	69	73	66	71
Kidneys															
Embryonal nephroma		2	1	2	1	1	0	3	1	1	2	1	1	3	1
Fibrosarcoma		0	0	0	0	0	1	0	0	0	0	0	0	0	0
Liver															
Bile-duct adenoma		0	0	1	0	0	0	0	0	0	1	0	0	0	0
Bile-duct carcinoma		0	0	0	0	0	0	0	0	0	1	0	0	0	0
Lymphatic tissue															
Lymphosarcoma (all types)		4	2	2	1	5	2	5	2	3	2	2	3	5	0
Lung															
Broncho-alveolar adenoma		1	0	0	0	0	0	1	0	0	0	1	0	0	0
Pancreas															
Islet-cell adenoma		0	0	0	0	0	0	0	0	0	0	0	0	1	1
Brain															
Sarcoma		0	0	0	1	0	0	0	0	0	0	0	0	0	0
Adrenal															
Pheochromocytoma and cortical adenoma		9	1	4	4	9	11	10	6	5	3	0	3	1	4
Skin															
Squamous-cell carcinoma		0	1	1	3	0	0	0	0	0	0	0	0	0	0
Adnexal adenocarcinoma		0	0	1	1	0	0	0	0	0	0	1	1	0	0
Fibroma		1	0	0	0	0	0	1	0	1	0	0	0	0	0
Fibrosarcoma		0	0	0	0	0	2	0	1	0	0	2	1	0	0
Salivary glands															
Squamous-cell carcinoma		0	0	0	0	0	1	0	0	0	0	1	0	0	0
Testes															
Sarcoma		0	0	0	0	0	0	0	1	0	0	0	0	0	0
Sertoli-cell tumour		0	0	0	0	0	0	0	1	0	0	0	0	0	0
Prostate															
Sarcoma		0	0	0	0	0	0	0	0	1	0	0	0	0	0
Ovaries															
Adenocarcinoma		0	0	0	0	0	0	0	1	1	0	0	0	0	0
Granulosa-cell tumour		0	2	0	0	0	1	0	0	0	0	0	0	0	0
Uterus/cervix/vagina															
Leiomyoma		0	1	0	0	0	0	0	1	0	1	0	0	0	0
Myxosarcoma		0	0	0	0	0	0	0	0	0	0	0	0	1	0
Mammary galnds															
Fibroadenoma		9	2	8	5	3	6	8	3	6	4	5	2	5	9
Adenocarcinoma		0	0	0	0	0	0	1	0	0	0	0	0	0	0

*For identification of groups see Table 1.

nodular hyperplasia was not always clear. Most adrenal adenomas were considered to be phaeochromocytomas, but no special techniques were applied to demonstrate chromaffin granules.

Statistical evaluation

As explained in the Introduction, the objectives of this experiment were different from those in a carcinogen bioassay. Statistical analysis of the data according to Hoel & Walburg (1972) on the basis of survival data was not adequate; too few animals died from neoplasms for an efficient application of this technique. Another method developed by Pike (1966) and improved by Peto & Lee (1973) could not be used as it is based on the time of diagnosis of readily visible skin carcinomas in the living animal. In our experiment, many of the neoplasms were detected *post mortem* by histopathological evaluation.

Comparison of the incidence and types of neoplasms in the treatment and control groups and the relation of these findings to the sex of the animals are of fundamental interest. In a first approach, the treatment groups were therefore compared with the control for the males and females separately and together for the total neoplasms and for the malignant tumours alone. When a cell frequency of the 2×2 tables was smaller than 5, the exact Fisher's test was applied; otherwise the conventional chi-square test was used. In this approach the statistical inferences are based on a comparisonwise error rate and they are not independent because they involve the same control. On the other hand, the relatively small sample sizes result in an unavoidably weak test power. Accordingly the statistical inferences drawn from these comparisons should be considered as indicative only. In a second approach, advantage was taken of the fact that the thirteen treatments (controls excluded) may be considered as a factorial design where the technological variables are caffeine content, extraction yield, way of drying and decaffeination. As the response is the proportion of animals with certain types of tumour, a natural approach was to regress

Table 5. *Incidence and types of tumours in different organs of male rats in treated and control groups*

Organ and type of tumour	No. of rats...	No. of rats with named tumour in group*													
		A	B	C	D	E	G	H	J	K	L	M	O	P	S
		31	34	36	34	32	35	32	30	34	34	33	36	28	33
Kidneys															
Embryonal nephroma		2	1	0	1	1	0	0	0	0	1	1	0	0	1
Fibrosarcoma		0	0	0	0	0	1	0	0	0	0	0	0	0	0
Lymphatic tissue															
Lymphosarcoma (all types)		3	1	0	0	3	1	3	1	2	1	1	1	1	0
Lung															
Broncho-alveolar adenoma		1	0	0	0	0	0	1	0	0	0	1	0	0	0
Pancreas															
Islet-cell adenoma		0	0	0	0	0	0	0	0	0	0	0	0	1	0
Testes															
Sarcoma		0	0	0	0	0	0	0	1	0	0	0	0	0	0
Sertoli-cell tumour		0	0	0	0	0	0	0	1	0	0	0	0	0	0
Prostate															
Sarcoma		0	0	0	0	0	0	0	0	1	0	0	0	0	0
Brain															
Sarcoma		0	0	0	1	0	0	0	0	0	0	0	0	0	0
Adrenals															
Pheochromocytoma and cortical adenoma		5	1	3	2	5	5	4	3	4	2	0	1	1	3
Skin															
Squamous cell carcinoma		0	0	1	0	0	0	0	0	0	0	0	0	0	0
Adnexal adenocarcinoma		0	0	0	1	0	0	0	0	0	0	1	0	0	0
Fibroma		1	0	0	0	0	0	0	0	1	0	0	0	0	0
Fibrosarcoma		0	0	0	0	0	0	0	1	0	0	1	1	0	0
Salivary glands															
Squamous-cell carcinoma		0	0	0	0	0	1	0	0	0	0	1	0	0	0
	Total...	12	3	4	5	9	8	8	7	8	4	6	3	3	4

*For identification of groups see Table 1.

Table 6. *Incidence and types of tumours in different organs of female rats in treated and control groups*

Organ and type of tumour	No. of rats...	No. of rats with named tumour in group*													
		A	B	C	D	E	G	H	J	K	L	M	O	P	S
		36	39	37	34	37	39	38	35	36	38	36	37	38	38
Kidneys															
Embryonal nephroma		0	0	2	0	0	0	3	1	1	1	0	1	3	0
Liver															
Bile-duct adenoma		0	0	1	0	0	0	0	0	0	1	0	0	0	0
Bile-duct carcinoma		0	0	0	0	0	0	0	0	0	1	0	0	0	0
Lymphatic tissue															
Lymphosarcoma (all types)		1	1	2	1	2	1	2	1	1	1	1	2	4	0
Pancreas															
Islet-cell adenoma		0	0	0	0	0	0	0	0	0	0	0	0	0	1
Ovaries															
Adenocarcinoma		0	0	0	0	0	0	0	1	1	0	0	0	0	0
Granulosa cell tumour		0	2	0	0	0	1	0	0	0	0	0	0	0	0
Uterus/cervix/vagina															
Leiomyoma		0	1	0	0	0	0	0	1	0	1	0	0	0	0
Myxosarcoma		0	0	0	0	0	0	0	0	0	0	0	0	1	0
Adrenals															
Pheochromocytoma and cortical adenoma		4	0	1	2	4	6	6	3	1	1	0	2	0	1
Mammary glands															
Fibroadenoma		9	2	8	5	3	6	8	3	6	4	5	2	5	9
Adenocarcinoma		0	0	0	0	0	0	1	0	0	0	0	0	0	0
Skin															
Squamous cell carcinoma		0	1	0	3	0	0	0	0	0	0	0	0	0	0
Adnexal adenocarcinoma		0	0	1	0	0	0	0	0	0	0	0	1	0	0
Fibroma		0	0	0	0	0	0	1	0	0	0	0	0	0	0
Fibrosarcoma		0	0	0	0	0	2	0	0	0	0	1	0	0	0
	Total...	14	7	15	11	9	16	21	10	10	10	7	8	13	11

*For identification of groups see Table 1.

the logit transforms of these proportions on the independent variables. The model is as follows:

$$P = \left[1 + \exp\left(-\beta_0 - \sum_{i=1}^{4} \beta_i x_i\right)\right]^{-1}$$

where x_1 is the caffeine content in %, x_2 is the extraction yield in %, x_3 is the way of drying ($x_3 = 0$ is for freeze-drying, $x_3 = 1$ is for spray drying), and x_4 is related to the decaffeination ($x_4 = 0$ is not decaffeinated, $x_4 = 1$ is decaffeinated with methylene chloride).

The coefficients β_0 and β_i ($i = 1,\ldots 4$) in the model have been estimated by the iterative method of maximum likelihood described by von Berchtold & Linder (1973). [See also Plackett (1974).]

Comparison of the treatments against the controls

Considering the findings in males, none of the treatment percentages of neoplasms, either malignant and benign, or malignant tumours only are larger than the control percentages. On the other hand, treatments O, B and P (all three with high levels of caffeine) give percentages of neoplasms (malignant or benign) significantly smaller than the control at the 5% level. The probabilities of obtaining these results under the hypothesis of no effect are as follows (exact Fisher's test):

Treatment	*One-sided probability*
O	0·0033
B	0·0047
P	0·0137

For the females, treatments H, G and C give percentages of neoplasms (either malignant or benign) higher than the control value, but not significantly so even at the 10% level. The probabilities of obtaining these results under the hypothesis of no effect are as follows (uncorrected chi-square test):

Treatment	*Two-sided probability*
H	0·1585
G	0·8503
C	0·8853

All the treatments except S give higher percentages of malignant tumours than the control. Only treatment P, however, is significantly higher at the 5% level. The probabilities of obtaining the three highest percentages under the hypothesis of no effect are as follows (exact Fisher's test):

Treatment	*One-sided probability*
P	0·0174
H	0·0622
C	0·1058

Table 7. *Values of the estimates b_i of the coefficients β_i in the multiple regression model (all neoplasms)*

b_i	Males	Females	Males and females
b_0	−1·273	−0·433	−0·765
b_1	−0·161*	−0·138**	−0·143***
b_2	0·005	−0·005	−0·001
b_3	−0·145	0·390	0·177
b_4	0·078	−0·092	−0·034

Asterisks indicated that the coefficient is significantly different from zero at the 5% (*), 1% (**) and 0·1% (***) levels.

Table 8. *Values of the estimates b_i of the coefficients β_i in the multiple regression model (malignant neoplasms)*

b_i	Males	Females	Males and females
b_0	−3·750**	−2·598***	−3·035
b_1	−0·063	−0·041	−0·049
b_2	0·025	0·008	0·014
b_3	0·079	0·198	0·139
b_4	0·484	0·047	0·220

Asterisks indicate that the coefficient is significantly different from zero at the 1% (**) and 0·1% (***) levels.

When *many* comparisons are performed in the same experiment and when the statistical inferences are based on a comparisonwise error rate, a comparison may lead to the rejection of the null hypothesis even in the absence of any effect as the only result of the sampling variation. The dependence between the comparisons does not alter the validity of this statement. This should be borne in mind in connexion with the probability associated with treatment P.

Multiple regression

The values of the estimates b_i of the coefficients β_i, $i = 0, 1,\ldots 4$ in the model are given in Tables 7 and 8. In every situation the model is valid, as checked by the test of goodness-of-fit between the observed and calculated proportions. This dispenses with the need to introduce interaction terms in the model.

A coefficient $b_i > 0$, $i = 1,\ldots 4$, indicates that increasing the corresponding x_i will induce an increase of P, which can be considered as the risk of neoplasm incidence. When $b_i < 0$, $i = 1,\ldots 4$, increasing x_i will induce a decrease of this risk.

DISCUSSION

In the multiple regression approach, all the calculations were carried out with and without the average age at killing time as an additional explanatory variable. As the conclusions regarding the effects of the technological variables were not affected by the inclusion of the average age, only the results without this additional variable are given, for the sake of brevity.

The only technological variable that gave a significant effect was the caffeine content. According to the results of this experiment, increasing the level of caffeine decreased the risk of neoplasm incidence. The effect was more pronounced on the benign than on the malignant forms. This conclusion is in agreement with the results of the comparisons of the treatments against the control, which showed that the proportion of neoplasms was significantly lower in three treatments of male rats involving high levels of caffeine, i.e. treatments B, P and O (see above). This observation may be of interest, since caffeine modifies alkylation and radiation damage *in vitro* and *in vivo* and interacts with repair mechanisms of the DNA at still poorly understood molecular levels (Gaudin & Yielding, 1969; Hussain, Ehrenberg & Ahnstrom, 1976; Kato, 1974; Lang, 1975; Zajdela & Latarjet, 1973). In relation to the human situation, however, this

observation remains rather academic and any possible interpretations are speculative.

An important outcome of this experiment is the absence of any significant increase in the risk of neoplasm incidence when the coffee extracts are decaffeinated with methylene chloride, as disclosed by the non-significant coefficients b_4 in Tables 7 and 8. Although this observation, because of the technical design of the protocol, could not be based on dose-dependent results, the seven experimental groups treated with decaffeinated coffee provide considerable evidence of an absence of carcinogenic effects. Theoretically, carcinogenesis is irreversible and progresses with time, and there is no indication of a subthreshold dose as far as the primary effects at the molecular level are concerned, but secondary effects—the multiplication and proliferation of cancer cells—are possibly influenced by dose and time, so that at low doses, induction times may be beyond the life expectancy of the species (Druckrey, 1967; Jones & Grendon, 1975). Therefore one can assume that the use of lower dose levels in this experiment would not have changed our conclusions. This conclusion is also supported by the observation that this strain of rat is sensitive to treatment effects, as demonstrated by the observed effects of caffeine. On the other hand, in this experiment, maximum tolerated dose levels of regular and decaffeinated instant coffees were used, ranging from 2·9 g/kg for males to 3·5 g/kg in females and providing a comfortable equivalence of 70–80 cups/day for man. Product H, one of the six decaffeinated coffees, was the only treatment in this investigation that yielded a somewhat higher tumour prevalence and somewhat increased incidences of certain neoplasms (Tables 2–6). The values, however, were close to those of the control group and were in no instance significant. They have to be considered, therefore, as biological variation.

In evaluating the types and incidence of neoplasms according to the sex distribution, it should be pointed out that the higher incidence of malignant neoplasms in the females of group P probably stems from the high incidence of embryonal nephroma. This well-known tumour is, however, not sex linked. It was classified as malignant because, in some animals, the size of the tumour caused death, but it did not metastasize. There was no embryonal nephroma in female controls, but males of the control group showed the opposite pattern. The statistical interpretation supports the view that this significance is the result of sampling variation rather than a true effect. The spontaneous level of neoplastic incidence in control rats was fairly high, but tissue sensitivity to carcinogens appears not to be directly related to the spontaneous tumour rates, as was shown by Bhalchandra & Meier (1976) in three genetically different mouse strains.

In conclusion, the data from our 2-yr feeding study of regular and decaffeinated instant coffees in rats, using maximum tolerated dose levels, indicate no increased risk of formation of neoplasms, and treatments providing high caffeine levels even decreased the incidence of neoplasms in both males and females.

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EVALUATION OF ACUTE AND SHORT-TERM ADMINISTRATION OF 2,4,5-TRICHLORO-PHENOXYACETATE WITH RESPECT TO RENAL PROXIMAL TUBULAR TRANSPORT

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Abstract—Previous studies in rats demonstrated that a single dose (45–90 mg/kg) of 2,4,5-trichlorophenoxyacetate (2,4,5-T) reduced the transport of organic acids and bases in slices of renal cortex taken from the treated animals. Upon repeated daily administration of 2,4,5-T to rats, the daily rate of excretion of this herbicide increased after initial retention until 100% of the daily dose administered was excreted within 24 hr. However, the ability of renal slices to transport organic ions was not facilitated during this period of increased excretion; in fact, a significant depression in transport was still measured. Additionally, the high degree of binding of 2,4,5-T by renal cortical homogenates from untreated rats was not altered by pretreatment of the rats with 90 mg 2,4,5-T/kg. Single or repeated administration of the herbicide at the lower dose of 20 mg/kg did not alter the transport of organic ions significantly. From these data and others, it appears that the susceptibility of rats to the toxicity of 2,4,5-T is directly related to the ability of the animals to excrete high doses of this herbicide.

INTRODUCTION

The organic acid herbicide 2,4,5-trichlorophenoxyacetate (2,4,5-T) has been shown to alter the transport of organic acids and bases in the proximal tubules of the kidney (Berndt & Koschier, 1973; Erne & Sperber, 1974; Hook, Bailie, Johnson & Gehring, 1974; Hook, Cardona, Osborn & Bailie, 1976; Koschier & Berndt, 1976a). The renal impairment of transport of 2,4-dichlorophenoxyacetate (2,4-D) and tetraethylammonium (TEA) appeared to be competitive in nature (Berndt & Koschier, 1973; Hook *et al.* 1974; Koschier & Berndt, 1976b,c). Although *in vivo* secretion of 2,4,5-T by the renal proximal tubules appears to be minimal (Erne & Sperber, 1974; Hook *et al.* 1976), this herbicide is excreted predominantly by renal mechanisms. As the dose of 2,4,5-T administered was increased, the percentage of the dose excreted in the urine within 24 hr decreased. When the herbicide was administered on a daily basis, however, the daily excretion of 2,4,5-T increased steadily until 100% of the daily dose was excreted (Koschier & Berndt, 1976a).

The present study explored the extent to which repeated doses of 2,4,5-T impaired transport in the renal proximal tubules. The effect of low doses of 2,4,5-T on renal function was also determined.

EXPERIMENTAL

Test materials. Analytical-grade 2,4,5-T (AGR 86187) was obtained from Dow Chemical Co., Midland, Mich. Radiolabelled compounds (^{14}C-labelled α-aminoisobutyrate (AIB), TEA, 2,4-D, *p*-aminohippuric acid (PAH) and 2,4,5-T) were supplied by Amersham/Searle, Arlington Heights, Ill., or New England Nuclear, Boston, Mass.

2,4,5-T pretreatments and excretion studies. Adult male Sprague-Dawley rats were used throughout these studies. For the *in vitro* studies of organic-ion transport and accumulation, groups of four rats were pretreated with daily sc injections of 90 mg 2,4,5-T/kg for 8 days or daily sc injections of 20 mg/kg for 1, 8 or 16 days, and were killed 24 hr after the last injection. These doses were administered in 70% ethanol, the injection volume being approximately 0·9 or 0·2 ml/kg. Control rats received an appropriate volume of the ethanol vehicle. Renal tissue from rats given 90 mg 2,4,5-T/kg for 8 days was examined by light microscopy.

To investigate the possibility that the change in excretory rate of 2,4,5-T observed following daily administration in earlier experiments (Koschier & Berndt, 1976a) could have been caused by an ethanol-induced change in the rate of sc absorption, four rats were given ^{14}C-labelled 2,4,5-T in a daily ip dose of 90 mg/kg in a corn oil–ethanol (85:15, v/v) vehicle (injection volume 0·9 ml/kg) for up to 9 days, and the urinary excretion of radioactivity was measured by liquid scintillation spectrometry.

Possible effects of the route of administration were studied by comparing the urinary excretion of radioactivity and its retention in the renal cortex following administration of a labelled dose of 90 mg 2,4,5-T/kg in the corn oil–ethanol vehicle by the sc, ip and oral routes to groups of four rats. A similar group was given 90 mg [^{14}C]PAH/kg in 0·9% saline (pH 7·4) by ip injection.

Renal-slice preparation and incubation. The rats were killed by cervical dislocation and tissues were

placed immediately into cold Krebs–Ringer phosphate solution (pH 7·4), containing 1 mM-calcium and 5 or 40 mM-potassium (Umbreit, Burris & Stauffer, 1957). Renal cortical slices were prepared free-hand and incubated in the Krebs–Ringer phosphate buffer. The incubation solutions also contained the designated organic ion at an initial concentration in the medium of 10^{-5} M. The slice accumulation of these compounds was followed by monitoring the uptake of tracer amounts (0·02 μCi/ml) of radiolabelled organic ions. Renal-slice accumulation of organic ions was measured at the end of incubation, which was performed at 25°C in a Dubnoff metabolic shaker with a 100% oxygen atmosphere. The ^{14}C concentration was determined in aliquots of media or whole-tissue homogenates by standard liquid scintillation methods, including external standard quench connections when necessary (Bransome, 1970; Kobayashi & Maudsley, 1974). The uptake data are expressed as the slice-to-medium ratio, i.e. the concentration of radioactivity in the tissue (cpm/g) divided by that in the bathing solution (cpm/ml).

Tissue-binding studies. Studies on the tissue binding of the herbicide in homogenates of renal cortex from rats pretreated with 90 mg 2,4,5-T/kg for 1, 3 or 5 days were performed with Amicon-50 filter cone membranes. A 10% renal cortical homogenate in the Krebs–Ringer buffer was incubated with 2,4,5-T containing a ^{14}C-tracer for 120 min, and was then poured into a filter cone suspended in a large centrifuge tube. Each cone was centrifuged at approximately 25°C for 5–20 min at 900 **g**, until about 20% of the homogenate had filtered through the cone. Aliquots of both filtrate and original homogenate were assayed for radioactivity. [^{14}C]2,4,5-T (0·02 μCi/ml) was present in all homogenate incubations and sufficient unlabelled material was added to yield concentrations between 10^{-6} and 9×10^{-4} M.

Data evaluation. The data were analysed statistically using Student's *t* test or a paired *t* test depending on experimental design (Sokal & Rohlf, 1969 & 1973). The level of significance was chosen as $P < 0{\cdot}05$.

Table 1. *Organic ion transport in renal tissue after eight daily sc injection of 90 mg 2,4,5-T/kg*

Organic ion	Radioactivity accumulation in renal slices† from: Untreated rats	2,4,5-T-treated rats	Difference (%)
2,4-D	29·43 ± 0·71	9·81 ± 1·29*	−66·7%
TEA	10·72 ± 0·76	8·94 ± 0·89*	−16·6%
AIB	1·71 ± 0·24	1·66 ± 0·13	−2·9%

†Renal slices were incubated for 2 hr in Krebs–Ringer phosphate buffer containing 40 mM-K. Lactate (10^{-2} M) was present in the 2,4-D incubations.
Values are slice-to-medium ratios, i.e.

$$\frac{\text{concn of radioactivity in tissue (cpm/g)}}{\text{concn of radioactivity in medium (cpm/ml)}}$$

The data are presented as means ± SEM for 3–5 determinations. Those marked with an asterisk differ significantly (paired *t* test) from the corresponding control value: $*P < 0{\cdot}05$.

RESULTS

In renal slices from rats given 90 mg 2,4,5-T/kg sc for 8 days, the transport of 2,4-D, an organic acid, was reduced significantly (−66·7%) and that of TEA, an organic base, was also reduced (−16·6%) compared with the situation in renal slices from control rats, but the accumulation of AIB, a non-metabolizable amino acid, was unaffected by the repeated treatment with 2,4,5-T (Table 1).

Previously only large doses of 2,4,5-T were tested and these were shown to alter renal transport significantly. In further studies to test the sensitivity of these transport systems to inhibition by 2,4,5-T, the renal-slice transport of organic ions was found to be unaffected by pretreatment with a single sc dose of 20 mg/kg given 24 hr before the experiment (Fig. 1a). When rats were pretreated for 8 or 16 days with this small dose of 2,4,5-T, only minor alterations in slice transport were measured 24 hr after the final injection, whether or not the metabolic substrate, lactate, was present in the medium (Figs 1b & 2).

When labelled 2,4,5-T was administered ip in a corn oil–ethanol vehicle, two rats showed the same

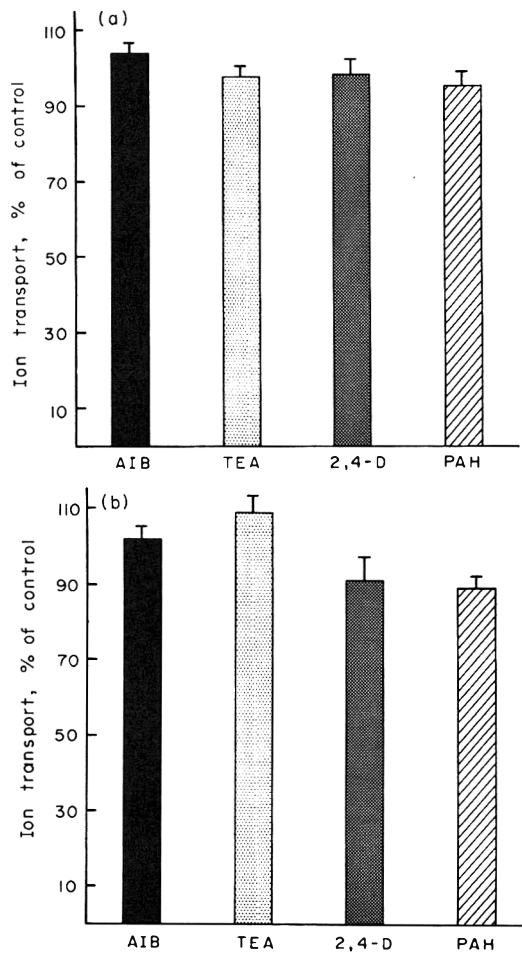

Fig. 1. Transport of organic ions by renal slices from rats pretreated with 2,4,5-T in (a) a single sc dose of 20 mg/kg and (b) eight daily sc doses of 20 mg/kg ($n = 4$). Slices were incubated for 2 hr in a Krebs–Ringer phosphate buffer containing 40 mM-K. Lactate (10^{-2} M) was present in the incubation media containing PAH or 2,4-D.

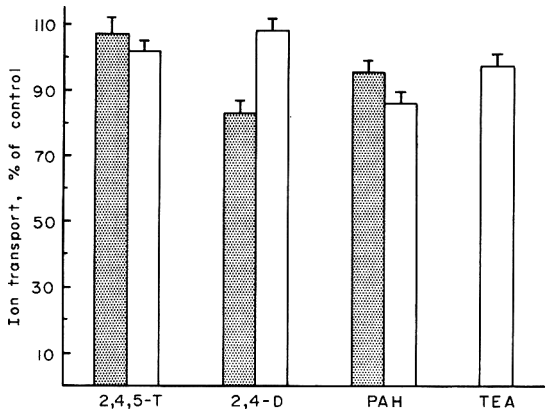

Fig. 2. Transport of organic ions by renal slices from rats pretreated with 16 daily sc doses of 20 mg 2,4,5-T/kg (n = 4). Slices were incubated for 2 hr in a Krebs–Ringer phosphate buffer containing 5 mM-K, with 10^{-2} M-lactate (▨) or with no metabolic substrate (□).

excretion pattern (Fig. 3) as was reported for rats given sc injections in ethanol (Koschier & Berndt, 1976a); after some initial retention, the excretion rate increased and, by day 6, all of the daily dose of radioactivity was excreted within 24 hr. However, the other two rats showed severe impairment of excretion during the first few days of herbicide administration and died on days 3 and 5, respectively. Table 2 summarizes the data on the renal excretion of 2,4,5-T and its derivatives after administration of a dose of 90 mg/kg in the corn oil–ethanol vehicle by various routes. Neither excretion nor renal retention was affected by the route. [^{14}C]PAH administered in saline at the same dose level as the 2,4,5-T was excreted to a greater degree, although the kidney cortex retained approximately 10 μg/g. The renal cortical concentration of 2,4,5-T (350 μg/g) was approximately 3–4 times greater than was found in animals receiving 2,4,5-T in an ethanol vehicle (Koschier & Berndt, 1976a).

In the tissue-binding study (Fig. 4), the percentage of 2,4,5-T bound in the homogenates was high at all concentrations of 2,4,5-T studied and was unaffected by pretreatment of the rats with the herbicide for 1–5 days.

Light-microscopic examination of renal tissue was performed on rats that received 90 mg 2,4,5-T/kg for 8 days. On day 9, renal tissue was prepared for staining with eosin–hematoxylin or periodic acid–Schiff reagent. Comparison of these tissues with tissue from control rats failed to reveal any alterations of the kidney attributable to pretreatment with 2,4,5-T.

DISCUSSION

The acute impairment of renal proximal tubular transport caused by 2,4,5-T appears to continue upon repeated administration of the herbicide. The transport of 2,4-D and TEA was depressed significantly by treatment with large (90 mg/kg) doses of this herbicide for 8 days. The previously reported increase in renal excretion after repeated administration of 2,4,5-T did not coincide with an increased ability of renal slices to transport organic ions.

The administration of lower doses of 2,4,5-T (e.g. 20 mg/kg) appeared to have no effect on renal transport. Repeated administration of this dose level for 8–16 days did not alter acid or base transport significantly. In addition, no facilitation of 2,4,5-T transport was measured even after 16 days of pretreatment. Since the transport of organic ions did not change

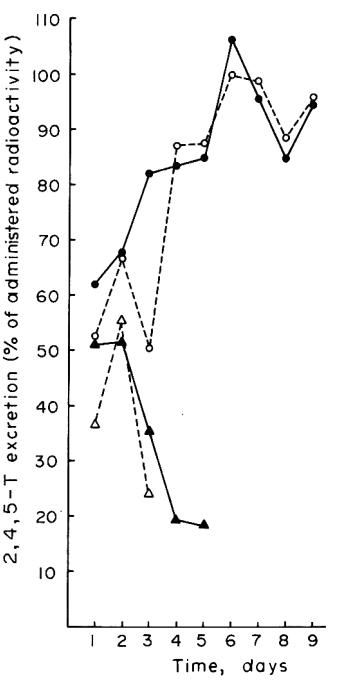

Fig. 3. Excretion of [^{14}C]2,4,5-T following its administration to rats in doses of 90 mg/kg/day in a corn oil–ethanol vehicle (0·9 ml/kg) for 9 days or until the rats died. Excretion values, given for four individual rats, were determined by liquid scintillation spectrometry.

Table 2. *Disposition of 2,4,5-T and PAH 24 hr after administration of a dose of 90 mg/kg to rats by various routes*

Organic acid and vehicle	Dosage route	Amount excreted in urine (% of dose)	Concn in renal cortex (μg/g tissue)
2,4,5-T in corn oil–ethanol	Subcutaneous	45·5 ± 3·6	375 ± 17·0
	Intraperitoneal	49·3 ± 3·6	346 ± 54·8
	Oral	53·0 ± 4·8	358 ± 30·05
PAH in 0·9% saline	Intraperitoneal	82·2 ± 1·62	9·25 ± 0·4

Values are means ± SEM for groups of four rats.

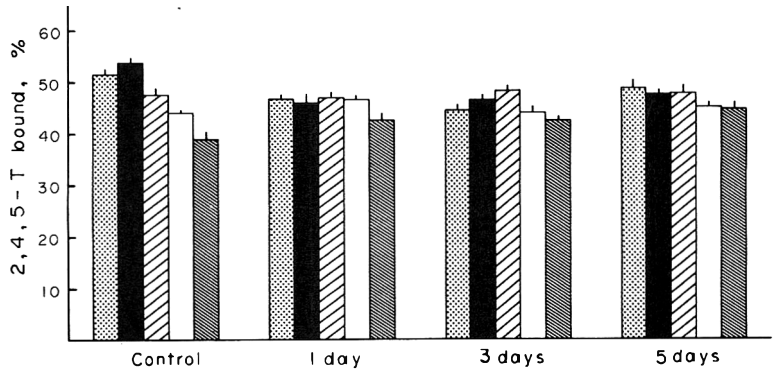

Fig. 4. Binding of 2,4,5-T to renal cortical homogenates from groups of four rats pretreated with 90 mg 2,4,5-T/kg daily for 1, 3 or 5 days. Control animals received no pretreatment. The homogenate incubations contained 2,4,5-T in molar concentrations of 3×10^{-7} (▨), 10^{-6} (■), 10^{-5} (▨), 10^{-4} (□) and 9×10^{-4} (▧).

with the 2,4,5-T excretion pattern, another mechanism must be responsible for increasing the short-term excretion of 2,4,5-T.

Two possible factors which could have an effect on the renal handling of 2,4,5-T were explored in this study: the first concerned the high tissue binding exhibited by 2,4,5-T, and the second dealt with possible alterations in the rate of 2,4,5-T absorption due to the route of administration and composition of the vehicle. Since tissue binding has been shown to be a factor (and may be a major factor) in the action of certain other inhibitors of organic-ion transport, including probenecid and cyanine dye no. 863 (Berndt, 1966 & 1967; Rennick, Kandel & Peters, 1956; Volle, Peters & Green, 1960), it was important to demonstrate that the high degree of tissue binding of 2,4,5-T was unchanged upon repeated administration of this herbicide. From these data and others, it appears that once a high concentration of 2,4,5-T is reached in the renal cortex, the transport of organic ions is inhibited until the herbicide is cleared from this tissue. The finding that the route of administration and the composition of the vehicle appeared to have little effect on the excretion pattern of 2,4,5-T in healthy animals makes it unlikely that the excretion pattern reflects alterations in the rate of absorption.

Whatever the mechanism for the reversal of the depressed renal excretion of 2,4,5-T, the conclusion that low doses of 2,4,5-T do not alter significantly the transport of organic ions in rats can be drawn from the current studies. However, this observation does not mean that low doses of 2,4,5-T could not have a significant environmental impact. For example, the rate of clearance of 2,4,5-T in substantially less in man than in the rat (Piper, Rose, Leng & Gehring, 1973); human whole-body and plasma rates of clearance are, respectively, 60 and 20% of the values for the rat. In addition, as little as 12 mg/kg has been shown to produce vomiting and weight loss in primates (Dougherty, Herbst & Coulston, 1975). Such susceptibility to the effects of pesticides is not uncommon in primates. For example, the LD_{50} of DDT in the rat is 250 mg/kg, whereas man shows signs of illness at 10 mg/kg (Matsumura, 1975).

The response of experimental animals to 2,4,5-T is also variable. Only minor toxic effects have been reported in animals receiving repeated doses of this herbicide, but severe maternal and foetal toxicities have been noted in mice at a daily dose of 120 mg/kg (Highman, Gaines & Schumacher, 1976; Highman, Gaines, Schumacher & Haley, 1976). The high degree of 2,4,5-T toxicity in mice may not be a simple case of increased susceptibility of the myocardium, bone marrow and other target tissues to this herbicide. Severe toxic effects were found in some mice, which became very ill or died as a result of 2,4,5-T treatment, but some mice receiving the same 2,4,5-T dose were unaffected by the herbicide. This variation could be related directly to the finding that rats receiving a high dose of 2,4,5-T (Fig. 3) show varying excretory patterns and toxicity. Rats able to excrete 2,4,5-T showed no apparent degree of toxicity at the end of the experiment except a reduction in weight gain, as reported previously (Koschier & Berndt, 1976a). The rats that showed a low excretory rate lost weight and died after building a large body store of the herbicide. A similar excretion–toxicity pattern was observed by Erne (1966) in a study in which some animals tolerated the daily administration of 50 mg 2,4-D/kg and eliminated this herbicide at a greater rate than animals that showed a progressive poisoning.

In conclusion, the excretory pattern of 2,4,5-T is of paramount importance with respect to the short-term toxicity of large doses of this herbicide. Although some questions remain on the environmental impact of low doses (10–20 mg/kg) of 2,4,5-T, the low concentration and short persistence of this herbicide in the environment (Kenaga, 1975; Matsumura, 1972 & 1975) ensure that such a dose level would be reached only in the case of accidental poisoning or occupational exposure.

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MYCOTOXINS IN FOODSTUFFS. X. PRODUCTION OF CITRININ BY *PENICILLIUM CHRYSOGENUM* IN BREAD

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Abstract—*Penicillium chrysogenum* produced citrinin on whole wheat bread, whole wheat bread with wheat germ, whole wheat bread with linseed, whole rye bread with shredded wheat and rye bread with wheat flour, but two other moulds (Pencillium sp. and *Aspergillus niveus*), known to be able to form this mycotoxin, were not able to do so on these types of bread. The highest yields, found in whole wheat bread, were in the range of 0·2–0·4 μg/g bread. The growth of *P. chrysogenum* and (to a less marked degree) the toxin production were influenced by the total acid content (*Säuregrad*), which had to be less than about 9 for activity to be maintained, and by the temperature. The optimal temperatures for growth and for citrinin formation were 30 and 25°C, respectively.

INTRODUCTION

In 1931, Hetherington & Raistrick described the isolation of a yellow crystalline compound, which they named 'citrinin', from *Penicillium citrinum*. Further studies revealed that this substance was also produced by other species of Penicillium and other moulds, notably *P. expansum* (Ciegler, Mintzlaff, Machnick & Leistner, 1972; Haese, 1963), *P. viridicatum* (Ciegler, Fennell, Sansing, Detroy & Bennett, 1973; Krogh, Hasselager & Friis, 1970; Scott, van Walbeek, Harwig & Fennell, 1970b; Scott, van Walbeek, Kennedy & Anyeti, 1972), *P. claviforme* (Ciegler *et al.* 1972), *P. palitans* (Scott *et al.* 1972), *P. steckii* (Jabbar & Rahim, 1962), *P. notatum* (Betina, Nemec, Kutková, Balan & Kováč, 1964), *P. lividum, implicatum, citreoviride, jenseni* and *fellutanum* (Pollack, 1947), *Aspergillus terreus* (Raistrick & Smith, 1935) and *A. candidus* (Damodaran, Ramadoss & Shanmugasundaram, 1973; Timonin, 1942). Even *Crotalaria crispata*, a higher flowering plant from Australia, produces citrinin in its leaves (Ewart, 1933). Brown, Cartwright, Robertson & Whalley (1948) established the structure of citrinin as a bicyclic phenol derivative (Fig. 1).

Citrinin has attracted much attention because of its bacteriostatic properties against staphylococci and other Gram-positive and Gram-negative organisms in dilutions between 1:15,000 and 1:50,000 (Heatley & Philpot, 1947; Kavanagh, 1947; Oxford, 1942). Treatment of *Staphylococcus aureus* with the toxin resulted in an inhibition of respiratory enzymes (Michaelis & Thatcher, 1945). Moreover, signs of severe toxicity to phytopathogenic fungi (Verona & Gambogi, 1952) and higher plants, notably the wilting of shoots of bean, cotton and sorghum (Damodaran, Kathirvel-Pandian, Seeni, Selvam, Ganesan & Shanmugasundaram, 1975) have been described.

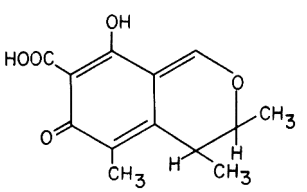

Fig. 1. Structure of citrinin.

As a consequence of its inhibitory action on bacteria, citrinin was suggested as a powerful new antibiotic (Bastin, 1949 & 1952; Blanpin, 1959), until toxicity studies in animals showed that citrinin was too toxic for such use. In their studies on the pharmacology of citrinin, Ambrose & DeEds (1945 & 1946) found typical parasympathetic effects, such as miosis, salivation, increased bronchial secretion and lachrymation. The LD_{50} values were for rats (sc and ip) 67 mg/kg body weight, for mice (sc and ip) 35 mg/kg, for guinea-pigs (sc) 37 mg/kg and for rabbits (iv) 19 mg/kg (Ambrose & DeEds, 1946). Furthermore, dilatation of blood vessels, a fall in blood pressure and tachyphylaxis were reported. Severe kidney damage was caused in pigs and rats (Friis, Hasselager & Krogh, 1969; Krogh *et al.* 1970) and in dogs (Carlton, Sansing, Szczech & Tuite, 1974) after oral administration of the mycotoxin. Japanese authors revealed that citrinin, produced by *P. citrinum*, is responsible for the 'yellowed rice' mycotoxicosis in animals (Saito, Enomoto & Tatsuno, 1971).

Several analytical procedures have been developed for the detection of citrinin. They are based upon the yellow fluorescence of the toxin under a UV lamp of 360 nm (Damodaran *et al.* 1973; Hald & Krogh, 1973; Scott, Lawrence & van Walbeek, 1970a).

The natural occurrence of citrinin in foodstuffs has been reported mainly from Canada and Denmark, suggesting that the toxigenic Penicillium species have a competitive advantage in cooler climates (van Walbeek, 1973). In Denmark, Krogh and his co-workers described the detection of citrinin in barley which caused chronic kidney degeneration when fed to pigs and rats (Krogh *et al.* 1970). In regions with a high incidence of porcine nephropathy, a naturally occurring kidney disease, 9% of samples of cereals used

as feed for bacon pigs contained citrinin, always together with the mycotoxin ochratoxin A (Krogh, Hald & Pedersen, 1973). In Canada, Scott *et al.* (1970b & 1972) found citrinin in 13 of 29 samples of heated grain (wheat, rye, oats and barley) at levels of 0·07–80 ppm. In the USA, citrinin-producing strains of *P. citrinum* and *P. viridicatum* have been isolated from dried beans (Mislivec, Dieter & Bruce, 1975) and from country-cured ham (Wu, Ayres & Koehler, 1974), and in Germany, Ciegler *et al.* (1972) isolated 422 Penicillium strains from 44 mould-ripened dry sausages and detected an ability to form citrinin in ten of the isolates. Malting and brewing experiments revealed a complete degradation of added crystalline citrinin during the malting process, so that no detectable amounts were present in the wort (Krogh, Hald, Gjertsen & Myken, 1974).

Several of the citrinin-forming species of Penicillium and Aspergillus listed above may be found in flour (Graves & Hesseltine, 1966; Hesseltine & Graves, 1966) and in cereal products (Reiss, 1973a; Senser, 1969). Therefore, the ability of several toxigenic moulds to form citrinin in different kinds of bread and the influence of environmental factors on this formation were studied.

EXPERIMENTAL

Fungi. Three moulds known to produce citrinin, namely *P. chrysogenum* (Sp 465; isolated from a raw sausage in Germany), Penicillium sp. (Sp 385) and *A. niveus* (Krogh strain), were kindly supplied by Prof. Dr. L. Leistner, Kulmbach. Stock cultures were maintained on malt-extract agar (Difco).

Bread samples. The following kinds of bread were used as substrate: whole wheat bread (*Grahambrot*; 6·4), whole wheat bread with wheat germ (*Weizenkeimbrot*; 6·4), whole wheat bread with linseed (*Leinsamenbrot*; 5·5), whole rye bread (*grobes rheinisches Vollkornbrot*; 11·2), whole rye bread with shredded wheat (*Felkebrot*; 4·0) and rye bread with wheat flour (*Bauernschnitten*; 7·2), the figures in parentheses being the total acid content (*Säuregrad*) determined by titration of a methanolic bread extract with 0·1 N-NaOH against phenolphthalein.

For evaluating the influence of the acidity of the substrates, two further samples of whole rye bread with shredded wheat were produced with a total acid content of 6·5 and 10·4, respectively, and used for a comparative test. The various types of bread were sliced, packed in cellulose foil (GEB 300; Kalle AG, Wiesbaden) and sterilized in hot steam.

Inoculation and incubation of the bread packages. The first slice of each package was inoculated with spores of one of the mould isolates and the package was incubated at 25 ± 1°C. In order to study the influence of various temperatures on growth and toxin formation, packages of whole wheat bread were inoculated with *P. chrysogenum* and incubated at 2, 10–12, 20, 30, 35 and 40°C. The incubations were always performed in the dark and three packages were used in each test.

Determination of fungal growth. Every 24 hr, the diameter of the fungal colonies on the first slice of bread was measured through the transparent foil.

Semiquantitative determination of citrinin. A 1-g piece of the first slice, with adhering hyphae and spores, was extracted for 10 min with 5 ml chloroform, the most suitable solvent (Neely, Ellis, Davis & Diener, 1972). The chloroform was decanted and evaporated, the residue was dissolved in 1 ml chloroform, and 2 µl of this solution was spotted on a precoated silica-gel sheet (Polygram SIL N-HR from Macherey-Nagel & Co., Düren), the chromatogram being developed with toluene–ethyl acetate–90% formic acid (6:3:1, by vol.) (Damodaran *et al.* 1973; Scott *et al.* 1970b). Citrinin spots were detected by their yellow fluorescence under long-wave ultraviolet radiation at 360 nm (Blak-Ray UVL-21). The standard used was 2 µl of a solution of 0·1 mg citrinin (provided by Prof. Dr. L. Leistner) in 2 ml chloroform.

The semi-quantitative determination of the toxin present in the spots was performed with a Kodak gray scale under ultraviolet radiation at 360 nm (author's unpublished data), a procedure based upon the disappearance of the fluorescence of the individual spots under different shades of gray and used successfully in the determination of aflatoxins (Reiss, 1973b) and sterigmatocystin (Reiss, 1975a). The detection limit was 0·01 µg.

RESULTS

Growth of the fungi on the different types of bread

P. chrysogenum grew well on whole wheat bread, whole wheat bread with wheat germ, whole wheat bread with linseed and rye bread with wheat flour but not on whole rye bread (Fig. 2). Penicillium sp. and *A. niveus* formed new colonies in a similar manner, but these were smaller in diameter than those of *P. chrysogenum*.

Influence of total acid content on the growth of P. chrysogenum

Only *P. chrysogenum* was able to form citrinin on the various types of bread and therefore the influence

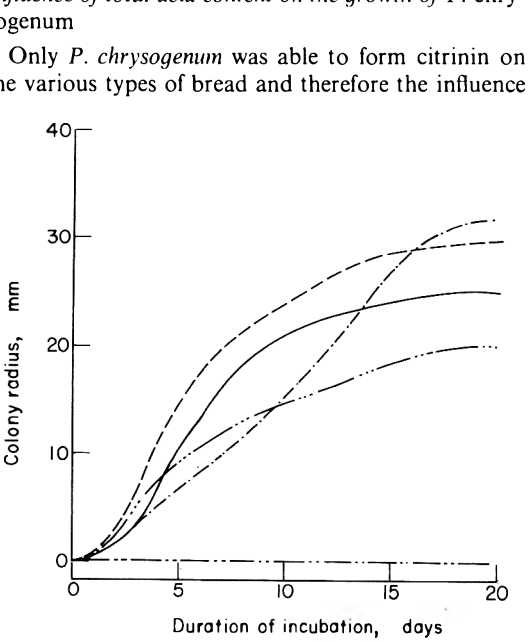

Fig. 2. Growth of *P. chrysogenum* on whole rye bread (—·—·—), whole wheat bread (— — —), whole wheat bread with wheat germ (——), whole wheat bread with linseed (— —) and rye bread with wheat flour(—·—·—).

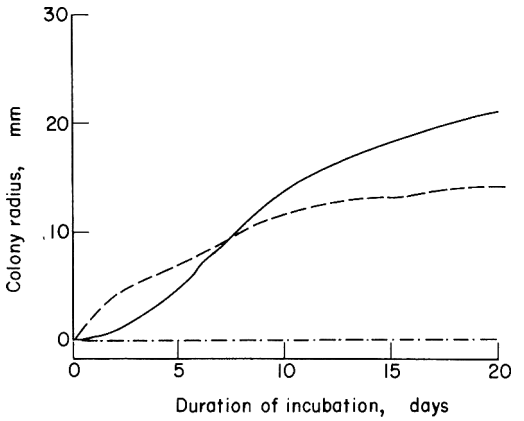

Fig. 3. Growth of *P. chrysogenum* on samples of whole rye bread with shredded wheat with a total acid content of 4·0 (— —), 6·5 (——) and 10·4 (— · —).

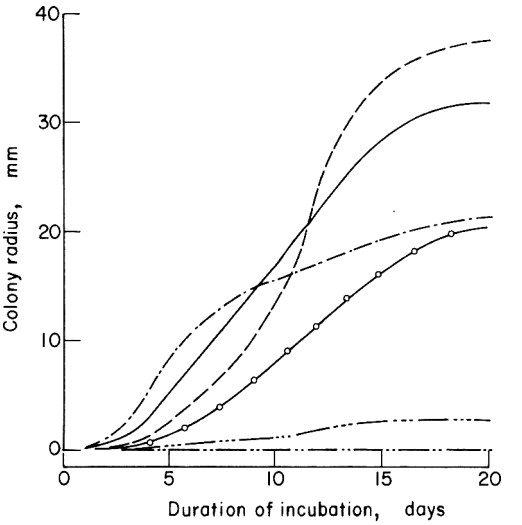

Fig. 4. Growth of *P. chrysogenum* on whole wheat bread at 2 (— · · —), 10–12 (— · —), 20 (—○—), 25 (— · — ·), 30 (— —), 35 (——) and 40°C (— · · —).

of the acidity of the substrate was investigated only with this organism. The optimal total acid content in whole rye bread with shredded wheat was about 6·5, whereas lower and higher acidity reduced the fungal growth (Fig. 3).

Influence of incubation temperature on the growth of P. chrysogenum

The optimal temperature for the growth of *P. chrysogenum* on whole wheat bread was 30°C (Fig. 4). At 20, 25 and 35°C the mould developed smaller colonies. No growth occurred at 2 or 40°C and very little at 10–12°C.

Formation of citrinin

Only *P. chrysogenum* was able to form citrinin on the different types of bread (Table 1). Penicillium sp. and *A. niveus* developed colonies but produced no toxin. The amount of citrinin detectable in the bread slices increased with an increase in incubation time from 10 to 20 days. The total acid content of the substrate obviously had no influence on the formation of citrinin. The optimal temperature for citrinin production by *P. chrysogenum* was 25°C (Table 2).

DISCUSSION

P. chrysogenum grew and formed citrinin on different types of bread made from whole wheat and whole rye. A comparison of Figs 2 and 3 with Table 1 shows that the formation of citrinin is influenced by the composition of the substrate and is not merely dependent upon the growth of the fungus as expressed by the radius of its colonies. After 10 days, *P. chrysogenum* developed the largest colonies on whole wheat bread with linseed but produced only 0·01–0·02 μg citrinin on this bread, far less than on whole wheat bread and whole rye bread with shredded wheat, on which the mould developed smaller colonies.

As in the case of other mycotoxins (Reiss, 1973c, 1975b, c & 1976), the total acid content, over a wide range, did not markedly affect the toxin production. The fungal growth, however, was influenced by the acidity of the substrate, as is shown in Fig. 3.

The optimal temperature for the growth of *P. chrysogenum* on whole wheat bread was 30°C and that

Table 1. *Formation of citrinin by* P. chrysogenum *on different types of bread*

Type of bread	Total acid content	Duration of incubation (days)	Formation of citrinin (μg/g bread)
Whole wheat bread	6·4	10	0·02–0·04
		20	0·2–0·4
Whole wheat bread with wheat germ	6·4	10	0·01–0·02
		20	0·2–0·4
Whole wheat bread with linseed	5·5	10	0·01–0·02
		20	0·06–0·08
Whole rye bread with shredded wheat	4·0	10	0·08–0·1
		20	0·06–0·08
	6·5	10	ND
		20	0·02–0·04
Rye bread with wheat flour	7·2	10	0·01–0·02
		20	0·08–0·1

ND = Not determined

Table 2. *Formation of citrinin by* P. chrysogenum *on whole wheat bread at different incubation temperatures*

Incubation temperature (°C)	Formation of citrinin (μg/g bread) after	
	10 days	20 days
10–12	ND	0·01–0·02
20	0·02–0·04	0·02–0·04
25	0·06–0·08	0·2–0·4
30	0·06–0·08	0·08–0·1
35	ND	0·08–0·1

ND = Not determined

for the formation of citrinin was 25°C. These results agree with the report of Panasenko (1967) that the optimal temperature for the growth of this mould lay between 25 and 28°C and with the report of Wu *et al.* (1974) that the optimal temperature range for citrinin production by *P. viridicatum* on country-cured ham was 25–30°C. In the study on the growth of *A. versicolor* and the formation of sterigmatocystin on bread (Reiss, 1976), the optimum temperature for toxin production was similarly found to be lower than that for mycelial growth.

P. chrysogenum is a ubiquitous mould occurring consistently in soil and detectable in bread and pastries among other foodstuffs (Raper & Thom, 1949; Senser, 1969). Consequently infection of bread by this organism and the formation of citrinin cannot be discounted. Because of the pronounced toxicity of this mycotoxin, the possible hazard to health of contamination by citrinin-producing moulds should be taken into consideration in hygienic measures instituted for the preparation and handling of bakery products.

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SAFETY TESTING OF ALKYL POLYETHOXYLATE NONIONIC SURFACTANTS. I. ACUTE EFFECTS

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Abstract—Acute toxicity tests were carried out on two alkyl polyethoxylate surfactants ($CH_3 \cdot [CH_2]_{11-12} \cdot [OCH_2 \cdot CH_2]_6 \cdot OH$, or $C_{13}E_6$ and $CH_3 \cdot [CH_2]_{13-14} \cdot [OCH_2 \cdot CH_2]_7 \cdot OH$, or $C_{14}E_7$) and on two detergent formulations containing 33% of either of the alkyl polyethoxylate (AE_x) surfactants. The acute oral toxicity was low in rats, dogs and monkeys. Emesis was produced in the dogs and monkeys and probably accounted for the generally lower activities of the test compounds in these species compared with the rat. Pharmacological responses (convulsions and/or depression) were seen in ip studies in mice, rats and monkeys; however, the doses required for these effects were generally in the lethal range. When the surfactants were given orally, pharmacological effects were not seen unless lethal doses were given.

The acute dermal toxicity of these materials was low in rabbits and guinea-pigs but moderate skin irritation was produced under occluded patches after 24 hr (concentrations up to 10%). Patch tests on human skin produced only minor irritation after 4 hr at concentrations of 25 or 100%. Young guinea-pigs immersed to chest level for 4 hr in 25% AE_x developed only slight irritation. AE_x surfactants and formulations were severely irritating to the rabbit eye but had a much less severe and more transient effect on the monkey eye. Rat studies indicated a low inhalation toxicity.

In general, these results suggest that $C_{13}E_6$ and $C_{14}E_7$ would not involve significant risk to man under conditions of predicted use and potential misuse.

INTRODUCTION

Tridecylhexaethoxylate ($C_{13}E_6$) and tetradecylheptaethoxylate ($C_{14}E_7$) are alkyl polyethoxylate (AE_x) surfactants used at present in heavy-duty laundry-detergent formulations and having a potential use in dishwashing detergents. Because of potential human exposure to the ingredients either during manufacture or in consumer use of products containing them, we have carried out an extensive animal testing programme concerned particularly with dermal administration.

Early in the development of these substances as ingredients in laundry products we became aware that similar AE_x compounds had been used medicinally to treat stomach ulcers (Hochrein & Schleicher, 1951; Strack, 1950) and respiratory infection in infants (Larkin, 1957) and as spermicidal creams (Berberian, Gorman, Drobeck, Coulston and Slighter, 1965). They may also have analgesic action (Schulz, Harz, & Soehring, 1953) as well as local anaesthetic and systemic anaesthetic effects (Zipf, Wetzels, Ludwig & Fredrich, 1957). We therefore performed several additional experiments on the pharmacological actions of $C_{13}E_6$ and $C_{14}E_7$.

Following these acute studies, subchronic dermal and oral animal studies as well as repeated insult patch tests for skin sensitization in man were performed (Brown & Benke, 1977).

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EXPERIMENTAL

Test materials. $C_{13}E_6$ ($CH_3 \cdot [CH_2]_{11-12} \cdot [OCH_2 \cdot CH_2]_6 \cdot OH$) was prepared by base-catalysed ethoxylation of *n*-dodecanol and *n*-tridecanol, using 6 mol ethylene oxide/mol alcohol. (These materials contained at least 70% normal alcohol with lesser amounts of 2-substituted alcohols. Substitution was predominantly methyl or ethyl.) The AE_x produced contained 42% C_{12} and 58% C_{13} and an average ethoxylate (E) chain-length of 6·5 (range 0–14). $C_{14}E_7$ ($CH_3 \cdot [CH_2]_{13-14} \cdot [OCH_2 \cdot CH_2]_7 \cdot OH$) was prepared in similar fashion with a mixture of *n*-tetradecanol and *n*-pentadecanol. The reaction product contained 58% C_{14} and 42% C_{15} alkyls and an average ethoxylate chain-length of 7·0 (range 0–13). For both materials there was a normal distribution of ethoxylates. $C_{13}E_6$ and $C_{14}E_7$ were incorporated at a level of 33% into experimental detergent formulations A and B, respectively. The balance of these formulations was composed of water, ethanol, anionic surfactants, neutralizers, perfumes and colouring agents.

Acute oral toxicity. Formulations A and B and 33% aqueous solutions of $C_{13}E_6$ and $C_{14}E_7$ were administered orally to fasted adult Wistar rats (190–300 g) in groups having equal proportions of each sex. Observations for signs of toxicity were made at short intervals (15 min to 1 hr) during the first 8 hr and daily for 2 wk. At this time, the LD_{50} values were calculated by the Thompson (1947) and Weil (1952) methods. The oral toxicities of $C_{14}E_7$ and B were also determined in fasted weanling Cox rats (22–24 days old and weighing 40–60 g) and in adult males (200–300 g). For these studies the Miller &

Tainter (1944) method of LD_{50} determination was used.

The oral toxicities of these materials were also determined in fasted beagle dogs weighing 7·5–13 kg (one of each sex per group). Dogs were observed continuously for 4 hr and then twice daily for 7 days. In separate tests, ED_{50} values (dose producing emesis in 50% of the dogs treated) were determined. Four dogs (two of each sex) were dosed at each of four dose levels.

In a range finding study, pairs of 1·8–3·7 kg juvenile rhesus monkeys were given steadily increasing doses of $C_{13}E_6$ (0·5, 0·6, 0·75, 1·0, 1·25 and 1·5 g/kg $C_{13}E_6$ as a 25% aqueous solution) or formulation A (1·5, 1·8, 2·15, 3·0 and 4·5 g/kg) by gavage at approximately 3-day intervals. Subsequently, single doses (6–20 g/kg) of formulation A were given to six pairs of rhesus monkeys (one pair per dose level). A single rhesus monkey was given 10 ml/kg of a 33% $C_{14}E_7$ solution (3·3 g/kg) by gavage and four pairs of rhesus monkeys were given 5, 10, 15 or 20 g formulation B/kg. Pairs of 2·7–3·0-kg cynomolgus monkeys were given single oral doses of 6·7 or 10 g $C_{14}E_7$/kg or 10 or 18 g formulation B/kg. All pairs of monkeys comprised one of each sex. Animals were observed for any signs of toxicity or pharmacological effects during the ensuing 8-hr observation period and then twice daily for 14 days.

Intraperitoneal toxicity. To assess possible pharmacological actions of AE_x compounds and to detect species differences in susceptibility, ip toxicity was determined in ICR mice, Cox rats, Hartley guinea-pigs, rhesus monkeys and cynomolgus monkeys. The treated animals were observed continuously during the first 4–6 hr, and then several times daily for 14 days. LD_{50} values were obtained for $C_{13}E_6$ and formulation A in both mice and rats, and for $C_{14}E_7$ in pre-weanling, weanling and adult rats. Lethal dose ranges were determined for $C_{14}E_7$ and formulation B in mice, for $C_{14}E_7$ in cynomolgus monkeys and for $C_{13}E_6$ and formulation A in rhesus monkeys.

Clinical chemistry. Groups of six fasted adult male Cox rats (240–260 g) were dosed ip with 235 mg/kg of $C_{13}E_6$, 300 mg/kg of $C_{14}E_7$, or the vehicle (physiological saline, all at 10 ml/kg). These doses were chosen to produce maximum elevation of serum enzymes without causing immediate death. Similar numbers of rats were dosed orally with 1·0, 1·5 or 2·5 g/kg of AE_x or the saline vehicle. Rats were killed under ether anaesthesia by exsanguination from the inferior vena cava. The blood was allowed to clot for 30 min at 25°C and then 30 min at 5°C. After a 5-min 2000-***g*** centrifugation at 25°C, the straw-coloured serum was transferred to clean test tubes for assay. Ornithine carbamoyl transferase (EC 2.1.3.3; OCT) was assayed by the method of Drotman (1975), sorbitol dehydrogenase (EC 1.1.1.14; SDH) by the method of Gerlach (1965), and α-hydroxybutyrate dehydrogenase (EC 1.1.1.27; α-HBD) using Abbott Reagent Kit no. 6052-03 and the method described by Rosalki & Wilkinson (1964) and Ellis & Goldberg (1971). Creatinine phosphokinase (EC 2.7.3.2; CPK) was measured by the method of Rosalki (1967) using Abbott Reagent Kit no. 6047-03. One international unit (IU) of SDH or α-HBD is defined as the amount of enzyme that will oxidize 1 μmol NADH/min at 30°C; one IU of CPK is the amount of enzyme that will reduce 1 μmol NAD/min at 30°C; one IU of OCT is the amount of enzyme that will arsenolyse 1 μmol citrulline/hr at 30°C. Blood urea nitrogen (BUN) was determined by the method of Marsh, Fingerhut & Miller (1965) and serum protein by the biuret method (Weichselbaum, 1946). Serum glucose was assayed by a linked enzyme method (Magar & Farese, 1965).

For urine collection, rats were housed for 16–18 hr in stainless-steel metabolism cages. Urinary protein, glucose and pH were determined using Combistix (Ames Company, Division of Miles Laboratories, Elkhart, Ind.) and specific gravity was determined with a refractometer. Urine was examined microscopically for cells.

Intravenous toxicity. This route of administration was used, in unfasted rats and guinea-pigs, to provide a large, rapidly distributed systemic load.

To study possible toxic or pharmacological effects on the heart, several experiments *in vivo* in cats were performed. Groups of four anaesthetized (sodium pentobarbitone) cats, weighing 2·5–3·5 kg, were dosed iv with increasing doses (1, 10 or 30 mg/kg) of $C_{13}E_6$ or $C_{14}E_7$. Using two six-channel Grass polygraphs, the blood pressure, inotropic activity, heart rate and electrocardiogram (ECG) were monitored. Blood pressure was recorded directly from the femoral artery with a Statham P23AC strain gauge. Inotropic activity was studied (using artificially ventilated thoracotomized cats) by suturing a strain gauge arch (John A. Warren, Charleston, S.C.) to the right ventricle. ECGs were recorded with platinum-needle electrodes. Heart rate was recorded with a Grass EKG and tachygraph preamplifier (5P4). The interactions of $C_{13}E_6$ or $C_{14}E_7$ (several dose levels) with norepinephrine (0·5 μg/kg), isoproterenol (0·1 μg/kg) or histamine (0·1 μg/kg) were also studied at various time intervals.

Acute dermal toxicity. A modification of the Draize (1959) method was used to evaluate acute percutaneous toxicity. Young adult New Zealand rabbits or Hartley guinea-pigs were evenly distributed (by weight) into groups with three males and three females in each. Hair was clipped from an area 160 cm^2 on each animal's back (approximately 10% of the total body surface) 24 hr before treatment. The backs of three animals (two females, one male) were abraded with the clippers (making minor scratches through the stratum corneum but not producing bleeding) and three (two males, one female) were left intact. The test materials were applied with a tongue depressor and spread over the test sites, covered with 8-ply gauze and a rubber sleeve and taped securely with several wrappings of 3-inch Elastoplast tape. Harnesses (Newmann, 1963) were used to prevent removal of the dose. At least four dosage groups were used. After 24 hr, the dressings and residual test material were removed and the application sites were examined for local skin reactions. Daily observations for deaths, skin reactions and behavioural abnormalities were made for 14 days. Weights were then recorded and autopsies were performed. LD_{50} values were calculated by the method of Litchfield & Wilcoxon (1949).

Inhalation toxicity. Five different aerosol concen-

trations of $C_{13}E_6$ and $C_{14}E_7$ were produced by an atomizer (Spraying Systems: No. 1/8 JBC, with a No. 2050 (modified) fluid nozzle, and a No. 64 air nozzle operated with filtered compressed air). Groups of ten adult male Sprague–Dawley rats were exposed to each AE_x concentration. A constant aerosol concentration was maintained throughout the 4-hr exposure period in a 56-litre glass test chamber (10 litre/min flow rate). Exposure concentrations were calculated using samples removed from the chamber at intervals during the exposure. For $C_{13}E_6$, the calculated concentrations were: 0·28, 0·63, 1·48, 3·65, and 9·28 mg/litre. The calculated $C_{14}E_7$ concentrations were: 0·9, 1·4, 2·3, 2·7 and 3·0 mg/litre. Animals were observed continuously for toxic effects during exposure. Any animals that died and all those killed on day 14 were autopsied and the following tissues were weighed and examined histologically: lung, trachea, liver, kidney, heart, thyroid, adrenal, gonad, spleen, brain, stomach, intestine, bone and eye.

Anaesthetic and analgesic effects. Local anaesthesia was tested in adult rats by sc injection of the test material (50, 100 or 300 mg/kg) into the shaved hip or centre-back area. Using a heated probe (maintained at 46°C by a rheostat), the time necessary to produce a positive response when compared to the opposite hip (saline injected) or to the predose response was used as a measure of the local anaesthetic effect. Tests were performed at various times after dosing. Procaine HCl, a known local anaesthetic, was used as a positive control.

Systemic analgesia was determined in mice following ip injections of $C_{13}E_6$ (100 mg/kg) or formulation A (300 mg/kg). Mice were tested 15, 30, 60 and 90 min after dosing by placing them on a Corning ceramic hotplate (maintained at 80–85°C), and the time necessary to produce stress (increased movement) was recorded. The method was similar to that of Woolfe & MacDonald (1944). Immediately after this test the tails of the mice were immersed to 75% of their length in water at 55–60°C and the time required for movement resulting from discomfort from the heat was recorded. Morphine sulphate, a narcotic analgesic drug, was used as the positive control.

Acute eye-irritation studies. In these studies, minor modifications (Nixon, 1971) of the method of Draize (1959) were used. Rhesus monkeys and New Zealand albino rabbits were tested. In all cases, 0·1 ml of 10 or 100% aqueous solutions were used. Applications to rabbit eyes were made directly into the conjunctival sac. Scores were made at 1 hr, 1, 2, 3, 4 and 7 days, and weekly for 5 wk or until the eyes were normal. Monkey eyes were treated by direct application onto the corneal surface. Scores were made at 1, 2, 3, 4, 7, 9, 11 and 14 days and then weekly until eyes were normal.

Acute skin-irritation studies. The irritation produced by a single dermal application of $C_{13}E_6$ (0·5 ml of a 1% solution) or $C_{14}E_7$ (0·5 ml of a 10% solution) was determined in New Zealand albino rabbits (2·2–2·8 kg). The test materials were applied to abraded and intact clipped sites on the back of each animal (six animals for each material). Sites were covered with felt pads (0·75 × 1 in.) and the animals were then wrapped with 3 in. Elastoplast tape. After 24 hr the patches were removed. The scoring method of Draize (1955) was used to obtain a primary irritation index (PII). Scoring was done at both 24 and 72 hr after application of the dose.

In an acute immersion study, two juvenile guinea-pigs (one male and one female, 225 g each) were subjected to a single 4-hr chest-level immersion exposure in a 25% $C_{14}E_7$ solution at 40°C. A restraining basket (Opdyke & Burnett, 1965) prevented oral ingestion and kept the animals in position. Animals were observed for signs of effect during the immersion and at frequent intervals for 2 wk.

In human skin-irritation studies, groups of 5–10 subjects were exposed on the arm to the test materials (0·4 or 0·5 ml) under 0·875 in. diameter Webril® occluded patches. The concentrations used were either 10 or 25% solutions of AE_x or undiluted formulations A or B. With the exception of one 24-hr exposure with $C_{13}E_6$, the exposures were 4 hr.

RESULTS

Acute oral toxicity

Results of oral toxicity tests in several animal species are shown in Table 1. In all cases, LD_{50} values for $C_{13}E_6$ and $C_{14}E_7$ were equal to or greater than 1·4 g/kg and for A and B were equal to or greater than 4·7 g/kg, indicating a low order of toxicity.

In rats the primary signs of toxicity were CNS depression, loss of righting reflex, diarrhoea and piloerection. Dose-response curves for loss of righting reflex essentially overlapped those for lethality. Gastrointestinal irritation and loss of electrolytes and fluid were the primary toxic manifestations. Most deaths occurred within 2 days of treatment, although some deaths occurred as late as day 6. Autopsies revealed discolouration of gastric or intestinal mucosa or both, with prominence of serosal blood vessels. Polyuria and a bloody discharge around the mouth and nose were seen in most rats. No appreciable toxicological differences among the four test materials were seen although weanling Cox rats were significantly less susceptible to the lethal effects of formulation B than adult Cox rats (LD_{50} 11·6 compared with 7·9 g/kg).

Emesis and diarrhoea were the only effects observed in beagle dogs dosed by gavage with 1·65 g $C_{13}E_6$/kg, 5 g A/kg or 10 g B/kg. Higher doses were not tested. A slight hyperaemia of the gastric mucosa was the only unusual finding at autopsy (at 7 days). $C_{14}E_7$ was not tested for oral toxicity or emetic response in dogs. ED_{50} values for A, B and $C_{13}E_6$ in dogs were 0·63, 1·0 and 0·1 g/kg, respectively.

In the range-finding study with monkeys given steadily increasing doses of $C_{13}E_6$ and formulation A, as in the dog the only effects observed were emesis and diarrhoea. Subsequent administration of single doses of formulation A between 6 and 20 g/kg to six pairs of rhesus monkeys failed to establish a lethal dose range. Emesis and diarrhoea were seen at all dose levels but were most marked above 9 g/kg. All animals exhibited signs of weakness and sedation, especially at doses of 15, 17·5 and 20 g/kg. One animal died 14 hr after receiving 15 g/kg, apparently from a

®Registered trade name of Kendall Co., Walpole, Mass.

Table 1. *Acute oral toxicity of $C_{13}E_6$, $C_{14}E_7$ or formulations A and B in rats, dogs and monkeys*

Test animal	Approximate age	Acute oral LD_{50} (g/kg)			
		$C_{13}E_6$	$C_{14}E_7$	A	B
Rat (Wistar)	Adult	2·1 (1·6–2·5)*	3·3 (2·7–4·1)*	6·7 (6·0–7·4)*	6·1 (5·2–6·9)*
Rat (Cox)	Weanling	—	2·6 ± 0·1†	—	11·6 ± 1·1†
Rat (Cox)	Adult	1·4 (1·2–1·7)*	—	4·7 (4·2–5·2)*	7·9 ± 1·2†
Dog (Beagle)	Adult	> 1·65‡	—	> 5‡	> 10‡
Monkey (rhesus)	Juvenile	> 1·5‡	> 3·3‡	> 20‡	> 20‡
Monkey (cynomolgus)	Juvenile	—	≥ 10‡	—	≥ 18‡

*LD_{50} and 95% confidence interval calculated by computer using a program for methods of Thompson (1947) and Weil (1952). (n > 40).

†LD_{50} ± standard error calculated by method of Miller and Tainter (1944). (n = 12–34, mixed sexes for weanling rats, males only for adult rats).

‡Highest dose tested (see text for numbers of animals in these studies).

complication following aspiration of vomitus (indicated at autopsy and confirmed by histological examination). The remaining animals appeared normal by 24 hr. One animal from each of the seven dosage groups was autopsied 14 days after dosing. There was no evidence of any gross or histopathological changes caused by the test materials.

Mild diarrhoea was the only observed toxic effect of a 10-ml/kg dose of a 33% $C_{14}E_7$ solution on the single rhesus monkey so treated. Emesis and diarrhoea occurred in all animals given 5, 10, 15 or 20 g formulation B/kg, but occurred more rapidly in animals receiving higher doses. The main effects were similar to those seen with formulation A. By 24 hr all animals except the female in the 20-g/kg group had recovered. This monkey died 3 hr 30 min after dosing from aspiration of vomitus (confirmed at autopsy and substantiated by histological examination).

In cynomolgus monkeys, emesis and diarrhoea were seen 30–60 min after doses of 6·7 g $C_{14}E_7$/kg or 10 g formulation B/kg. By 24 hr, the two monkeys in each of these groups were normal. Doses of 10 g $C_{14}E_7$/kg and 18 g formulation B/kg resulted in emetic effects within 30 min in one of two monkeys in each group. In the other monkey in each of these groups, emesis was delayed until 4 and 2 hr, respectively, after dosing. Both of these monkeys were very depressed and weak and died without recovering from the initial depression. Autopsies were conducted on the two monkeys that died. Of the nineteen tissues examined histologically, the gastro-intestinal tract and lungs were the principal organs affected. The lung changes were indicative of acute oedema and the presence of parasitic lung mites (*Pneumonyssus simicola*). Acute diffuse haemorrhagic enteritis was seen in various parts of the intestinal tract.

Acute ip toxicity

The results of acute ip toxicity tests of $C_{13}E_6$, of $C_{14}E_7$ and of formulations A and B in several animal species are shown in Table 2. All test materials produced marked neuropharmacological signs. Doses in the lethal range produced (within 10–30 min) depression, ptosis and varying degrees of ataxia followed by gasping and loss of righting reflex. Tonic convulsions were produced, but not at less than lethal doses.

In rodents, most deaths occurred within 2–4 hr or between 2 and 4 days. The early deaths occurred during a period of depression (2–4 hr after dose), and were seen with doses generally exceeding the LD_{50}. The deaths that occurred in monkeys treated ip followed this pattern (see below). The delayed deaths that were seen in rodents always followed an apparently normal recovery from the initial effects of the AE_x. By days 2–4 abdominal swelling and diarrhoea were commonly seen, although the animals continued to eat normally. Vascular effects were suggested by

Table 2. *Acute intraperitoneal toxicity of $C_{13}E_6$, $C_{14}E_7$, and formulations A and B in mice, rats, guinea-pigs and monkeys*

Material	LD_{50} (mg/kg)*				
	Mice	Rats	Guinea-pigs	Rhesus monkeys	Cynomolgus monkeys
$C_{13}E_6$	177 (154–205)	209 (184–223)	—	≥ 333 < 667	—
A	309 (264–350)	410 (379–470)	—	≥ 1750 < 2000	—
$C_{14}E_7$	> 100 < 200	209 (184–223)	100†	—	> 250 < 300
B	> 100 < 200	—	—	—	—

*LD_{50} values and their 95% confidence intervals calculated by methods of Thompson (1947) and Weil (1952). (n = 40 to 60). Lethal dose ranges (LDRs) in mice established with 10–20 animals. Rhesus LDRs estimated using two animals for each material, using increasing doses. Cynomolgus LDRs performed using five dose levels (one animal at each).

†Highest dose tested. Four guinea-pigs per group dosed at 15, 25, 50, 75 and 100 mg/kg to establish a response level.

the presence of whole erythrocytes in the peritoneal fluid (in autopsied animals) and by the presence of blood around the nose and mouth. The animals died without signs of neurological involvement. Autopsies of rats given ip doses of $C_{13}E_6$ and $C_{14}E_7$ revealed numerous adhesions involving several internal organs (pancreas, spleen, liver and intestines), and the stomach and small intestine were usually distended with haemorrhagic fluid. In oral doses of 2·5 g/kg, $C_{13}E_6$ or $C_{14}E_7$ produced similar but less severe changes. Oral doses of 1·5 g/kg produced only fluid accumulation, while 1·0 g/kg had little effect. In addition, rats given ip doses near the LD_{50} had as much as a 50% decrease in blood volume when killed within 24 hr of the dose.

In guinea-pigs dosed ip at 50–100 mg/kg, ptosis and depression were observed within 1 hr of dosing. By 4 hr all animals appeared normal. Higher doses were not given. Animals were killed (but not autopsied) after 14 days.

Of two rhesus monkeys, one survived an ip dose of 1·75 g/kg of formulation A, but the other died after a dose of 2·0 g/kg. Depression and convulsions occurred by 30 min and death by 1 hr. Single ip doses of 330 mg $C_{13}E_6$/kg (1 ml 33% $C_{13}E_6$/kg) were given to two other rhesus monkeys. Although initially ataxic, one monkey recovered while the other died. Five dose levels of $C_{14}E_7$ were given ip to cynomolgus monkeys (one per dose level). Doses of 300, 400 and 600 mg/kg resulted in deaths at 9 hr, 4·5 hr and 43 min, respectively. Convulsions were seen with 400 and 600 mg/kg only. In each of these animals, ataxia, depression, reduced touch response and hypothermia were seen. Doses at 200 and 250 mg/kg did not result in observable signs of toxicity or pharmacological action. No treatment-related evidence of a target organ was seen grossly at autopsy or histologically in either species of monkey.

As shown in Table 2, species differences in susceptibility to ip doses of these materials were small except for rhesus monkeys, which were several times less susceptible to formulation A than were mice or rats. Neuropharmacological signs of toxicity, with the exception of slight depression, were not seen in animals given less than lethal doses.

To determine the effect of age on the response to an AE_x surfactant, pre-weanling (14–15 days), weanling (22–24 days) and adult (8–10 wk) Cox rats were tested ip for susceptibility to $C_{14}E_7$. LD_{50} values and their 95% confidence intervals for these three age groups were 197 (173–213), 213 (160–243) and 209 (184–223) mg/kg, respectively. These values were not significantly different from each other.

Clinical chemistry

These ip and oral studies were performed to obtain more information about target-organ toxicity.

Intraperitoneal administration of $C_{13}E_6$ *and* $C_{14}E_7$. The clinical chemistry results at 0–48 hr after ip doses of $C_{13}E_6$ and $C_{14}E_7$ are shown in Table 3. The serum enzymes (CPK, α-HBD and SDH) and blood glucose increased considerably during the first 2 hr after treatment with $C_{13}E_6$ or $C_{14}E_7$, but by 24 hr these values had decreased markedly. Total protein values were not affected. BUN values also increased rapidly after dosing and were at their highest recorded values by 24 hr and 48 hr after $C_{13}E_6$ and $C_{14}E_7$ administration, respectively.

No unusual changes in specific gravity, pH or protein or in the results of microscopic examination were found in urine from these same animals. Glucosuria (defined as >0·1% urinary glucose) was noted at 24 hr in three of six $C_{13}E_6$-dosed rats and four of six $C_{14}E_7$-dosed rats. By 48 hr, glucosuria was not detectable.

Oral administration of $C_{13}E_6$ *and* $C_{14}E_7$. Effects of oral administration of $C_{13}E_6$ and $C_{14}E_7$ on clinical chemistry are shown in Tables 4 and 5, respectively. Approximately 1·5–3-fold elevations in CPK, α-HBD, glucose and BUN were seen within 6 hr of administration of 1 g $C_{13}E_6$/kg (Table 4). Glucose, protein and BUN values were slightly less elevated after dos-

Table 3. *Serum chemistry values for male rats dosed ip with 235 mg* $C_{13}E_6$*/kg or 300 mg* $C_{14}E_7$*/kg*

Time (hr)	CPK* (IU/litre)	α-HBD* (IU/litre)	SDH* (IU/litre)	Glucose* (mg/dl)	Total protein* (g/dl)	BUN* (mg/dl)
			Saline			
0–48	57 ± 3	11 ± 7	15 ± 1	104 ± 3	6·7 ± 0·1	14·5 ± 0·2
			$C_{13}E_6$			
0	58 ± 6	102 ± 31	15 ± 3	101 ± 9	6·8 ± 0·1	15·4 ± 1·3
0·5	3307 ± 883	499 ± 124	31 ± 5	160 ± 30	6·3 ± 0·3	18·8 ± 1·8
2	13,461 ± 1274	1642 ± 30	67 ± 12	264 ± 13	6·4 ± 0·4	28·0 ± 1·6
24	165 ± 52	429 ± 115	40 ± 9	146 ± 10	7·2 ± 0·4	37·2 ± 19·0
48	79 ± 30	189 ± 87	24 ± 15	109 ± 2	6·7 ± 0·5	29·2 ± 14·0
			$C_{14}E_7$			
0	79 ± 13	95 ± 17	16 ± 2	108 ± 14	6·6 ± 0·1	15·1 ± 0·7
0·5	4651 ± 959	654 ± 93	46 ± 8	154 ± 13	6·3 ± 0·1	18·1 ± 0·9
2	5544 ± 424	2008 ± 137	89 ± 7	226 ± 16	6·8 ± 0·3	24·8 ± 0·7
24	2514 ± 1183	1325 ± 285	61 ± 10	105 ± 13	6·0 ± 0·3	75·8 ± 18·0
48	259 ± 119	217 ± 37	16 ± 7	125 ± 0·3	6·7 ± 0·3	92·0 ± 33·0

CPK = Creatinine phosphokinase α-HBD = α-Hydroxybutyrate dehydrogenase
SDH = Sorbitol dehydrogenase BUN = Blood urea nitrogen

*Values are means ± SEM. For surfactant-treated groups n = 6; since no significant differences were seen among the control groups killed at various times, all values were pooled and n = 50.

Table 4. *Serum chemistry values for rats dosed orally with $C_{13}E_6$*

Time (hr)	CPK* (IU/litre)	α-HBD* (IU/litre)	SDH* (IU/litre)	Glucose* (mg/dl)	Total protein* (g/dl)	BUN* (mg/dl)
			Saline			
—†	57 ± 3	111 ± 7	15 ± 1	104 ± 3	6·7 ± 0·1	14·5 ± 0·2
			1·0 g $C_{13}E_6$/kg			
0	43 ± 4	94 ± 24	12 ± 1	90 ± 10	7·0 ± 0·1	15·6 ± 0·6
6	91 ± 31	241 ± 73	19 ± 3	159 ± 25	7·4 ± 0·3	38·5 ± 5·4
24	47 ± 7	118 ± 13	11 ± 3	132 ± 9	6·9 ± 0·2	27·0 ± 6·8
48	43 ± 4	74 ± 10	12 ± 1	107 ± 9	6·7 ± 0·1	14·2 ± 1·6
72	83 ± 19	234 ± 88	16 ± 3	77 ± 9	6·6 ± 0·1	16·4 ± 1·1
96	35 ± 2	86 ± 10	9 ± 2	100 ± 6	6·6 ± 0·2	22·5 ± 3·2
			1·5 g $C_{13}E_6$/kg			
0	40 ± 5	69 ± 21	11 ± 1	90 ± 10	8·1 ± 0·2	14·8 ± 0·6
4	115 ± 10	149 ± 12	15 ± 1	351 ± 29	9·5 ± 0·4	45·3 ± 4·5
6	144 ± 11	416 ± 52	29 ± 2	171 ± 20	6·6 ± 0·2	41·3 ± 6·4
8	74 ± 15	146 ± 25	13 ± 2	195 ± 50	8·6 ± 0·4	50·0 ± 8·0
24	124 ± 54	148 ± 34	18 ± 2	179 ± 39	8·0 ± 0·2	49·4 ± 10·0
48	47 ± 12	202 ± 64	14 ± 2	91 ± 9	5·9 ± 0·4	20·8 ± 3·0
			2·5 g $C_{13}E_6$/kg			
0	32 ± 7	77 ± 14	17 ± 3	114 ± 7	7·1 ± 0·2	15·4 ± 0·5
6	136 ± 29	202 ± 67	20 ± 2	222 ± 54	9·1 ± 0·4	43·8 ± 6·1
24	52 ± 17	108 ± 27	22 ± 6	270 ± 20	6·7 ± 0·4	27·2 ± 4·3
48	73 ± 6	215 ± 30	21 ± 3	141 ± 9	4·9 ± 0·2	31·2 ± 2·8

*Mean values ± standard error (n = 6). See Table 3 for abbreviations.
†No significant differences were seen among control groups killed at various times, therefore all values were pooled (n = 50).

ing with 1 g $C_{14}E_7$/kg (Table 5); however, notable increases in CPK or α-HBD values were not seen. All values returned to near control levels by 48 hr after a 1·0-g/kg dose of $C_{13}E_6$ or $C_{14}E_7$. All serum enzymes and other measurements increased after 1·5- and 2·5-g/kg doses of $C_{13}E_6$ and $C_{14}E_7$. By 48 hr after 2·5 g $C_{13}E_6$/kg, CPK, α-HBD and BUN were still elevated (Table 4). In this group and in the 1·5 g

Table 5. *Serum chemistry values for rats dosed orally with $C_{14}E_7$*

Time (hr)	CPK* (IU/litre)	α-HBD* (IU/litre)	SDH* (IU/litre)	Glucose* (mg/dl)	Total protein* (g/dl)	BUN* (mg/dl)
			Saline			
—†	57 ± 3	111 ± 7	15 ± 1	104 ± 3	6·7 ± 0·1	14·5 ± 0·2
			1·0 g $C_{14}E_7$/kg			
0	80 ± 28	192 ± 90	11 ± 3	102 ± 7	7·2 ± 0·1	17·2 ± 0·0
6	73 ± 14	133 ± 26	16 ± 1	144 ± 10	8·6 ± 0·5	26·5 ± 2·2
24	53 ± 17	124 ± 18	14 ± 1	156 ± 24	7·2 ± 0·3	45·4 ± 9·1
48	63 ± 9	123 ± 45	17 ± 6	105 ± 11	7·0 ± 0·1	17·6 ± 1·4
72	60 ± 8	115 ± 25	10 ± 2	86 ± 7	6·3 ± 0·2	16·1 ± 0·9
96	66 ± 15	134 ± 46	9 ± 2	132 ± 26	6·8 ± 0·2	16·4 ± 0·9
			1·5 g $C_{14}E_7$/kg			
0	54 ± 15	137 ± 56	17 ± 5	101 ± 13	6·4 ± 0·2	17·6 ± 1·8
6	121 ± 30	225 ± 69	26 ± 1	143 ± 16	8·0 ± 0·8	44·8 ± 19·0
24	52 ± 7	126 ± 38	12 ± 2	165 ± 26	6·9 ± 0·7	81·0 ± 24·0
48	98 ± 22	430 ± 136	24 ± 1	86 ± 4	6·2 ± 0·1	21·2 ± 33·0
72	45 ± 9	112 ± 28	13 ± 3	75 ± 6	5·9 ± 0·2	18·7 ± 0·2
96	33 ± 7	106 ± 6	15 ± 4	106 ± 3	6·8 ± 0·6	23·0 ± 5·0
			2·5 g $C_{14}E_7$/kg			
0	77 ± 14	188 ± 57	25 ± 3	120 ± 10	5·8 ± 0·4	13·8 ± 1·4
6	118 ± 19	345 ± 98	20 ± 5	169 ± 10	7·9 ± 0·4	51·2 ± 2·0
24	73 ± 14	195 ± 37	23 ± 2	235 ± 32	5·3 ± 0·2	17·4 ± 13·1
48	118 ± 4	356 ± 47	27 ± 4	175 ± 16	5·3 ± 0·3	50·8 ± 10·0
72	244 ± 56	726 ± 85	29 ± 3	103 ± 8	4·7 ± 0·5	40·8 ± 4·5
96	67 ± 26	255 ± 139	27 ± 6	105 ± 11	4·1 ± 0·3	24·1 ± 7·0

*Mean values ± standard error (n = 6). See Table 3 for abbreviations.
†No significant differences were seen among control groups killed at various times, therefore, all values were pooled (n = 50).

$C_{13}E_6$/kg group, 72 and 96 hr samples were not obtained. CPK, α-HBD and BUN were elevated 72 hr after 2·5 g $C_{14}E_7$/kg and between 24 and 48 hr after $C_{13}E_6$. This was observed at several dose levels.

There were no changes in values for urinary protein, pH or specific gravity for any of the groups of rats dosed orally with $C_{13}E_6$ or $C_{14}E_7$. Glucosuria was detected in two of five rats by 24 hr after 2·5 g $C_{13}E_6$/kg and 48 hr after 2·5 g $C_{14}E_7$/kg. In animals killed at other times, glucosuria was not seen.

Intravenous toxicity

The acute iv LD_{50} values for $C_{14}E_7$ in weanling and adult rats and adult guinea-pigs were 104 ± 5, 68 ± 8 and 40 ± 5 mg/kg (±SEM), respectively. The Miller & Tainter (1944) method of LD_{50} calculation was used. Deaths usually occurred within 5–10 min. Signs of toxicity included gasping, depression and loss of righting reflex. Gradual depression in rate and depth of respiration was the immediate cause of death. Convulsions were not seen in these studies.

Clinical chemistry studies of animals dosed ip demonstrated elevations in the serum enzymes CPK and α-HBD (discussed earlier), suggesting possible cardiac involvement (Cornish, 1971). To test further for heart effects, several additional experiments were performed using anaesthetized cats. Intravenous administration of $C_{13}E_6$ and $C_{14}E_7$ (at 1, 10 and 30 mg/kg) resulted in a marked fall in blood pressure and inotropic activity and in some animals a mild decrease in heart rate. $C_{13}E_6$ was about three times as effective as $C_{14}E_7$. At the lowest dose level (1 mg/kg) only a slight reduction in blood pressure was seen. Doses between 1 and 10 mg/kg produced a decrease in blood pressure of short duration (2–4 min), while higher doses (10–30 mg/kg) produced deaths in some cats. Neither AE_x produced arrhythmia. At 10 mg/kg, bradypnoea and hypoventilation were seen. Both $C_{13}E_6$ and $C_{14}E_7$ slightly attenuated the pressor effects of norepinephrine. In addition, a 20-mg/kg iv dose of $C_{13}E_6$ or $C_{14}E_7$ produced an incomplete reversal of the blood pressure effects of 0·1 μg/kg isoproterenol. The AE_x materials, however, had no dramatic effects on the physiology or pharmacology of the cardiovascular system.

Acute dermal toxicity

Since human skin exposure may occur both in manufacturing and in the home, the AE_x materials were evaluated in animal dermal studies. Undiluted and 33% solutions were used. The results (Table 6) demonstrated that the 33% AE_x solutions had a similar toxicity to undiluted samples. No gross behavioural effects nor signs of pharmacological activity, other than hypoactivity, were seen in the animals that died. Undiluted $C_{13}E_6$ and $C_{14}E_7$ produced severe primary skin irritation in rabbits, but only transient mild irritation in guinea-pigs. In rabbits, sloughing of the superficial dermis began on day 7 and was still apparent by day 14. Hair growth was returning to normal by this time on most animals. Three of the six rabbits treated with 2 ml undiluted $C_{14}E_7$/kg died. Autopsies revealed severe pulmonary congestion. Microscopically, acute diffuse bronchopneumonia was seen but this was not related to the test. With 33% $C_{13}E_6$, moderate irritation proceeding to escharosis was the only toxic effect observed. The LD_{50} values for 33% $C_{13}E_6$ and $C_{14}E_7$ are shown in Table 6. Evidence of systemic toxicity was not seen on histological examination. Formulation A produced effects similar to those with undiluted $C_{13}E_6$.

Inhalation toxicity

The approximate LC_{50} (single 4-hr exposure) for either AE_x was between 1·5 and 3·0 mg/litre (actual measured concentration). The precise LC_{50} could not be estimated since for each material the death rate was either 0 or 100% in all but one or two groups of rats. The majority of deaths occurred 2–5 days after exposure. Laboured breathing, rales, corneal opacities and decreased activity were the primary effects observed. Those signs, other than eye effects, were transient at lower concentrations, but in some animals in the high-dose groups they persisted for several days.

All animals were autopsied at 14 days or at death. Eyes and lungs were the primary target organs. Because of the very high concentrations used, severely irritating effects (corneal opacities) were produced in nearly all test animals. Eye-irritation studies are described later. Lungs from rats dying from the exposures were dark red and smaller than normal. When sliced, blood was not exuded and lung-to-body weight ratios were unchanged. Therefore, atelectasis was indicated as the cause of death. Decreased weight gains were seen in surviving rats, although organ-to-body weight ratios were similar to those of controls. Sections of liver, kidney, lungs, myocardium, trachea and eyes were examined histologically. Vacuolation and hyperplasia of the corneal epithelium in about 50% of test animals was the only test-related lesion seen.

Anaesthetic and analgesic effects

Local anaesthetic effects were tested by sc injections into the hip or back of rats followed after various intervals by application of a heated probe. Doses of 100 mg $C_{13}E_6$/kg and 300 mg formulation A/kg were as effective as 100 mg procaine/kg in delaying or preventing pain-induced responses. Little or no local anaesthetic activity was seen with 50 mg $C_{13}E_6$/kg. Formulation A at 150 mg/kg was as effective as 50 mg procaine/kg. Both $C_{13}E_6$ and procaine lacked activity

Table 6. *Acute dermal toxicity of $C_{13}E_6$, $C_{14}E_7$ and formulation A in rabbits and guinea pigs*

Test material	Concentration	LD_{50} (95% confidence interval) ml/kg*	
		Rabbit	Guinea pig
$C_{13}E_6$	Undiluted	>2·0	>2·0
$C_{13}E_6$	33%	2·0 (0·8–5·1)	—
$C_{13}E_6$	33% (repeat)	2·7 (1·3–5·9)	—
A	Undiluted	>2·0	>2·0
$C_{14}E_7$	Undiluted	≈2·0†	>2·0
$C_{14}E_7$	33%	2·0 (0·8–5·0)	—
$C_{14}E_7$	33% (repeat)	2·7 (1·3–5·9)	—

*LD_{50} calculated by method of Litchfield and Wilcoxon (1949).

†Three of six rabbits at this dose died.

at 25 mg/kg and formulation A was inactive when tested at 75 mg/kg.

Systemic analgesia was tested in mice using hot-plate and tail-flick methods following ip administration. Some analgesic action was noted with $C_{13}E_6$ at 100 mg/kg but not at 75 mg/kg. At a dose of 100 mg/kg, $C_{13}E_6$ produced an increased average response time and decreased response, compared to controls. Also with 100 mg $C_{13}E_6$/kg, a partial loss of righting reflex and convulsions were seen at 5 and 10 min, respectively. At a dose level of 300 mg/kg, formulation A showed analgesic effects in the hot-plate method, but not in the tail-flick method. (For comparison, the minimum effective level of morphine in both assays was 30 mg/kg.)

Acute eye-irritation study

Both AE_xs and their respective formulations were tested for irritation of rabbit eyes as part of their routine safety evaluation. Like many other detergent formulations in current use (Bierbower, Seabaugh, Osterberg & Hoheisel, 1975), these materials produced severe irritation when tested in rabbit eyes in undiluted form, and many of the treated eyes did not clear within 35 days. Maximum average scores (MAS) calculated according to Draize (1959) were 59·3 for $C_{13}E_6$ and 54·0 for $C_{14}E_7$. For the formulations, MAS values could not be determined. When 10% dilutions were used, or when eyes were rinsed after application of undiluted AE_x, moderate irritation (reversible) was produced (MAS values ranged from 10 to 35).

Tests were also conducted on monkey eyes, possibly a better model on which to predict eye hazards in man (Buehler & Newmann, 1964). Monkey eyes were considerably less sensitive to the irritant effects of formulations A and B, and no permanent effects were produced even with undiluted and unrinsed test materials. The MAS (Draize, 1959) for formulations A and B were 20·5 and 20·0, respectively, and eyes cleared within 14 days.

Acute skin-irritation study

Rabbit studies were conducted prior to dermal studies in man. A 1% solution of $C_{13}E_6$ and a 10% solution of $C_{14}E_7$ were tested for skin irritation using a rabbit closed-patch test (tests not done concurrently). $C_{13}E_6$ was mildly irritating under these conditions, producing a Primary Irritation Index (PII) score of 1·6. $C_{14}E_7$ was only moderately irritating under these test conditions (PII = 4·2) despite being tested at a ten-fold greater concentration than was used in the $C_{13}E_6$ test.

The human skin irritation produced by all of the test materials was also evaluated by occluded patch testing (Table 7). Only slight erythema was produced by a 24-hr exposure to a 10% solution of $C_{13}E_6$. Three alternate-day exposures of 4 hr/day to 25% $C_{14}E_7$ produced negligible or slight irritation. When undiluted $C_{14}E_7$ was used, only negligible or slight irritation was seen. A single 4-hr exposure to undiluted formulation A or B resulted in negligible irritation, while 25% formulation B produced no skin irritation.

In a separate study, in which two guinea-pigs were immersed to chest level in 25% $C_{14}E_7$ solutions for 4 hr, no pharmacological or toxicological signs were observed during the immersion or during the next 2 hr. Animals behaved normally and weight gains were normal during the observation periods. The only effect seen at autopsy was a very slight degree of skin fissuring.

DISCUSSION

From the results of extensive single-dose testing of two alkyl polyethoxylate (AE_x) surfactants ($C_{13}E_6$ and $C_{14}E_7$) and the two detergent formulations containing 33% of either of these materials (formulations A and B, respectively), it is possible to assess the potential acute hazards associated with consumer use (and misuse) of these formulations as well as with conceivable acute industrial exposure to these materials.

Acute oral ingestion by adults or children would appear to represent a minimum hazard. The formulations A and B were "slightly toxic" as defined by Gosselin, Hodge, Smith & Gleason (1976) when administered orally to rats or dogs and "practically nontoxic" in both of the species of monkeys tested; A and B might have proved to be "practically nontoxic" to dogs had higher doses been tested. Emesis and diarrhoea were the primary effects seen in dogs and monkeys, and this probably contributed to the lower susceptibility of these animals compared to rats. Oral LD_{50} values in rats for AE_xs are in the same range as those for linear alkyl benzene sulphonate and a series of other anionic and nonionic surfactants (Calandra & Fancher, 1969) used by the detergent industry. Although signs of pharmacological activity were observed in the rat and monkey LD_{50} studies,

Table 7. *Human patch testing of $C_{13}E_6$, $C_{14}E_7$ and formulations A and B*

Test material	Concentration	Volume applied (ml)	Duration of exposure (hr)	No. of subjects	Degree of irritation*
$C_{13}E_6$	10% aq.	0·5	24	8	Slight
A	Undilute	0·4	4	5	Negligible
$C_{14}E_7$	Undilute	0·4	4†	10	Negligible to slight
$C_{14}E_7$	25% aq.	0·4	4†	10	Slight
B	Undilute	0·4	4	5	Negligible
	25% aq.	0·4	4	5	None

*All examinations for irritancy were performed 24 hr after removal of patch.
†Subjects exposed on three alternate days, 4 hr per day; skin examined 24 hr after last treatment.

doses required for the production of these effects were near or exceeded lethal levels.

When the compounds were given ip, little difference in susceptibility was observed between rats and mice. In those cases where the data permit comparison, rhesus and cynomolgus monkeys appeared to be distinctly less susceptible.

Clinical chemical studies were designed to detect toxic effects on target organs. The most striking effect of $C_{13}E_6$ and $C_{14}E_7$ when dosed ip was the increase in serum enzyme used as indicators of heart damage, namely α-HBD and CPK (Cornish, 1971). The high level of serum CPK that occurred 2 hr after dosing could have been due to release of this enzyme by skeletal muscle as well as the heart. However, α-HBD increased concomitantly, suggesting heart muscle damage (Elliot, Jepson & Wilkinson, 1962). In addition, BUN concentrations were also elevated at 2 hr after dosing, and were even higher at 48 hr. Blood urea increases during congestive cardiac failure, but may also be a complication of low blood volume (Henry, 1974), which was observed at high doses of $C_{13}E_6$ and $C_{14}E_7$. The clinical chemical studies conducted on rats dosed ip suggested that there were also effects on the liver (serum SDH increase) and kidney (glucosuria and elevated BUN). The ip route of administration is not realistic in terms of human exposure, but may be useful for studies in rats on the mechanism of action. For comparison, similar studies using the oral route were conducted in rats. The first oral dosing study (Table 5, $C_{13}E_6$ at 2·5 g/kg) was terminated at 48 hr, since at this dose death usually occurred just after this time. SDH values were not greatly affected, suggesting that liver may not be a target organ. Effects on heart tissue were again indicated by increased α-HBD and CPK. Urinalysis indicated no discernible toxic effects on the kidney. Of the serum parameters measured, only α-HBD was increased after dosing with 1 g $C_{13}E_6$/kg, although observed elevations were only slightly above the normal range. At this dose of $C_{14}E_7$ only the BUN values were elevated, suggesting that increases in BUN are primarily related to effects in the kidney rather than the heart, since α-HBD and CPK were normal.

In contrast to these results in rats, studies in cats designed specifically to measure the effects of the $C_{13}E_6$ on the physiology and pharmacology of the heart demonstrated that this material does not produce any significant cardiac effects.

The AE_xs tested produced pharmacological activity in animals, but very high doses were required for these effects. The dose levels (sc or ip) of $C_{13}E_6$ necessary for local anaesthetic or systemic analgesic effects were at least 25–50% of the ip LD_{50} doses (see Table 2). In consideration of the probable human routes of exposure to AE_xs or their formulations (i.e. oral and dermal), and the doses necessary for toxic and pharmacological effects by these routes, it is not felt that the pharmacological actions of $C_{13}E_6$ and $C_{14}E_7$ are of significance under the usual or accidental conditions of exposure anticipated for liquid detergent formulations.

Acute rabbit dermal toxicity studies using $C_{13}E_6$ and $C_{14}E_7$ showed that 33% solutions had similar toxicities to the undiluted materials. It is possible that the undiluted test samples were unable to penetrate the skin because of micelle formation. Formulation A (containing 33% $C_{13}E_6$) was no more toxic to rats when given orally than a 33% $C_{13}E_6$ solution, suggesting that the toxic effects of the formulation could be accounted for entirely by the toxicity of the nonionic AE_x surfactant ingredient. The production of skin irritation in rabbits under these extreme test conditions, and the resulting stress was probably the major cause of the deaths seen in these studies. This is suggested by a failure to find evidence of organ damage (other than skin) by histopathological examination. This acute dermal toxicity test is not directly applicable to any reasonable human exposure, although it is useful for determining the relative irritation potential of different substances. The relative proportion of body surface exposed (10%), the contact time (24 hr) and type of exposure (occluded and abraded skin) certainly exaggerate any possible human exposure. Rabbit-skin patch tests with $C_{13}E_6$ and $C_{14}E_7$ showed these materials to be capable of mild-to-moderate skin irritation. Comparable patch test studies in man (using a more realistic 4-hr exposure rather than 24 hr) showed that $C_{13}E_6$, $C_{14}E_7$, A and B were non-irritating or only slightly irritating.

The production of fine aerosols of $C_{13}E_6$ and $C_{14}E_7$ in either manufacturing or home-use situations is unlikely. In any case, the concentrations necessary to produce lethal effects in rats would be extremely high. Deaths were not seen at doses below 1 mg/litre (1000 mg/m^3). In addition, low vapour pressures of AE_xs would prevent vaporization. Inhalation should therefore not present an acute safety problem for AE_x surfactants.

Eye-irritation studies demonstrated rather striking differences between rhesus monkeys and rabbits in response to AE_x materials. Lachrymation is more efficient in monkeys than in rabbits and may be partially responsible for the observed differences. Buehler & Newmann (1964) have reported that the monkey eye is more comparable (in response to many irritants) to the human eye than is the rabbit eye. Monkey-eye irritation produced by formulation A and B was moderate and transient even when the applied material was not rinsed from the eye. When tested in rabbit eyes, both the AE_xs and their respective formulations produced severe eye irritation; however, under the same test conditions nearly one-half of the 152 marketed detergent products tested by Bierbower *et al.* (1975) were also severe rabbit-eye irritants.

In summary, the results presented here suggest that $C_{13}E_6$, $C_{14}E_7$ and their formulations A and B would not involve significant risk to man under conditions of predicted use or potential misuse involving single or very limited exposures. Subchronic tests results are the subject of a separate paper (Brown & Benke, 1976).

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SAFETY TESTING OF ALKYL POLYETHOXYLATE NONIONIC SURFACTANTS. II. SUBCHRONIC STUDIES

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Abstract—The alkyl polyethoxylates $CH_3 \cdot [CH_2]_{11-12} \cdot [OCH_2 \cdot CH_2]_6 \cdot OH$ (or $C_{13}E_6$) and $CH_3 \cdot [CH_2]_{13-14} \cdot [OCH_2 \cdot CH_2]_7 \cdot OH$ (or $C_{14}E_7$) were evaluated for subchronic oral toxicity (using rats) and percutaneous toxicity (using rabbits), and for human contact sensitization and skin mildness. When fed to rats for 91 days, a diet containing 1% $C_{14}E_7$ produced only increased liver-to-body weight ratios while a diet containing 1% $C_{13}E_6$ also produced growth depression. No systemic toxicity was produced in 4- or 13-wk percutaneous tests of $C_{13}E_6$, $C_{14}E_7$ or heavy-duty laundry-detergent formulations containing 33% of either of these surfactants, but all these treatments produced varying degrees of skin irritation and eruptions. Under the greatly exaggerated exposure conditions of the repeated insult patch test, a low incidence of skin hyper-reactivity to $C_{13}E_6$ was produced. However, tests using over 500 human volunteers produced no evidence suggestive of sensitization to formulations containing 33% alkyl polyethoxylates. Usage tests of these polyethoxylate detergents in which the condition of the skin was monitored by dermatologists demonstrated that these products are comparable in mildness with other detergent products having years of safe marketing experience.

INTRODUCTION

The toxicity testing that must be carried out on new chemicals before they are used in consumer products can be used not only to assess whether their use or potential misuse would pose a hazard to the consumer, but also to provide a basis for recommendations for the safe handling of the raw materials in industry. Linear alkyl polyethoxylates (AE_xs) and formulations containing 33% AE_x have been tested extensively, and the acute testing programme has been reviewed in the preceding paper (Benke, Brown, Walsh & Drotman, 1977). Subchronic percutaneous and oral toxicity (using rabbits and rats, respectively) as well as human skin-sensitization and irritation tests are the subjects of this report.

EXPERIMENTAL

Test materials. The $C_{13}E_6$ ($CH_3 \cdot [CH_2]_{11-12} \cdot [OCH_2 \cdot CH_2]_6 \cdot OH$), $C_{14}E_7$ ($CH_3 \cdot [CH_2]_{13-14} \cdot [OCH_2 \cdot CH_2]_7 \cdot OH$ and formulations used in these studies were prepared as described by Benke *et al.* (1977).

Subchronic oral toxicity. Three separate studies of 91 days' duration were conducted using three different strains of rats. In each study, groups of 20 male and 20 female weanling (50–70 g) albino rats, uniformly distributed by weight, were fed *ad lib.* a standard laboratory chow diet containing various levels of test material. Each study included a control group that received only the standard chow. Feed consumption and body weights were recorded weekly. At 91 days, all animals were autopsied. An intermediate 4-wk autopsy was conducted in one study. In each study, five males and five females were randomly selected from each group for histological examination. Tissues examined microscopically were liver, spleen, pancreas, kidney, stomach, intestine, bladder, heart, lung, gonad, adrenal, lymph node, skin, bone marrow, thyroid, brain, pituitary, spinal cord, eye, salivary gland, tongue, nerve and muscle. (Only the first 15 tissues listed were examined in the low-level $C_{13}E_6$ study.) Weights of major organs were determined, and haematological studies (haemoglobin, haematocrit, complete blood count) were conducted at death. Clinical chemistry determinations (total protein, albumin, calcium, inorganic phosphorus, glucose, blood urea nitrogen, uric acid, creatinine, alkaline phosphatase, γ-glutamyl transpeptidase, serum glutamic–pyruvic transaminase and serum glutamic–oxaloacetic transaminase) were also conducted in the $C_{14}E_7$ study.

Subchronic percutaneous toxicity. Modifications of the Draize (1959) method were used to evaluate the potential toxic effect of repeated dermal applications of the various test materials. Young adult (2–3 kg) New Zealand albino rabbits were randomly divided into groups of six animals (three males and three females). An area on the back corresponding to approximately 10% of the overall body surface of each animal (about 160 cm^2) was clipped free of hair. The treatment sites of animals in the 4-wk tests were lightly abraded before each application (until cumulative irritation obviated this need) using a clipper head

in such a manner that the stratum corneum was penetrated but bleeding was not produced. Treatment sites of animals in the 13-wk tests were not abraded. Clipping was repeated as necessary throughout the treatment periods. Solutions of test materials or deionized water (control) were applied daily (5 days/wk) at a dose of 2 ml/kg body weight and spread over the clipped area. Rabbits were placed in harnesses (Newmann, 1963) to prevent ingestion of the test material.

The animals were observed daily for deaths, general appearance and signs of systemic toxicity. Body weights were determined weekly. All surviving animals were autopsied at the end of the test period. Haematological tests were performed, kidney and liver-to-body weight ratios were calculated, and tissues were taken for microscopic examination. These included parathyroid, trachea, ureter, urethra and seminal vesicles and prostate or uterus and vaginal wall, in addition to the tissues taken in the oral toxicity studies.

Animals treated for long periods with these materials at highly exaggerated levels were apparently very susceptible to massive bacterial invasion. To permit the identification of any direct toxicity of the AE_x or its formulation, a further 13-wk study was conducted under conditions that were intended to reduce the likelihood of microbiological cross-contamination and interference. Precautionary procedures for the 'special' handling groups included physical isolation of the study, pre-study elimination of animals with *Staphylococcus aureus* on the test site, special decontamination of workers, confinement of food and bedding to be used in the treatment room, use of only disposable or disinfected equipment and clothing, and monitoring of airborne contamination. Similar groups were tested in a separate room using 'routine' procedures. Animals in all groups were sampled weekly (test site, untreated skin and nares) for *S. aureus*, and bacterial colonies were identified.

Guinea-pig immersion. A method similar to that proposed for guinea-pig immersion by Opdyke & Burnett (1965) was used. A group of ten Hartley guinea-pigs (five males, five females, weighing 150–180 g) was used. Animals were positioned head-up in restrainers which were placed in 600 ml beakers containing approximately 250 ml of a 10% aqueous solution of test material. This volume was sufficient to permit chest-level exposure. Plexiglas® collars were used to prevent ingestion of the test solution. The temperature of the solutions was maintained at 40°C. Control animals (two males, two females) were immersed in water only. Daily 4-hr exposures were conducted for five consecutive days. Animals were rinsed and patted dry with cloth towels after each immersion, and were observed for signs of a toxic or pharmacological response during exposure and for the following 2 hr. The animals were killed and autopsied 3 days after the last exposure. The same tissues were taken for histopathology as in the subchronic oral toxicity studies.

Handwashing. Panels of 16 human volunteers each washed their hands for 30 sec, three times daily for 3 wk (excluding weekends) with 5 ml of either formulation A or B (undiluted). Skin was examined for irritation throughout the exposure period prior to each daily exposure to the test materials. When increased irritation was seen, the use of hand cream (without antibacterial additives) was permitted *ad lib.* For bacteriological sampling, areas (1 in.^{2}) the palm and dorsum of each hand were scrubbed with sterile, distilled water. The swabs were streaked immediately on Baird-Parker and blood agar media. Pre-test samples were taken for 2 wk prior to exposure, and the hands were sampled on the first and last day of each test week. A recovery of *S. aureus* from either hand was scored as a positive for that subject.

Contact sensitization. Repeated insult patch tests were conducted on groups of human volunteers according to the method of Griffith (1969). Subjects wore occlusive patches containing solutions of test material for 24-hr periods on alternate days, three times/wk for 3 wk. Concentrations used were the highest levels (0·25–2·5% aqueous solutions) that did not produce uncomfortable levels of skin irritation in the test participants at the season of the year when the test was conducted. Seventeen days after the final induction patch, challenge applications were made on the induction site and on a previously unexposed contralateral site. Test sites were graded after 48 and 96 hr for evidence of skin sensitization.

Home usage: clinical mildness studies. Groups of volunteer housewives used formulation A or B for all their hand dishwashing for 2-wk periods. Before and after this usage period, their hands were examined by dermatologists for signs of dryness or irritation according to the method of Suskind & Whitehouse (1963). Different degrees of hand-skin condition were graded on a scale of 1–10 and were evaluated statistically by analysis of variance.

Microbiology: animal tests. In subchronic percutaneous studies, test materials were washed from the test sites after 6 hr of exposure and the sites were towel-dried. Skin test sites (particularly areas of lesions) were then sampled with calcium alignate swabs moistened with sterile distilled water. An area of skin not exposed to the test material (the nape of the neck) was sampled similarly as a control reference. All swab samples were immediately streaked on both Baird-Parker and blood agar plates; cultures were incubated for 36 hr at 37°C. Previous screening studies had indicated that the only human pathogenic organism present in discernible numbers was β-haemolytic *S. aureus*. Consequently, Baird-Parker plates were considered the primary culture and the blood agar plates were used to demonstrate haemolysis. Colonies showing typical *S. aureus* morphology were re-streaked on blood agar and mannitol soy agar plates and inoculated into coagulase plasma for confirmation of identification. The criteria for identification were: (1) β-haemolysis on blood agar, (2) growth on mannitol, (3) growth on Baird-Parker medium with the ability to clear egg yolk, (4) production of coagulase and (5) Gram-positive staining with microscopic morphology typical of *S. aureus*.

RESULTS

Subchronic oral toxicity

In the first study, $C_{13}E_6$ was fed to Charles River CD rats at levels of 0, 5, 50 and 500 ppm in the diet for 91 days. No effects were produced on growth, food consumption, feed conversion efficiency, body weights

or organ-to-body weight ratios. Haematological values were within the normal range. The only possibly significant lesion found was mild degeneration of some of the seminiferous tubules in two males from the highest dose level. Because this lesion is commonly found in the rat and may result from a number of causes (Berg, 1967, Ribelin; 1963), it is doubtful whether it was directly related to $C_{13}E_6$ administration. These lesions were not seen in rats given 20-fold higher dietary levels.

In another study, commercial samples of $C_{13}E_6$ from two suppliers were fed to Cox rats at dietary levels of 1000, 5000 or 10,000 ppm for 91 days. There were no differences between the effects of the two test materials, and therefore only data from the current supplier are presented (Table 1). A dose-related, palatability factor was believed to be responsible for differences in final body weights. Feeding efficiency was not altered, but the liver-to-body weight ratios showed a dose-related increase. No biologically significant haematological changes were produced, and no test-related lesions were seen grossly at autopsy. Histologically, the only lesion of possible significance was the presence of eosinophilic material in kidney tubules and/or tubule cells. These effects were seen in all groups including controls, but were more prevalent in animals on test diets and appeared to be dose-related. This lesion has been seen previously in tests of other materials conducted by our laboratory. It is associated with the Cox rat and appears to be related to non-specific stress. Similar lesions were not observed in Sprague–Dawley rats fed $C_{14}E_7$.

A similar 91-day study was conducted with $C_{14}E_7$ using Sprague–Dawley albino rats fed diets containing 1000, 5000 or 10,000 ppm (Table 1). It should be noted that the lower feed conversion efficiencies recorded for $C_{14}E_7$ compared with $C_{13}E_6$ in Table 1 reflect the much higher initial weights of the Sprague–Dawley rats used in this study compared with the Cox rats used in the $C_{13}E_6$ study. After 28 days, five males and five females were selected at random from each group for haematological studies, clinical chemistry, urine analysis, autopsy and histological examination of 24 tissues. All remaining rats were killed at wk 13 and examined grossly. Terminal haematological and histological studies, clinical chemistry and urine analyses were performed on ten randomly selected animals (five males and five females) from each group. Single clinical chemistry values—slightly elevated γ-glutamyl transpeptidase (24 IU/litre) and markedly elevated fasting serum glucose (310 mg/100 ml)—outside the normal range were observed in one female and one male, respectively, at the intermediate dose level. These could neither be correlated with nor confirmed by other chemistry values or histopathology, and their biological significance is unclear. No treatment-related changes were seen in any of the other determinations that were made except an increase in liver-to-body weight ratio. Histologically these livers were normal.

Subchronic percutaneous toxicity

Eleven subchronic percutaneous toxicity studies were conducted with $C_{13}E_6$, $C_{14}E_7$ or their formulations. The results are summarized in Table 2.

$C_{13}E_6$ and formulation A

Skin irritation was the only compound-related effect detected in studies with $C_{13}E_6$ or its formulation A. Occasional deaths occurred, but no evidence of compound-related effects was seen either grossly or microscopically. No abnormalities were seen in body-weight gain, haematology, organ weights and gross and microscopic pathology in the surviving animals except for evidence of skin irritation.

$C_{14}E_7$ and formulation B

Applications of 20 mg $C_{14}E_7$/kg/day or 60 mg formulation B/kg/day produced only slight-to-moderate skin irritation (Table 2). No other abnormalities were

Table 1. *Final body weights, feed conversion efficiency and liver-to-body weight ratios for rats fed $C_{13}E_6$ and $C_{14}E_7$ for 13 wk*†

Compound and dietary level (ppm)	Final body weight (±SEM)		Feed conversion efficiency‡		Liver-to-body weight ratio§	
	Male	Female	Male	Female	Male	Female
$C_{13}E_6$‖						
0	429 ± 7	256 ± 6	0·185	0·132	3·08	3·18
1000	418 ± 8	250 ± 4	0·188	0·129	2·95	3·13
5000	400 ± 7**	239 ± 4*	0·188	0·136	3·40	3·42*
10,000	377 ± 8***	203 ± 3***	0·192	0·125	3·82**	4·06***
$C_{14}E_7$¶						
0	499 ± 15	250 ± 7	0·156	0·083	3·51	3·41
1000	473 ± 11	297 ± 23	0·143	0·094	3·59	3·63
5000	479 ± 11	278 ± 6	0·140	0·078	3·27	3·89
10,000	461 ± 11	270 ± 7	0·152	0·093	3·90*	4·18**

†Figures for weight gain and feed efficiency are means for the 20 rats/group. Body-weight ratios are for the five randomly selected rats/group.
‡Body-weight gain (g)/feed consumed (g).
§Expressed as % of body weight.
‖Cox CD rats, initial weight 65–75 g.
¶Sprague–Dawley rats, initial weights 136–150 g (males) and 122–139 g (females).
Figures marked with asterisks differ significantly (one-way analysis of variance test; Scheffe, 1952) from the corresponding controls: *$P < 0·05$; **$P < 0·01$; ***$P < 0·001$.

Table 2. *Subchronic percutaneous toxicity of alkyl polyethoxylates and their formulations in rabbits*

Material	Dose (mg/kg/day)	Duration (wk)	Deaths	Degree of skin irritation
$C_{13}E_6$	50	4	0/6	SM
	50	13	1/6	SM
Formulation A	60	13	0/6	SM
	200	13	1/6	SV
$C_{14}E_7$	20	4	0/6	SM
	50	4	1/6	MP/Pap*
	20	13	0/5	M
	50	13	3/6	MP/Pap*†
Formulation B	60	13	0/6	SM
	200	13	6/6	MP/Pap*†
	200	13	1/12	SV/Pap*

SM = Slight–moderate M = Moderate MP = Moderate–pronounced
SV = Severe Pap = With papular eruptions
*Similar skin lesions were observed on control animals treated with water.
†These studies were conducted simultaneously with a single water-control group. One death occurred in the control group during the treatment period. Septicaemia was the apparent cause of death.

seen. However, increasing the dose level increased dermal irritation, which was complicated by bacterial infections.

A 4-wk test of $C_{14}E_7$ at a dose of 50 mg/kg/day (2·5% aqueous solution) produced moderate or pronounced skin irritation (erythema, oedema, thickening, scaliness, encrustation and fissuring with subsequent desquamation and/or exfoliation). Red, papular eruptions were observed in the treatment area of all test animals in this test after 1 wk of treatment. Similar lesions and cutaneous abscesses appeared on control animals after 2·5 wk. One death occurred in the test group and was considered to be a result of treatment stress related to acute infectious disease. An additional test animal had a slight body-weight loss throughout the test period and was unhealthy in appearance. No other compound-related effects were seen.

Moderate or pronounced skin irritation was also produced in the 13-wk test of $C_{14}E_7$ at this dose. Red, papular eruptions appeared in both test and control animals (comparable to effects seen in the 4-wk study). Three test animals and one control succumbed to infectious disease after at least 1 month of treatment. Septicaemia was seen microscopically, and was the probable cause of death since no other effects were observed that could be related to compound administration.

Formulation B (10% aqueous solution) tested for 13 wk at a dose of 200 mg/kg/day (66 mg $C_{14}E_7$/kg/day) produced moderate or pronounced dermal irritation and red, papular eruptions. Five animals died between days 27 and 91. The sixth was killed on day 55 in a moribund condition. The deaths appeared to be caused by infectious septicaemia possibly in combination with treatment stress. Examination of the

Table 3. *Incidence of* S. aureus *on the skin of rabbits and guinea pigs in 13-wk percutaneous toxicity study*

Duration of application (wk)	Animals/group† positive for *S. aureus* on the test site					
	Normal rabbits		'Special'‡ rabbits		'Special'‡ guinea-pigs	
	Formulation B	Water	Formulation B	Water	Formulation B	Water
1	1	0	3	0	0	0
2	0	0	3	0	0	0
3	2	0	4	0	1	0
4	3	0	3	0	0	0
5	4	0	5*	0	0	0
6	4§	1	6*	0	0	0
7	5*	0	5*	0	1	0§
8	5*	0	5*	0	0	0
9	2	0	6*	1	0	0
10	5*	0	6*	0§	0	0
11	3	0	6*	1	0	0
12	2	0	6*	1	0	0
13	4	0	4	2	0	0

†Each group consisted initially of six animals.
‡'Special' conditions were designed to minimize bacterial contamination.
§Death of one animal from unrelated cause.
Figures marked with asterisks differ significantly (*$P < 0·05$) from comparable controls.

treatment solutions revealed contamination by Gram-negative rods. Extensive infection (both local and systemic) by *S. aureus* was demonstrated in all groups in this study. There were no gross or microscopic findings in any of these animals suggestive of direct chemical toxicity.

A 10% aqueous solution of formulation B (200 mg/kg/day; 66 mg $C_{14}E_7$/kg/day) was administered for 13 wk to two groups of six rabbits ('routine' and 'special' handling) and a 'special-handling' group of six young adult Hartley albino guinea-pigs (three males, three females). Corresponding control groups were treated with water only. The only grossly observable effect of the test material was the production of skin irritation, which was severe in the rabbit. Skin eruptions, similar to those in previous studies, occurred on treatment sites of rabbits in both treatment groups after 4 wk and persisted throughout the study. Similar but less severe lesions appeared on rabbits in both 'routine' and 'special' handling control groups later in the study. Moderate skin irritation (not accompanied by papular eruptions) occurred in guinea-pigs. Thickening of the outer epithelium was seen microscopically in both species and was accompanied by a mild inflammatory reaction in the rabbit. Any internal lesions noted at autopsy were opened aseptically and swabbed: blood agar and Baird-Parker plates were streaked. No other unusual or test-related lesions were observed. All haematological values were within normal limits.

The results of the microbiological testing showed that a high incidence of bacterial (*S. aureus*) colonization was specific to the experimental rabbit groups only (see Table 3). After treatment for 5–7 wk, statistically significant increases in *S. aureus* incidence were seen in both test groups compared to their respective control groups. Special handling to reduce contamination did not have a detectable effect on the microbiological profiles of these animals, as shown by chi-square analysis. Colonization was limited to the skin and did not result in either systemic infection (as judged by pathogen-free visceral fluid and liver homogenate) or any detectable effect on the animals' health as judged by gross and microbial examination at autopsy. All internal lesions were negative for *S. aureus*.

Guinea-pig immersion

Ten guinea-pigs were immersed in 10% aqueous solutions of $C_{14}E_7$ for 4-hr daily exposures for 5 days. Fissured skin was noted on all test animals at autopsy. The only compound-related lesions seen microscopically were mild-to-moderate subacute dermatological changes characterized by hyperkeratosis, acanthosis and infiltration of the superficial dermis (predominantly by lymphocytes and plasma cells with scattered heterophils present in some sections). No lesions indicative of sytemic toxicity were observed under these conditions.

Handwashing

Because of the *S. aureus* involvement in the percutaneous toxicity studies using rabbits, it was necessary to assess the possibility of skin colonization by this organism subsequent to human exposures. The two panels of 16 subjects (male and female) whose hands were exposed to undiluted test formulations were sampled for *S. aureus*. None of the members of the two panels of 16 handwashing panelists using formulations A or B (half of each panel) were found to have *S. aureus* on their hands after the study if it was not present before the study. The reverse case (*S. aureus* present before but not after) was seen in three of 16 subjects using A and ten of 16 subjects using B (a significant change, $P < 0.05$, chi-square test). Thus, in contrast to the results seen in rabbits, washing with formulation B reduced the incidence of *S. aureus* on the hands. Seven subjects using A and four using B were negative before and after handwashing, while six using A and two using B were positive for *S. aureus* both before and after the study. Under the exaggerated exposure conditions of this test, the production of slight skin irritation was noted; however, there were no signs of infectious lesions as had been observed in the dermal studies in the rabbit. None of the panelists had to discontinue the test because of irritation.

Contact sensitization

Repeated insult patch tests were conducted with $C_{13}E_6$ (176 subjects), $C_{14}E_7$ (144 subjects) and formulations containing these materials (212 and 227 subjects for formulations A and B, respectively).

Hyper-reactivity to $C_{13}E_6$ (2·5% aqueous solution) appeared to have been induced in one subject. This subject subsequently used formulation A (undiluted for hand rubbing heavily soiled laundry and for routine machine laundering) for 14 wk without developing a dermatitis. Patch testing with a non-irritating (0·75%) aqueous solution of formulation A similarly produced no sign of sensitization after this period of usage. Sensitization was not seen in any subjects in tests of 2·5% $C_{14}E_7$ or 0·25–1% formulation B.

It appears that under the greatly exaggerated conditions of the repeated insult patch test, a low level of hyper-reactivity could be induced by $C_{13}E_6$. Formulations containing 33% $C_{13}E_6$, however, have not produced evidence of sensitization under comparable conditions in any of 212 subjects.

Home usage clinical mildness studies

Three studies were conducted in groups of housewives using formulations A or B or a marketed product. A total of 342 housewives used formulation A and 753 used formulation B for dishwashing for

Table 4. *Home usage clinical mildness studies*

Test no.	Test product	No. of subjects	Average skin grade*
1	Formulation A	248	7·1
	Formulation B	242	7·2
	Marketed product 1	244	6·9
	Marketed product 2	256	7·0
2	Formulation A	94	7·6
	Marketed product 2	88	7·4
3	Formulation B	511	7·3

*Analysis of variance demonstrated no significant differences ($P < 0.05$) among average product grades within each test. Skin grade scale: 10–1, where 10 = perfect and 1 = very poor skin condition.

a 2-wk period. Examinations by dermatologists before and after this usage period revealed that the use of these test materials produced comparable skin mildness scores (Table 4). Statistical evaluation by analysis of variance demonstrated that formulations A and B were comparable to existing marketed products in their effect on the skin.

DISCUSSION

Formulations A and B are liquid detergents intended for use on heavily stained laundry items, with specific emphasis on greases and oils. The recommended uses for these products are in the washing machine (at a concentration of approximately 0·1%) and for pretreating stains (hand application of undiluted product) prior to laundering.

Although $C_{13}E_6$ and $C_{14}E_7$ are not currently used in products intended for dishwashing, the potential for such use in the future as well as the possible misuse of laundry products suggested the need for subchronic oral toxicity evaluation. The ingestion of diets containing 1% of the AE_x for 91 days produced only increased liver-to-body weight ratios ($C_{13}E_6$ and $C_{14}E_7$) or only growth depression thought to be related to diet palatability ($C_{13}E_6$).

Repeated skin contact with AE_xs in detergent products is certainly anticipated, and the effects of these exposures have been assessed in terms of percutaneous systemic toxicity, contact sensitization and skin irritation.

Animal subchronic percutaneous exposures lasting for periods of 1 or 3 months produced no observable systemic toxicity at AE_x doses up to 50 mg/kg/day or at doses of the formulations up to 200 mg/kg/day. The only observed effects were related to skin irritation. Increased susceptibility to *S. aureus* infection, seen at high dose levels resulting in several deaths, appeared to be specific for the rabbit, guinea-pigs being unaffected. Human use of these formulations did not increase *S. aureus* in the area of contact. On the contrary, a lesser incidence of this pathogen was observed following hand-washing with formulation B.

Because the conditions used in the subchronic percutaneous toxicity tests were in excess of predicted human exposure (even in misuse of the product for dishwashing), we feel that there would be little or no risk with regard to systemic toxicity through use of these materials. As mentioned below, home usage tests indicated that these products were comparable in mildness to currently marketed products.

Using our patch-testing conditions, commercially prepared $C_{13}E_6$ produced a skin reaction suggesting sensitization in 1 of 176 subjects. No skin reactions were seen in 439 volunteers patch tested with either formulation A or B. Skin reactions can be seen, however, in patch tests in some individuals who do not react under normal conditions of product use. The one individual showing a positive patch-test reaction to $C_{13}E_6$ was given formulation A (containing 33% $C_{13}E_6$) for home use. No problems were encountered through normal use of this product for 14 wk, suggesting either that the patch-test conditions were more severe than exposures during normal use, or that this subject was a false positive in the earlier test. Subsequent repatching using formulation A in this individual failed to indicate that sensitization had been produced.

Although slight skin irritation (dryness and redness) has been demonstrated under exaggerated laboratory conditions, formulations containing high levels of alkyl ethoxylates are as mild as other products (intended for similar use) having years of safe market experience. The results of home usage tests indicate that no significant skin irritation is associated with the use of two formulations containing 33% $C_{13}E_6$ or $C_{14}E_7$.

In conclusion, the results of the various subchronic tests conducted indicate that products containing $C_{13}E_6$ or $C_{14}E_7$ are safe for human use. In our judgement, the risks are extremely low with regard to the exposures that are likely to result from either normal use or possible misuse.

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PRIMARY SENSITIZATION POTENTIALS OF SOME HALOGENATED SALICYLANILIDES AND THEIR CROSS-SENSITIVITY IN GUINEA-PIGS

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Abstract—Contact sensitization potentials of some halogenated salicylanilides were studied in guinea-pigs after topical immunization with the compounds in acetone with the aid of heat-killed *Mycobacterium tuberculosis* as an adjuvant. When challenged and rechallenged with the inducer allergen, the treated animals had the following responses: strong to 4′-monobromosalicylanilide (4′-MBS) and 3,3′,4,5-tetrachlorosalicylanilide (TCSA), weak to strong to 4′,5-dibromosalicylanilide (4′,5-DBS), weak to moderate to 3,5-dibromosalicylanilide (3,5-DBS), negligible to weak to 5-bromosalicylanilide (5-MBS) and 3,4′,5-tribromosalicylanilide (TBS), and negligible to 2′,3,4′,5-tetrabromosalicylanilide (tetra-BS). The relative contact sensitization pattern after repeated challenges was TCSA > 4′-MBS > 4′,5-DBS > 3,5-DBS > 5-MBS > TBS > tetra-BS, and closely parallels the pattern in man. When each immunized animal group was challenged with the inducer allergen plus six other halogenated salicylanilides, the guinea-pigs strongly sensitized to 4′-MBS showed a weak cross-sensitivity to 4′,5-DBS and TCSA. All other groups had no cross-sensitivity or a negligible response to all other halogenated salicylanilides. When guinea-pigs were immunized with a topical application of TBS in soap or in acetone and challenged with TBS in aqueous soap, they showed a strong sensitization.

INTRODUCTION

Numerous natural and synthetic chemicals, including drugs, cosmetics ingredients and industrial chemicals or by-products used in clothing or household goods, ultimately reach man and animals through the skin. Some of these chemicals are primary irritants or sensitizers (Birmingham, 1959; Cronin, 1967; Epstein & Maibach, 1966; Fisher, 1967; Fisher, Pascher & Kanof, 1971; Hjorth & Trolle-Lassen, 1963; Klauder, 1962; Masters, 1960; Rothenborg & Hjorth, 1968; Schorr, 1971) and also cause phototoxicity or photoallergy in man (Epstein, 1971; Fisher, 1967). Although hairless mice (Fitzpatrick, Pathak, Magnus & Curwen, 1963; Ison & Blank, 1967; Ison & Davis, 1969; Rothe & Jacobus, 1968) and rats, rabbits, miniature swine and guinea-pigs (Morikawa, Nakayama, Fukuda, Hamano, Yokoyama, Nagura, Ishihara & Toda, 1974; Sams, 1966; Sams & Epstein, 1967; Stott, Stasse, Bonomo & Campbell, 1970) develop phototoxic skin reactions, only guinea-pigs have been shown to have photoallergic responses (Harber, Targovnik & Baer, 1967 & 1968; Morikawa, 1971; Morikawa *et al.* 1974; Vinson & Borselli, 1966).

Many antibacterial agents, especially the halogenated salicylanilide germicides used in soaps, can cause phototoxicity and/or photoallergy (Harber *et al.* 1967 & 1968; Morikawa *et al.* 1974; Vinson & Borselli, 1966), and some of the halogenated salicylanilides have recently been banned from use in cosmetics for this reason (*Federal Register*, 1975). These salicylanilides are known also to cause primary contact sensitization (Calnan, Harman & Wells, 1961; Harber *et al.* 1967 & 1968; Osmundsen, 1970; Wilkinson, 1962). It has been suggested that the halogenated photoallergens are first photodecomposed with loss of one or more halogens to active primary contact sensitizers that will cause sensitization in man and animals (Coxon, Jenkins & Walfi, 1965; Morikawa *et al.* 1974; Willis & Kligman, 1968a). Cross-sensitivity among these halogenated compounds has frequently been reported (Agrup, Fregert & Ovrum, 1969; Baughman, 1964; Burry, 1967 & 1968; Chung & Carson, 1975 & 1976; Chung & Giles, 1972; Chung, Giles & Carson, 1970; Crow, Wilkinson & Osmundsen, 1969; Harber, Harris & Baer, 1966; Harber *et al.* 1967; Marzulli & Maibach, 1973; Morikawa *et al.* 1974; Osmundsen, 1970), but it is not clear whether the cross-sensitivity is due mainly to the photodecomposition products or to the similarity of chemical structures. Humans and guinea-pigs that were photosensitized to one chemical tended to show more cross-sensitivity to other halogenated chemicals on challenge with ultraviolet irradiation than in the absence of this challenge (Harber *et al.* 1967 & 1968; Morikawa, 1971; Morikawa *et al.* 1974; Vinson & Borselli, 1966). No adjuvants were used in the guinea-pig tests, and therefore the sensitization rates were too low for adequate evaluation and comparison with the clinical human data.

This paper reports the primary contact sensitization potentials of some of the halogenated salicylanilides and their mutual cross-sensitivity in guinea-pigs treated with an adjuvant and an allergen. In addition, a sample of soap containing 3,4′,5-tribomosalicylanilide (TBS) was tested for its sensitization potential.

EXPERIMENTAL

Chemicals. The pure compounds 4′-monobromosalicylanilide (4′-MBS), 5-monobromosalicylanilide (5-MBS), TBS and 2′,3,4′,5-tetrabromosalicylanilide

(tetra-BS) were obtained from Fine Organics, Inc., Lodi. N.J. Purified 4',5-dibromosalicylanilide (4',5-DBS) and 3,5-dibromosalicylanilide (3,5-DBS) were obtained from Lever Brothers Co., Edgewater, N.J., and 3,3',4',5-tetrachlorosalicylanilide (TCSA) was purchased from Geigy Pharmaceuticals, Ardsley, N.Y. The adjuvant (heat-killed *Mycobacterium tuberculosis* H37 Ra in oil) was diluted to a desired concentration with incomplete adjuvant, both adjuvants having been purchased from Difco Laboratories, Detroit, Mich.

Animals. Male Hartley albino guinea pigs (300-400 g) were used. They were fed Guinea Pig Chow obtained from the Ralston Purina Co., St. Louis, Mo.

Immunization. The method of immunization has been described in previous publications (Chung & Carson, 1975 & 1976; Chung & Giles, 1972; Chung *et al.* 1970). All guinea-pigs, including controls, were given 100 μg of the adjuvant in each of the four footpads, and within 4 hr 0·2 ml of a 2% acetone solution of each allergen was applied topically in the nuchal area for induction. The controls received only acetone without the allergen. For immunization with a soap containing 0·75% TBS and unidentified perfume ingredients, a 34% (w/v) aqueous solution was used. The controls received water.

Challenge. The rest period after the initial immunization (or previous challenge) ranged from 3 to 7 wk. For challenge, all guinea-pigs, including the controls, received 0·05 or 0·1 ml of several concentrations of the allergen or of six cross-reactants on both flanks, which had been closely clipped.

Reaction scoring. Details of the rating of skin sensitization reactions in guinea-pigs have already been described (Chung & Carson, 1975 & 1976; Chung & Giles, 1972; Chung *et al.* 1970). The skin reactions were rated from 0 to 6 depending on the degree of erythema, and readings were taken at 24, 48, 72, 96 and 144 hr or longer after challenge. A slight modification was introduced to improve the procedure, since not all animals gave peak reactions at the same time. The highest skin reaction for each site or animal was taken for each rating period, as shown in Table 1. The highest score was taken for the final rating.

Skin reactions of 3 and above were arbitrarily considered as positive, and those of 2 and below were considered as negative. The fractional response (FR) is the number of positive animals (or sites per concentration) related to the total number of animals (or sites per concentration) used. The fractional response index (FRI) is the ratio of the net FR (FR of treated animals minus FR of controls) to the net maximum FR (1 minus FR of controls) expressed as a percentage:

$$\text{FRI} = \frac{\text{FR of treated} - \text{FR of controls}}{1 - \text{FR of controls}} \times 100.$$

This expression is a general form for expressing frequency or incidence of allergenic sensitization under particular experimental conditions even when controls are not zero. When the controls are zero, FRI = FR × 100; this is a special case and becomes the conventional expression that clinicians prefer to use (Epstein & Maibach, 1966; Marzulli & Maibach, 1973 & 1974; Morikawa *et al.* 1974), often without proper controls, in order to avoid false positivity. However, false negativity will result when the challenge concentrations are too low or are not optimal.

Similarly, the intensity of the skin sensitization reaction to an allergen can be rated. The average intensity (AI) of the erythema reactions of a group of animals in response to an allergenic challenge is the ratio of the sum of the numerical scores to the total number of animals at a given challenge concentration. The average intensity index (AII) can be calculated as the ratio of the net AI of the treated animals to the net maximum AI of the skin reactions expressed as a percentage:

$$\text{AII} = \frac{\text{AI of treated} - \text{AI of controls}}{6 - \text{AI of controls}} \times 100.$$

This indicates the degree (or intensity) of the average skin sensitization in response to an allergenic challenge in a host. By nature this expression is more quantitative than the frequency expression. The maximum values 1 and 6 are arbitrary values depending on the rating scale or system. These values are presented without a percentage sign or unit.

Table 1. *A rating procedure utilizing the highest score during a challenge period*

Animal* no.	Skin reactions at (hr)				Highest score during a 144-hr period
	24	48	72	144	
1	0	0	0	0	0
2	4	4	4	3	4
3	0	0	0	0	0
4	0	0	0	0	0
5	0	0	0	0	0
6	0	2	2	3	3
7	0	2	4	2	4
8	4	3	1	1	4
9	0	0	0	0	0
10	0	0	0	3	3
11	0	0	0	2	2
12	0	1	0	0	1
13	0	0	0	0	0
14	3	2	0	0	3
15	0	0	0	0	0
16	0	0	0	0	0
17	0	0	0	1	1
18	0	0	0	0	0
19	0	0	0	0	0
20	5	4	4	3	5
21	0	0	0	2	2
22	0	0	0	0	0
23	4	4	0	0	4
24	0	0	0	0	0
Calculations†					
FR...	5/24	4/24	3/24	4/24	8/24
FRI (%)...	21	17	13	17	33
AI...	0·83	0·92	0·63	0·83	1·13
AII (%)...	14	15	11	14	19

*The guinea pigs were immunized and challenged with a topical application of 2% 4',5-DBS (4',5-dibromosalicylanilide) in acetone as described for the second experiment in Table 2. The eight control animals that were challenged similarly showed no skin reactions during the 144-hr observation period.

†For calculations of FR (fractional response), FRI (fractional response index), AI (average erythema intensity) and AII (average intensity index) see above.

The earlier definition of the ranges of AII values (Chung & Carson, 1975 & 1976) was modified slightly for convenience. AII values of 45 or more, between 30 and 44, and between 20 and 29 were arbitrarily considered as strong, moderate and weak sensitizations, respectively, without modification. The borderline (or very slight) sensitization range included values between 10 and 19 instead of between 15 and 19. The value range between 0 and 9 (instead of between 0 and 14) was considered as the negligible sensitization or non-allergenic range. FR and/or FRI values are listed for reference purposes but emphasis is placed on the more quantitative intensity data.

RESULTS

First challenge

After a rest period of about 4 wk following immunization, all guinea-pigs including the controls were challenged with 0·05 ml of 1 or 2% solutions of the inducing allergens in acetone. TCSA elicited a strong skin-sensitization rate but tetra-BS did not cause any sensitization (Table 2). TBS and 5-MBS elicited a negligible sensitization in the first experiment but a weak sensitization in the second experiment. In both experiments, 3,5-DBS caused a negligible skin reaction and 4′,5-DBS elicited a weak to very slight skin reaction.

Second challenge

When the animals were challenged for the second time, on the days indicated in Table 2, an appreciable increase in the sensitization rates occurred with several of the compounds. 4′-MBS was a strong sensitizer in both experiments, and 4′,5-DBS was a weak sensitizer in the first experiment and a strong sensitizer in the second. The AII value for TCSA was decreased in the second challenge but remained in the strong sensitization range. 5-MBS and 3,5-DBS elicited negligible and borderline reactions, respectively, in the first experiment, and weak and moderate reactions, respectively, in the second experiment. TBS and tetra-BS caused only negligible or no skin reactions. Although the reaction rates of each allergen varied in the two experiments, the relative sensitization rates of these compounds did not vary and were TCSA > 4′-MBS > 4′,5-DBS > 3,5-DBS > 5-MBS > TBS > tetra-BS. This pattern of relative contact sensitization rates follows data from man (Crow *et al.* 1969; Harber *et al.* 1966; Marzulli & Maibach, 1973; Willis & Kligman, 1968a) rather than earlier data from guinea-pigs (Morikawa, 1971; Morikawa *et al.* 1974).

TBS in soap

In 1971 an outbreak of skin rash was reported among the patients in one of the West Coast hospitals; the patients had all used a soap containing 0·75%

Table 2. *The primary sensitization potentials of various halogenated salicylanilides in guinea-pigs**

Immunizing and challenging compound	The highest skin reactions during a 144-hr period								
	First challenge with						Second challenge with		
	2%			1%			1%		
	FR	FRI	AII	FR	FRI	AII	FR	FRI	AII
Experiment no. 1									
4′-MBS	14/19	74	59	ND	—	—	18/19	95	81
5-MBS	2/20	10	8	ND	—	—	2/20	10	8
3,5-DBS	2/18	11	9	ND	—	—	4/18	22	13
4′,5-DBS	6/20	30	23	ND	—	—	6/20	30	20
TBS	2/20	5	3	ND	—	—	0/20	0	0
Experiment no. 2									
4′-MBS	13/25	52	38	12/25	48	39	21/25	84	51
5-MBS	6/25	24	19	6/25	24	15	14/25	56	29
3,5-DBS	2/23	9	9	1/23	4	6	17/22	77	43
4′,5-DBS	8/24	33	19	6/24	25	20	17/22	77	47
TBS	8/19	42	20	ND	—	—	2/19†	11†	6†
Tetra-BS	ND	—	—	0/20	0	0	1/19	5	4
TCSA	19/19	100	84	19/19	100	86	15/19	79	56

4′-MBS = 4′-Monobromosalicylanilide 5-MBS = 5-monobromosalicylanilide
3,5-DBS = 3,5-dibromosalicylanilide 4′,5-DBS = 4′,5-dibromosalicylanilide
TBS = 3,4′,5-tribromosalicylanilide tetra-BS = 2′,3,4′,5-tetrabromosalicylanilide
TCSA = 3,3′,4′,5-tetrachlorosalicylanilide ND = Not done

*Each guinea-pig, including controls, received 100 μg adjuvant (*Mycobacterium tuberculosis*, H37 Ra, heat-killed and suspended in oil) in four foot-pads and, within 4 hr, 4 mg of allergens in acetone were applied topically. Challenges were made by topical application of 0·05 ml of a 2% acetone solution. The first and second challenges for experiment no. 1 were made on days 26 and 86, respectively, after the initial immunization and for experiment no. 2 on days 29 and 88, respectively. The rating and scoring systems are given in Experimental and Table 1.

†Concentration used was 2% instead of 1%.

Table 3. *Sensitization potential of TBS in acetone and in an antibacterial soap*

Day-35 challenge		The highest skin reactions (during a 72-hr period) of guinea pigs immunized with:					
		Antibacterial soap (25·5 mg TBS in water)			TBS (2 mg in acetone)		
Vehicle	Content of TBS (mg)	FR	FRI	AII	FR	FRI	AII
Aqueous soap: 17%	12·75	9/9	NA	53	10/10	NA	59
8·5%	6·4	9/9	100	42	10/10	100	70
4·3%	3·2	2/9	22	29	8/10	80	53
2·2%	1·6	2/9	22	28	6/10	60	40
Acetone	1·0	7/10	64	24	4/10	28	10
	0·5	4/10	40	30	3/10	13	21
	0·2	1/10	10	10	1/10	10	12
	0·1	0/10	0	7	0/10	0	13

TBS = 3,4',5-Tribromosalicylanilide NA = Not applicable

*All guinea-pigs, including controls, received the adjuvant in four foot-pads and, within 4 hr, 20 guinea-pigs received a topical application of 0·2 ml 34% aqueous soap solution, while 20 received 0·2 ml 1% TBS in acetone in the nuchal area. On day 35 each group was challenged either with 0·1 ml of various concentrations of aqueous soap or with TBS in acetone. The rating and scoring systems are given in Experimental and Table 1.

(w/w) TBS and unidentified perfume ingredients. A sample of this soap was tested, along with pure TBS, for sensitization potentials and the results are shown in Table 3. Both the soap-immunized and TBS-immunized groups showed strong contact-sensitization rates with the highest challenge concentration of soap in water but the TBS-immunized group had a higher AII value than the soap-immunized group. When animals were challenged with various concentrations of TBS in acetone, the soap-immunized groups showed slightly higher sensitization rates than did the TBS-immunized groups. However, since the number of animals was too small for the difference to be significant, it can only be said that TBS in either soap or acetone is an effective contact sensitizer and that a higher TBS concentration in soap is required for equivalent induction and elicitation of contact sensitization in guinea-pigs. Contact sensitization potentials of these halogenated salicylanilides in guinea-pigs (Morikawa *et al.* 1974; Tables 2 and 3) and man (Willis & Kligman, 1968a; Marzulli & Maibach, 1973) seem to vary considerably from experiment to experiment.

Cross-sensitivity

In addition to being topically challenged for the second time with 0.05 ml of a 1% solution of the inducing allergen in acetone (two sites), the guinea-pigs used in the second experiment shown in Table 2 were also challenged with six other halogenated salicylanilides (one site each). The results of cross-reactivity are shown in Table 4. The guinea-pigs that were immunized with 4'-MBS showed a weak and a very weak cross-reactivity to TCSA and 4',5-DBS, respectively. The groups immunized with TCSA, 5-MBS, 3,5-DBS, 4',5-DBS, TBS or tetra-BS had either only very negligible or no cross-reactivity to any of the other salicylanilides. Although the groups immunized

Table 4. *Mutual cross-sensitivity of halogenated salicylanilides**

Immunizing (or inducing) substance	Cross-sensitivity of cross-reactants (highest values during a 144-hr period)													
	4'-MBS		5-MBS		3,5-DBS		4',5-DBS		TBS		Tetra-BS		TCSA	
	FRI	AII	FRI	AII	FRI	AII	FRI	AII	FRI	AII	FRI	AII	FRI	AII
4'-MBS	*84*	*51*	0	0	20	10	36	19	0	0	0	0	52	27
5-MBS	4	2	*56*	*29*	14	6	4	2	14	6	0	0	14	6
3,5-DBS	18	10	9	7	*77*	*43*	18	9	17	7	9	5	9	5
4',5-DBS	9	4	9	4	9	4	*77*	*47*	0	0	0	0	0	0
TBS	0	0	ND	ND	0	0	0	0	*11*	*6*	0	0	0	0
Tetra-BS	0	0	ND	ND	0	0	0	0	0	0	*5*	*4*	0	0
TCSA	5	3	ND	ND	0	0	5	4	16	8	5	4	*79*	*56*

4'-MBS = 4'-Monobromosalicylanilide 5-MBS = 5-Monobromosalicylanilide 3,5-DBS = 3,5-Dibromosalicylanilide
4',5-DBS = 4',5-Dibromosalicylanilide TBS = 3,4',5-Tribromosalicylanilide tetra-BS = 2',3,4',5-Tetrabromosalicylanilide
TCSA = 3,3',4',5-Tetrachlorosalicylanilide

*See Experiment no. 2 in Table 2 for details. At the second challenge each guinea-pig was challenged with 0·05 ml of a 1% acetone solution of the immunizing substance plus the six other salicylanilides listed.

with 4′,5-DBS and TCSA were strongly sensitized (see the italicized values in Table 4) they showed negligible or no cross-sensitivity. Sensitization rates of the groups immunized with TBS and tetra-BS were too low for cross-reactivity to be evaluated properly. This unexpectedly high immunological specificity is in contrast to the photocontact cross-sensitivity reported in guinea-pigs and man (Agrup *et al.* 1969; Anderson, 1963; Baughman, 1964; Burckhardt, Burckhardt & Schwarz-Speck, 1957; Burry, 1967 & 1968; Chung & Giles, 1972; Crow *et al.* 1969; Epstein, Wuepper & Maibach, 1968; Harber *et al.* 1966, 1967 & 1968; Morikawa, 1971; Morikawa *et al.* 1974; Osmundsen, 1969 & 1970; Vinson & Borselli, 1966; Vinson & Flatt, 1962; Wilkinson, 1961; Willis & Kligman, 1968a,b).

DISCUSSION

The contact sensitization rates were apparently enhanced by the use of an adjuvant in immunizing guinea-pigs for contact sensitization to halogenated salicylanilides. This enhancement was shown by a comparison of our results with those obtained by other investigators who used neither adjuvant nor irradiation (Harber *et al.* 1967 & 1968; Morikawa, 1971; Morikawa *et al.* 1974), although our rates varied considerably from experiment to experiment and also from the first to the second challenges. The comparable data from a deliberate sensitization of normal human subjects by patch-testing also varied considerably (Marzulli & Maibach, 1973 & 1974; Willis & Kligman, 1968a). Our data, obtained by using guinea-pigs and an adjuvant, are more or less within the rates of human sensitization studies and the order of the relative sensitization rate of these germicides also seems very similar. Any drug or cosmetic product containing the halogenated salicylanilides TBS, 4′,5-DBS, 3,5′-DBS or TCSA is considered as a new drug or as an adulterated cosmetic (*Federal Register*, 1975), and therefore such cosmetics cannot be marketed. These and other germicides used in soaps tend to sensitize humans repeatedly in a massive manner, unlike human or animal experimental patch tests.

Even so-called normal human subjects have been exposed to a variety of chemicals through skin, foods and air, unlike the short-lived experimental guinea-pigs, and therefore primary sensitization of test subjects may not be truly primary. Hence, sensitive guinea-pig tests are desirable for routine premarketing and surveillance test purposes. Furthermore, many drugs and industrial, agricultural, household and cosmetic products contain halogenated aromatic compounds that may cause contact sensitivity, and especially photocontact cross-sensitivity, to these halogenated salicylanilides.

The cross-sensitivities among these salicylanilides under our experimental conditions were negligible or at most much lower than the data obtained in guinea-pigs sensitized by repeated topical applications of the allergen for a long period (Harber *et al.* 1967; Morikawa *et al.* 1974). Contact and photocontact cross-sensitivity elicited in sensitized guinea-pigs and man by photochallenges (i.e. ultraviolet irradiation challenges) have been reported to be fairly extensive (Agrup *et al.* 1969; Baughman, 1964; Burckhardt *et al.* 1957; Burry, 1968; Chung & Giles, 1972; Harber *et al.* 1966, 1967 & 1968; Morikawa *et al.* 1974; Osmundsen, 1969; Vinson & Borselli, 1966). This difference may be explained by one or more of the following possibilities: (1) repeated immunizations result in more cross-sensitivity of the hosts, probably because of chemodegradation and biodegradation by the hosts and their microbial population; (2) repeated topical applications of a photosensitizer for a long period may lead to many products of photodecomposition by room lights despite precautions; (3) photodecomposition of a sensitizer after repeated applications followed by ultraviolet irradiation may result in the production of many free-radical compounds which may become various active antigens for effective immunization and elicitation of both primary sensitization and cross-sensitivity. If the availability of the antigen (the hapten–skin protein conjugate) is the rate-limiting step in the immunization and elicitation of sensitization and cross-sensitivity with these halogenated salicylanilides, free-radical compounds and unstable intermediates produced by photodecomposition may result in the presence of larger amounts of antigens within a shorter time than would occur without irradiation, and would thus lead to a greater elicitation of skin reactions and also of primary sensitization. Experimental verification of these suppositions may not be impossible but will require delicate tests. Attempts along these lines have already been made. Some investigators put a strong emphasis on free-radical formation from irradiated photoallergens, with subsequent antigen formation (Agrup *et al.* 1969; Jenkins, Welti & Baines, 1964). Others have put forward the theory, with experimental evidence for the hypothesis of Burckhardt *et al.* (1957), that a photosensitizer is converted by photodecomposition, with loss of halogens, into another compound which in turn acts as the strong responsible allergen (Coxon *et al.* 1965; Willis & Kligman, 1968a). A third group of investigators is of the opinion that neither hypothesis alone is sufficient to explain all the experimental and clinical observations (Morikawa *et al.* 1974).

Use of the highest score during a rating period is a very convenient procedure when the peak elicitation time is different for different animals. Whether the peak times occur early or late is dependent on many factors, such as the degree of sensitization of the host, the dose and route of challenge, the vehicle and the nature of the allergen. This procedure is more valid when control values are very low. When control values are high, the skin-reaction readings are taken for as long as possible and the highest FRI or AII values are considered for the final decision. The highest value may occur in early readings, or sometimes at 72 or 96 hr or even later. If high toxic challenges are avoided, the new procedure may be more meaningful.

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SHORT PAPERS

ABILITY OF ADULT AND FOETAL RAT TISSUES TO METABOLIZE CHLORINATED FATTY ACIDS

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Summary—*In vitro* incubation of rat tissues with [^{36}Cl]dichlorostearic acid showed that skeletal muscle can dechlorinate lipids fastest followed by heart, liver, brain and kidney. The ability of muscle to metabolize chlorinated lipids exceeds its ability to metabolize unchlorinated [^{3}H]oleic acid, but the reverse applies with liver. Inclusions of 1% chlorinated corn oil in the diet of rats for 2 wk prior to homogenate studies reduced the ability of all tissues except the kidney to metabolize either [^{36}Cl]dichlorostearic acid or [^{3}H]oleic acid. The ability of 15–16-day foetuses to dechlorinate [^{36}Cl]-dichlorostearic acid was higher than that of maternal liver but lower than that of maternal muscle. Cardiac tissues metabolized [^{36}Cl]linoleic acid much faster than [^{36}Cl]dichlorostearic acid but there was little difference in the rate at which the fatty acids were metabolized by hepatic tissue.

Introduction

Chlorinated lipids resulting from the bleaching of flour are toxic to rats (Cunningham, Lawrence & Tryphonas, 1977). They have also been found to be absorbed from the digestive tract and distributed throughout the body, with a particularly long half-life in adipose tissue (Cunningham & Lawrence, 1976 & 1977a,b). Most of the chlorine of chlorinated lipids absorbed from the digestive tract is known to be converted eventually to water-soluble compounds and excreted in the urine but the relative dechlorinating abilities of the tissues has not previously been determined. Chlorinated lipids also cross the placenta and although the levels found in foetuses decline with time, it is not known whether the foetus can dechlorinate lipids (Cunningham & Lawrence, 1977c). Chlorinated linoleic and linolenic acids were absorbed from the digestive tract at a slower rate than chlorinated oleic acid (dichlorostearic acid) but much less of these lipids was found in tissues than would be expected from the amount absorbed (Cunningham & Lawrence, 1977a).

The present experiments were conducted to determine the degree to which various tissues dechlorinate fatty acids and the effect of prefeeding with chlorinated lipids. The ability of foetal tissue to dechlorinate lipids was investigated and a comparison was made of the ability of adult tissues to dechlorinate dichlorostearic acid and chlorinated linoleic acid.

Experimental

Materials. Chlorine-36 was purchased as 3·25 *N*-H^{36}Cl (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 and linoleic acids, (both 99% pure) were obtained from the Sigma Chemical Co., St. Louis, Mo. Chlorine, produced by the reaction of HCl and $KMnO_4$ (Holmes, 1941) was used as described previously (Cunningham & Lawrence, 1976) to chlorinate oleic and linoleic acids. Sufficient carrier Cl_2 was produced to result in 60% chlorination of both acids. After this reaction was complete, additional carrier Cl_2 was added to the linoleic acid to achieve complete chlorination. All surplus Cl_2 was removed in a rotoevaporator at 70°C for 30 min and then [^{3}H]oleic acid was added to both preparations. Since additional oleic acid and other fatty acids would also be provided by the tissue homogenates, no further attempt was made to equalize the concentration of chlorinated fatty acids with that of other fatty acids. The fatty acids were purified by thin-layer chromatography (TLC) using 0·5 mm silica gel G on glass plates and a solvent system of hexane–ethyl ether–acetic acid (75:25:1, by vol.). The lipids were streaked along the origin and oleic acid was located by exposing the edges of the plate to iodine vapour. [^{36}Cl]Dichlorostearic acid was located just below the oleic acid by using fluorescein indicator in the silica gel and by testing successive strips across the plate for ^{36}Cl activity. Several unidentified compounds were obtained from the chlorinated linoleic acid. Those at the R_F of oleic acid and just below it to a point 2 cm above the origin were recovered and hereafter will be referred to as chlorinated linoleic acid. Just before use, aliquots of the labelled fatty acids were combined with bovine serum albumin (1 mmol/11·4 mg albumin) in 5% solution by heating to 55°C.

Homogenates. Rats were stunned by a blow on the head and the required tissues (see below) were quickly removed and placed in buffered isotonic saline at 0°C. Weighed samples were homogenized in a teflon-glass homogenizer with 9 vols ice-cold 0·25 M-sucrose containing 0·001 M-EDTA (buffered at pH 7·4)/g tissue. The homogenates were filtered through cheesecloth into ice-cold beakers. The incubation medium was

Table 1. *Comparison of ability of homogenates of tissues of rats prefed chlorinated corn oil and controls to metabolize [^3H]oleic and [^{36}Cl]dichlorostearic acid (chlorinated oleic acid)*

Isotope	Pretreatment of rats	Amount of tracers converted to water-soluble material in 20 min† (μmol/g protein)				
		Liver	Kidney	Heart	Muscle	Brain
^3H	Control	3·50 ± 0·46	0·32 ± 0·04	2·33 ± 0·74	1·59 ± 0·81	0·34 ± 0·07
	Prefed‡	1·70 ± 0·10**	0·57 ± 0·07*	1·02 ± 0·25	0·32 ± 0.07**	0·18 ± 0·01
^{36}Cl	Control	0·92 ± 0·10	0·64 ± 0·14	3·51 ± 0·64	5·31 ± 2·73	0·92 ± 0·14
	Prefed‡	0·57 ± 0·07*	0·64 ± 0·14	1·87 ± 0·21**	1·73 ± 0·89	0·39 ± 0·07*

†Each incubation flask contained 1 ml of homogenate, 0·70 μmol [^{3}H]oleic acid (264,000 dpm) 1·06 μmol [^{36}Cl]dichlorostearic acid (27,500 dpm) and the remaining constituents of the medium, listed in the experimental section.

‡The prefed rats were given a diet containing 1% chlorinated corn oil (Cl_2:corn oil = 1:5, w/w); controls received the same diet with unchlorinated corn oil.

Values are means ± SEM for groups of five rats and those marked with asterisks differ significantly (Student's *t* test) from controls: *$P < 0·05$; **$P < 0·01$.

Table 2. *Comparison of the ability of homogenates of tissues of pregnant rats and their foetuses to metabolize [^3H]oleic and [^{36}Cl]dichlorostearic acid*

Isotope	Amount of tracers converted to water-soluble material in 20 min* (μmol/g protein)		
	Maternal liver	Maternal muscle	Foetuses†
^3H	$3{\cdot}82 \pm 0{\cdot}46^{A}$	$2{\cdot}01 \pm 0{\cdot}46^{a}$	$0{\cdot}92 \pm 0{\cdot}24^{a}$
^{36}Cl	$0{\cdot}64 \pm 0{\cdot}07^{a,b}$	$2{\cdot}83 \pm 0{\cdot}17^{B}$	$1{\cdot}21 \pm 0{\cdot}24^{a,b}$

*Each incubation flask contained 1 ml of homogenate, 0·70 μmole [^{3}H]oleic acid (264,000 dpm), 1·06 μmol [^{36}Cl]dichlorostearic acid (27,500 dpm) and the remaining constituents of the medium listed in the experimental section.

†Mean total weight of foetuses was 9·0 ± 1·6 g/pregnant rat (mean weight 351 ± 10 g).

Values are means ± SEM for groups of five rats. A statistically significant difference ($P < 0{\cdot}01$) between any two means determined by a Student's *t* test is indicated by different superscripts of the same letter, (e.g. 'A' is significantly greater than 'a').

similar to that used by Wittels & Spann (1968) and modified by Munro, Salem, Goodman & Hasnain (1971). Incubation was carried out at 37°C in 25-ml Erlenmeyer flasks shaking at 125 cycles per min. Each flask contained 2·0 ml of medium made up of the following (in μmol): phosphate buffer (pH 7·4), 90; $MgCl_2$, 2; succinate, 1·5; DL-carnitine, 0·1; ATP, 2·0; CoA, 0·13; [^{3}H]oleic acid (264,000 dpm) 0·70; [^{36}Cl]fatty acid (27,500 dpm), 1·06. One ml of tissue homogenate was added to each flask and the reaction was terminated 20 min later by the addition of 0·5 ml 50% citric acid. Blank determinations were made in the same manner except that either the citric acid was added to the incubation medium before adding the homogenate or the homogenate was boiled before incubation.

Analytical methods. The protein content of the homogenates was determined by the method of Gornall, Bardawill & David (1949). The contents of the incubation flasks were partitioned into lipid and aqueous phases by Folch extraction (Folch, Lees & Sloane Stanley, 1957). Aliquots of the aqueous phase were dissolved in Aquasol® and the activities of ^{3}H and ^{36}Cl were counted in a Beckman Model LS-230 liquid scintillation counter using an external standard for quench correction.

Experiment 1. This was designed to determine whether there was a difference in the ability of tissues to metabolize [^{36}Cl]dichlorostearic acid and [^{3}H]-oleic acid and whether the rate of metabolism was affected by prefeeding rats with chlorinated lipid. Ten 150-g male Wistar rats were divided into two groups, a control group fed commercial rat chow containing 1% added corn oil and a treated group fed rat chow with 1% chlorinated corn oil (Cl_2:corn oil = 1:5, w/w). After 2 wk the rats were killed and homogenates of the liver, kidney, heart, skeletal muscle and brain were incubated with [^{3}H]oleic acid and [^{36}Cl]dichlorostearic acid.

Experiment 2. The ability of foetal tissues to metabolize chlorinated lipids was determined using five 350-g rats at day 15 or 16 of pregnancy. Homogenates of foetuses and samples of maternal liver and skeletal muscle were incubated with [^{3}H]oleic and [^{36}Cl]-dichlorostearic acid.

®Registered trade name of New England Nuclear, Boston, Mass.

Experiment 3. A comparison was made of the ability of rat tissues to metabolize dichlorostearic acid and chlorinated linoleic acid. Homogenates of livers and hearts of each of five 200-g male rats were incubated simultaneously in two media, one containing [^{3}H]oleic acid and [^{36}Cl]dichlorostearic acid and the other containing [^{3}H]oleic acid and [^{36}Cl]linoleic acid.

Results

The amount of ^{3}H- and ^{36}Cl-labelled lipids converted to water-soluble compounds was used as an indicator of the ability of tissues to metabolize unchlorinated and chlorinated lipids. At no time did this exceed 30% of the [^{3}H]oleic acid or 6% of the ^{36}Cl-fatty acids present in the incubation medium and consequently the amount of substrate available did not become a limiting factor during the 20-min incubation period.

In Experiment 1, the 1% dietary level of chlorinated corn oil fed over a 2-wk period reduced body-weight gain by 47·5% and increased relative liver weight by 24%. Table 1 shows that this pretreatment with chlorinated corn oil significantly reduced the ability of liver and muscle to metabolize [^{3}H]oleic acid but slightly increased the conversion by the kidney. The ability to dechlorinate lipids was lower in heart, liver and brain of pre-treated rats. In all tissues except the liver more ^{36}Cl label than ^{3}H was converted to water-soluble material. Absolute comparisons were not possible since the amount of additional oleic acid available in the homogenate from the tissue lipids was not known.

Homogenates of liver from pregnant rats (Experiment 2) metabolized [^{3}H]oleic acid more rapidly than did either muscle or foetal tissue (Table 2). However, homogenates of muscle from the pregnant rats dechlorinated [^{36}Cl]dichlorostearic acid more rapidly than did either liver or foetal tissue. The dechlorinating ability of foetal tissue was still considerable, for it was almost double that of the adult liver. The foetus also metabolized [^{36}Cl]dichlorostearic acid as rapidly as [^{3}H]oleic acid.

In Experiment 3, liver homogenates of 200-g rats metabolized [^{3}H]oleic acid more rapidly than did heart homogenates but the latter metabolized [^{36}Cl]dichlorostearic acid faster than liver (Table 3). There was little difference in the ability of liver to

Table 3. *Ability of tissue homogenates* of liver and heart of rats to metabolize [^3H]oleic acid and either [^{36}Cl]dichlorostearic acid or [^{36}Cl]linoleic acid*

Isotope	Amount of tracers converted to water-soluble material in 20 min† (μmol/g protein)	
	[^{36}Cl]dichlorostearic acid	[^{36}Cl]linoleic acid
	Liver	
^3H	$9{\cdot}81 \pm 0{\cdot}56^{A}$	$9{\cdot}31 \pm 0{\cdot}84^{A}$
^{36}Cl	$1{\cdot}11 \pm 0{\cdot}05^{a,c}$	$1{\cdot}31 \pm 0{\cdot}14^{a,b}$
	Heart	
^3H	$2{\cdot}66 \pm 0{\cdot}31^{a}$	$2{\cdot}44 \pm 0{\cdot}35^{a,b}$
^{36}Cl	$2{\cdot}39 \pm 0{\cdot}35^{Cb}$	$6{\cdot}62 \pm 0{\cdot}67^{B}$

*Each incubation flask contained 1 ml of homogenate, 0·70 μmol [^{3}H]oleic acid (264,000 dpm), 1·06 μmol [^{36}Cl]dichlorostearic acid or [^{36}Cl]linoleic acid (27,500 dpm).

†Values are means ± SEM for groups of five rats. A statistically significant difference ($P < 0{\cdot}01$) between any two means determined by a Student's *t* test is indicated by different superscripts of the same letter, (e.g. 'A' is significantly greater than 'a').

metabolize [^{36}Cl]linoleic acid and dichlorostearic acid but heart metabolized [^{36}Cl]linoleic acid much faster than [^{36}Cl]dichlorostearic acid or [^{3}H]oleic acid.

Discussion

The present studies show that homogenates of all the tissues tested were able to metabolize chlorinated fatty acids. This supports earlier studies in which rats fed [^{36}Cl]lipids excreted large amounts of ^{36}Cl in the form of water-soluble compounds in the urine (Cunningham & Lawrence, 1976). It was suggested that, since in rats fed [^{36}Cl]lipids the levels of these lipids declined more rapidly in the liver than in other tissues, the liver was the most probable site for dechlorination. The present work shows that the heart and skeletal muscle have an even greater ability than the liver to dechlorinate lipids. Prior feeding of chlorinated lipids increased the size of the liver but tended to reduce its ability to dechlorinate them. The size of the other organs was not significantly affected by chlorinated corn oil but their dechlorinating ability was also reduced with the exception of the kidney.

The experiments with foetal tissue also support earlier *in vivo* work suggesting foetal dechlorination of lipids. It was observed that the foetus was capable of removing from its tissues chlorinated lipids which were introduced through placental transfer (Cunningham & Lawrence, 1977c). The present work shows that this removal was facilitated by dechlorination.

Very little orally administered chlorinated linoleic or linolenic acid is deposited in the tissues of rats, as compared with dichlorostearic acid (Cunningham & Lawrence, 1977a). The present studies with heart muscle suggest that this may be because they are dechlorinated more rapidly. LD_{50} studies show that chlorinated linoleic and linolenic acids are considerably more toxic than dichlorostearic acid (Cunningham & Lawrence, unpublished data). The extraordinary ability of heart muscle to metabolize chlorinated linoleic acid more rapidly than [^{3}H]oleic acid was unexpected.

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EXCRETION AND METABOLISM OF 2,4,5,2',5'-PENTACHLOROBIPHENYL IN THE SQUIRREL MONKEY

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Summary—Earlier investigations have shown that 2,4,5,2',5'-pentachlorobiphenyl is excreted in mouse faeces mainly as a hydroxylated derivative. To confirm the relevance to other species of these investigations in mice. the excretion and metabolism of 2.4.5.2'.5'-pentachlorobiphenyl by two squirrel monkeys have been studied. One monkey was dosed orally and the other iv with ^{14}C-labelled pentachlorobiphenyl and the faeces were collected daily for 21 days. The faecal excretion of radioactivity by the monkey was initially more rapid than that observed in the mouse and the faeces of the monkey contained two hydroxylated metabclites instead of the one formed in the mouse.

Introduction

It has been demonstrated that 2,4.5,2',5'-pentachlorobiphenyl is excreted by the mouse mainly in the faeces as a hydroxylated derivative (Berlin, Gage & Holm, 1975). Hydroxylation of polychlorinated biphenyls (PCBs) has also been observed in the rabbit (Gardner, Chen, Roach & Ragelis, 1975) and in the rat (Yamamoto & Yoshimura, 1973). The only published study on primates has demonstrated mono- and dihydroxylation of di- and trichlorobiphenyls (Greb, Klein, Coulston, Golberg & Korte, 1973). In order to extend and confirm the relevance to other species of our earlier investigations with mice, the excretion and metabolism of 2,4,5,2',5'-pentachlorobiphenyl, a PCB that has been identified in human fat (Jensen & Sundström, 1974), have been studied in two squirrel monkeys.

Experimental

The preparation administered was a mixture of ^{14}C-labelled 2,4,5,2',5'-pentachlorobiphenyl (from Mallinckrodt Chemicals) and the unlabelled compound (kindly supplied by Docent C. A. Wachtmeister of the Wallenberg Laboratory, Stockholm), the mixture being dispersed in the lipid phase of an aqueous emulsion (Berlin *et al.* 1975). A dose of 108 mg (47·7 μCi) was given by stomach tube to a male squirrel monkey (A), weighing 700 g, and an iv dose of 66·6 mg (18·1 μCi) was administered to a female (B), weighing 625 g. The monkeys were placed in metabolism cages with a wire-mesh grid to separate faeces, and a plastics funnel to collect urine. Urine and faeces were collected daily for 21 days and the daily excretion of radioactivity was recorded. After 21 days the monkeys were killed and samples of all the major tissues were taken for radioactivity determinations.

For the metabolism studies, faeces were dried, powdered, acidified with phosphoric acid (1 ml 23·3 mM-H_3PO_4 diluted with acetone/10 g faeces), dried again and extracted with CH_2Cl_2 in a Soxhlet apparatus for 8 hr. The CH_2Cl_2 fraction (F1) was extracted with 1 M-NaOH, the remaining CH_2Cl_2 fraction being designated F2 and the aqueous fraction being acidified to pH 2 with HCl and extracted with hexane to give a hexane fraction, F3, and an aqueous residue.

The methods for thin-layer and gas–liquid chromatography (TLC and GLC), the measurement of radioactivity, the purification of fraction F3 by column chromatography and for mass spectrometry (MS) were described previously (Berlin *et al.* 1975).

Results

Excretion

Figure 1 shows the amount of radioactivity excreted in the faeces of monkeys A and B over the 21-day period compared with that from the mouse.

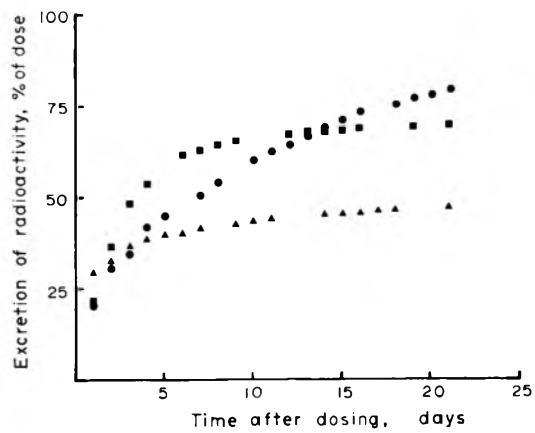

Fig. 1. Excretion of radioactivity in faeces of monkey A (▲), monkey B (■) and mouse (●) expressed as percentage of dose administered.

Table 1. *Excretion of radioactivity in faeces, expressed as percentage of total faecal excretion*

	Monkey A			Monkey B		
Sample Day...	1	2–9	1–9	1	2–9	1–9
Faeces	58·2	27·8	86·0	30·1	62·6	92·7
Fraction 1	52·8	25·7	78·5	18·8	35·4	54·2
Fraction 2	48·3	6·3	54·6	8·6	6·5	15·1
Fraction 3	—	17·2	17·2	10·6	32·0	42·6

The amount of radioactivity in faeces is expressed as the cumulative percentage of the dose administered. The recovery of the dose was incomplete, amounting to 70% in monkey B and 48% in monkey A, although at 21 days the rate of excretion was very slow. The total amount in urine over this period was 4·4% of the dose for monkey A and less than 0·3% for monkey B. Tissue analyses after 21 days showed radioactivity in all samples, with the highest amounts in the gall bladder, hair and body fat, but this amounted in all to less than 1% of the dose administered. After washing the hair with hexane, about 10% of the radioactivity was found in the hexane solution.

Metabolism

For the metabolism experiments the faecal output on day 1 and the bulked faeces collected on days 2–9 were investigated separately for each monkey. The amounts of radioactivity in fractions F1, F2 and F3, expressed as percentages of the total excreted in the faeces in 21 days, are shown in Table 1.

Monkey A. Thin-layer chromatography of the F2 fractions from day 1 and day 2–9 faeces showed a spot with R_F 0·6. A peak identical with that of the unchanged PCB was obtained by GLC; this was confirmed by MS on the day 2–9 sample. Fraction F3, when purified by silica-gel column chromatography, gave a double band on TLC with R_F 0·3–0·35. The fraction was further purified by treatment with fuming H_2SO_4 and silylated with *N,O*-bis(trimethylsilyl)acetamide. Combined GLC–MS gave two major peaks, with a characteristic Cl_5 cluster at *m/e* 412–420, corresponding to the molecular formula $C_{15}H_{13}OSiCl_5$, confirming the presence of phenolic derivatives in the untreated fraction.

Monkey B. The CH_2Cl_2 extract (F1) from the day 1 and day 2–9 faeces gave no indication of the presence of unchanged PCB by TLC, but did show two spots with R_F values of 0·3 and 0·35. These spots were also seen on TLC of fractions F2 and F3. The presence of phenolic metabolites in F2 must be attributed to the incomplete extraction of F1 with NaOH.

Table 1 shows that the extraction of radioactivity from the faeces of monkey B in fraction F1 was low. After extraction, the day 1 faeces were boiled for 6 hr with 3 M-HCl and re-extracted with hexane; this gave a further 0·4%. The faeces were further boiled with 3 M-H_2SO_4 and yielded 3·4% radioactivity on hexane extraction. This extract when examined by TLC gave the phenol spots with R_F values of 0·3 and 0·35.

Discussion

Two phenolic metabolites, found in the faeces of both monkeys, were probably the 3'- and 4'-hydroxy derivatives; the former of these was found in mouse faeces as a metabolite of 2,4,5,2',5'-pentachlorobiphenyl (Sundström & Wachtmeister, 1975). Gardner *et al.* (1973) also found 3- and 4-hydroxylated metabolites in their study of the metabolism of 2,5,2',5'-tetrachlorobiphenyl by the rabbit.

At the end of the experimental period, the rate of excretion of radioactivity in the faeces was low, and the total residual radioactivity in tissues was a small fraction of the dose administered. The relatively high apparent excretion of radioactivity in urine by monkey A may be attributable to leaching from faeces in the metabolism cage, as this monkey had rather loose stools. The low recovery of the dose must partly be attributed to the difficulty of removing the faeces completely from the metabolism cage. An additional factor contributing to the low recovery was the obvious excretion via the skin, revealed by the amount of 2,4,5,2',5'-pentachlorobiphenyl extracted from the fur. Figure 1 shows that faecal excretion by the monkey was initially more rapid than that previously observed in the mouse (Berlin *et al.* 1975), taking the ratio of the cumulative excretions at days 5 and 9 as an index; over this period only a small amount of unchanged PCB was excreted.

The high proportion of unchanged PCB in the faeces of monkey A on day 1 must be attributed to incomplete absorption after oral administration, as none was excreted by monkey B after iv injection. This incomplete absorption by monkey A may have been due to an intestinal disturbance.

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TERATOGENIC EVALUATION OF PIPERONYL BUTOXIDE IN THE RAT

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Summary—Groups of pregnant albino rats were treated by gavage with doses of either 300 or 1000 mg piperonyl butoxide/kg. The compound was administered daily from day 6 to 15 of gestation. Controls received the corn oil vehicle alone. There was no reduction in body-weight gain in the test groups during the dosing period; maternal growth over the entire gestation period was slightly reduced but no dams died. Numbers of corpora lutea, implantations, resorptions and viable foetuses per female were not altered by exposure to piperonyl butoxide. Complete external examination, as well as evaluation of internal and skeletal structure, revealed no structural alterations that might be related to chemical treatment. It is concluded that piperonyl butoxide is not teratogenic in the rat at levels below those that are toxic to the dams.

Introduction

Piperonyl butoxide (α-[2-(2-butoxyethoxy)ethoxy]-4,5-methylenedioxy-2-propyltoluene) is widely used as an insecticide synergist in oil solutions, aerosols, dusts, wettable powders and slurries. The acute oral toxicity of the chemical is low, generally exceeding 4 g/kg in animals (Lehman, 1948; Negherbon, 1959; Sarles, Dove & Moore, 1949). Repeated feeding of up to 5000 ppm to the rat for 17 wk produced liver enlargement and periportal hepatic cell hypertrophy with a minimal amount of fatty change and renal tubular pigmentation (Lehman, 1952). Sarles *et al.* (1949) found no residual tissue changes in the rat 3 wk after a series of six weekly doses of from 530 to 4240 mg piperonyl butoxide/kg body weight.

In 2-yr feeding studies, Sarles & Vandergrift (1952) found no significant growth suppression in male rats fed 100 ppm or in female rats fed 1000 ppm although either 10,000 ppm or 25,000 ppm resulted in a significant growth-rate reduction in both sexes. The death rate increased at the two highest feeding levels. Histological changes were detected in the liver at feeding levels of 10,000 ppm or higher.

Reproduction parameters such as mating performance, incidence of pregnancy and incidence of parturition were decreased at a feeding level of 10,000 ppm and reproduction did not occur among animals fed 25,000 ppm (Sarles & Vandergrift, 1952). In that study, there was no mention of careful examination of the resulting progeny for possible congenital malformations. The experiment reported here was designed to evaluate the teratogenic potential of piperonyl butoxide in the albino rat.

Experimental

Materials. Piperonyl butoxide (technical grade) was obtained from McLaughlin Gormley King Company, Minneapolis, Minn.

Animals and diet. The animals used were COBS random-bred albino rats impregnated at the Charles River Breeding Laboratories, Wilmington, Mass. Day 0 of gestation is defined as the day of sperm-positive vaginal examination and all rats were shipped by air to these laboratories the following day. Each animal was housed individually in a hanging stainless-steel rodent cage and was maintained on a standard pelleted feed, obtained from Ralston-Purina, St. Louis, Mo., with water provided *ad lib.* via demand-operated valves.

Experimental design. Preliminary pilot studies were conducted to determine dose levels at which increased maternal deaths and pharmacotoxic signs were not encountered. For this, 30 female animals were divided into five groups containing animals of approximately equal size and were treated with either 0, 100, 300, 1000 or 3000 mg piperonyl butoxide/kg body weight. The test material was dissolved in corn oil at concentrations that allowed the appropriate dose to be given in a standard volume of 1 ml/kg body weight. All doses were given by oral intubation for the treatment period, which lasted from day 6 to 15 of gestation. Control animals received the corn oil vehicle alone.

Each animal was weighed daily to allow accurate dosing and growth was monitored by recording these weights on days 6, 9, 12, 16 and 20 of gestation. Daily observations for pharmacotoxic responses were made during the study. On day 20 of gestation, animals were killed by chloroform asphyxiation. The uterine horns were examined for implantation sites, and the numbers of resorption sites, viable foetuses and corpora lutea in the ovaries were counted. Each foetus was given a complete external examination and was weighed after removal of excess amniotic fluid.

For the main study, female animals were divided into groups of 20 and were treated as above at dose levels of either 0, 300 or 1000 mg/kg. The method was identical to that of the pilot study with the addition of evaluation of skeletal development (Hurley, 1965) and of internal development (Wilson & War-

Table 1. *Reproduction and teratological data for rats—pilot study*

Dose (mg/kg/day)	No. of pregnant females examined	Maternal body-weight gain (g) for gestation days 6–15	No. of				Mean foetal body weight (g)	
			Corpora lutea†	Implantation sites†	Resorption sites	Foetuses†	Males	Females
0	5	75	63 (12·6)	48 (9·6)	2 (0·4)	46 (9·2)	3·8	3·9
100	5	74	63 (12·6)	61 (12·2)	3 (0·6)	58 (11·6)	4·3	4·0
300	6	73	76 (12·7)	67 (11·1)	7 (1·1)	60 (10·0)	4·3	3·0
1000	6	65	68 (11·3)	53 (8·8)	8 (1·3)‡	45 (7·5)	3·8	3·7
3000	2	39*	14 (7·0)	14 (7·0)	13 (6·5)	1 (0·5)	2·0	—

†Figures in parentheses refer to mean/female.
‡This figure resulted from the resorption of six of ten implantations in one female.
Figures marked with an asterisk differ significantly from the corresponding control values: $*P < 0·05$.

Table 2. *Reproduction and teratological data for rats given piperonyl butoxide*

Dose (mg/kg/day)	No. of pregnant females	Mean body-weight gain of dams (g) for gestation days		No. of					Foetal body weight (g)	
		6–15	6–20	Corpora lutea†	Implantation sites†	Resorption sites†	Foetuses	Abnormal foetuses†	Males	Females
0§	19	67	151	249 (13·1)	198 (10·4)	3 (0·2)	195 (10·2)	0 (0)	4·0	3·9
300	20	67	136*	234 (11·7)	191 (9·5)	12 (0·6)	179 (8·9)	2 (1·1)	4·0	3·9
1000	19	67	128*	243 (12·8)	175 (9·2)	14 (0·7)	161 (8·5)	2 (1·2)	4·0	3·9

†Figures in parentheses refer to mean/female.
‡Figures in parentheses refer to percentage of total foetuses.
§Mean and range values for ten similar control groups with $n = 20$ or more, evaluated during the same time period as this group: corpora lutea, 11·1 (10·0–12·8); implantation sites, 9·6 (8·9–10·8); resorption sites, 0·5 (0·3–0·7); foetuses, 9·2 (8·4–10·0) and abnormal foetuses, 1·8% (0·0–8·2%).
Figures marked with an asterisk differ significantly from the corresponding control values: $*P < 0·05$.

kany, 1966). Where possible, equal numbers of foetuses from each litter were selected for evaluation of either skeletal or internal development.

Differences in mean values were analysed by non-parametric methods (Lichfield & Wilcoxon, 1949).

Results and Discussion

In the pilot study, animals treated with up to 1000 mg/kg piperonyl butoxide showed normal weight gains during gestation and were free of gross signs of a toxic response to the chemical. The numbers of corpora lutea, implantation sites, resorption sites and viable foetuses and the external condition of the foetuses were unaltered by *in utero* exposure to the chemical (Table 1). One foetus, structurally normal but reduced in size, was recovered from a female in the 300-mg/kg group. The resorption sites in the 1000-mg/kg group were mainly in one female, which resorbed six of ten implantations. Among animals given 3000 mg/kg, a sharp reduction in body-weight gain occurred, two of six animals died during the dosing period (gross pathological changes were not apparent nor were there positive signs of pregnancy in these two animals), and the incidence of foetal resorption was markedly increased. A single viable foetus weighing 2.0 g was obtained from the two pregnant animals examined at this level. Thus dose levels of 300 and 1000 mg/kg were selected for the main study.

In the main study, no abnormal behaviour was observed among pregnant rats receiving either 300 or 1000 mg/kg, although body-weight gains during the entire gestation period were somewhat reduced (Table 2). Body weights of the test animals during the actual dosing period (days 6–15 of gestation) compared favourably with those of the controls.

The numbers of corpora lutea, implantation sites, resorption sites and foetuses, the foetal body weights and numbers of abnormal foetuses observed upon external examination are given in Table 2. None of these parameters showed statistically significant differences between control and test groups. The number of resorption sites/female in the two test groups reflects the resorption of a large part of her litter (eight of eleven foetuses) by one female in the 300 mg/kg group, and the resorption of an entire litter (seven implantations) by one in the 1000-mg/kg group. Body weights of foetuses from the test groups compared favourably with those of the controls.

All foetuses derived from females of the control group appeared normal upon external examination. In the 300-mg/kg group, two of 179 foetuses were abnormal; one was a runt and one displayed a haematoma. Two of 161 foetuses from the females treated with 1000 mg/kg were abnormal; one had an umbilical hernia and the other was a runt: These abnormalities are observed spontaneously in untreated (control) rats of this strain and the incidence and distribution within the two test groups does not suggest a treatment relationship.

The skeletal development of foetuses from all groups was similar. In the test groups, incidental findings such as incomplete ossification of the sternum and supernumerary ribs were observed with the same relative frequency as in the control group. Of the foetuses selected for skeletal examination, angulated ribs were seen in four (3·9%) control foetuses, three (3·2%) from the 300-mg/kg group and two (2·4%) from the 1000-mg/kg group. A common skeletal deviation, irregular ossification of the parietal bones, was seen in two of the foetuses examined in each test group (2·1 and 2·4% of those examined in the 300- and 1000-mg/kg groups, respectively).

Internal examination of the foetuses revealed no treatment-related deviations. Renal caudal ectopia was observed in three (3·2%), two (2·4%) and three (3·9%) of the foetuses examined from the control, 300- and 1000-mg/kg groups, respectively. A single instance of bilateral undescended testis was detected in the control group. Considerable variations in the size of the atrium, estimated visually, were seen in 14 (15·0%) of the control foetuses, 12 (14·7%) of the foetuses from the 300-mg/kg group and 20 (26·0%) of those from the 1000-mg/kg group. Changes in the size of the atrium reflect naturally occurring variations commonly observed in the strain of rat used and are not considered to reflect a response to the test chemical.

It is concluded that no teratogenic response is produced in rats following *in utero* exposure to the maximum doses of piperonyl butoxide which were not toxic to the dams.

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Review Section

BOOK REVIEWS

Residue Reviews: Residues of Pesticides and Other Contaminants in the Total Environment. Vol. 58. Edited by F. A. Gunther. Springer-Verlag, New York, 1975. pp. vi + 160. DM 45.90.

Residue Reviews: Residues of Pesticides and Other Contaminants in the Total Environment. Vol. 59. Edited by F. A. Gunther. Springer-Verlag, New York, 1975. pp. viii + 145. DM 41.00.

These two volumes of a well-known series are concerned almost exclusively with pesticides. Two thirds of the first is taken up with an extensive review of the metabolic fate of herbicides in experimental and farm animals, compiled from data published in the 1962–1973 period and derived primarily from *Chemical Abstracts*. The herbicides are divided into 16 groups on the basis of chemical structure, and in many cases tables are used to summarize available information on the possible or actual structure of metabolites in various animals, on the body fluids, tissues or excreta from which these metabolites have been isolated and on the analytical methods used in their identification. The tabulation of complex data is a practice beset with pitfalls, but the author of this review has wisely limited the scope of his tables, leaving the reader to derive from the text quantitative details on absorption, conversion and elimination as well as the critical evaluation of evidence supporting postulated structures of metabolites. Overall this approach seems to be very successful, and the review presents a considerable amount of information, as well as a large number of useful references.

The other review in this volume deals with the more specific but nevertheless important subject of factors affecting the metabolic fate, and thus the terminal residues, of insecticides applied to stored cereal grains. This paper, the third part of a series begun in "Residue Reviews" in 1967, surveys literature published in 1970–1974, a period of widespread change in the methods used for handling grain in bulk, and in the attitudes of governments, of the food industry, and indeed of the pesticide manufacturers themselves, to the control of pest infestation of grain stores.

Volume 59 contains several shorter contributions—two on analytical methods, one on the atmospheric dispersal of DDT compounds, and two of more direct toxicological interest. One of these relates the residue levels resulting from the control of woody and herbaceous weeds with 2,4,5-T to the toxicity of this compound and its derivatives in birds, and concludes that birds in areas treated with recommended doses of this herbicide should not be adversely affected at any stage of life. Finally an interesting review of "the more influential works" (100 in all) on the mechanisms by which DDT can cause reproductive failure in mammals and birds discusses both the effects of DDT compounds on gestation and egg-shell thinning, leading to death before birth or hatching, and the toxicity that directly affects the young as a result of transmission of the pesticide from the adult during lactation or via the egg.

Residue Reviews: Residues of Pesticides and Other Contaminants in the Total Environment. Vol. 60. Edited by F. A. Gunther. Springer-Verlag, New York, 1976. pp. ix + 160. DM 45.90.

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

The first volume of this pair follows standard practice for the series in carrying cumulative author and subject indexes for volumes 51–60 and an alphabetical list of subjects covered in these volumes. Also provided is a list of all papers included in volumes 1–60, identified by their short titles and year of publication.

The bulk of volume 60, some 100 pages, is concerned with the contact insecticide *O,O*-dimethyl *O*-(3-methyl-4-nitrophenyl) phosphorothioate, widely known as fenitrothion and marketed as Sumithion by Sumitomo Chemical Co. Ltd. of Japan. This company synthesized the compound in 1959 and members of its staff produced this survey, which compares the various methods available for the analysis of Sumithion in technical products and formulations and describes analytical procedures for residues on various crops, the properties and uses of commercial formulations and the biological properties of the insecticide.

This last topic occupies by far the longest chapter and presents, in considerable detail, data on the effectiveness of Sumithion in the fields of plant protection, grain and timber storage and public health. Less than a page, however, is spared for its possible adverse effects on beneficial insects and predators and its alleged phytotoxicity, notably to cruciferious vegetables and some varieties of apple. Furthermore, references to mammalian toxicity seem to be limited to LD_{50} values in mice and rats, tabulated for comparison with LD_{50} values in various insects, and to descriptions of Sumithion as "a safe and broad-spectrum insecticide" and as a compound "of low mammalian toxicity". Elsewhere there is a rather vague presumption about the presence of "not so high" levels of possible toxic metabolites. Further support for these statements would have been useful and

would have made this extensive survey more complete.

Inevitably, a contribution of this kind emanating from a clearly interested company runs the risk of being viewed to some extent as a promotional exercise. Nevertheless much of the information it presents would probably not be available to other authors and the potential value of this insecticide is underlined by WHO's interest in it as a possible agent for malaria control.

DDT, the mainstay of past achievements in the control of the malarial mosquito, is the chief subject of volume 61. Of particular relevance for a compound that has fallen from grace because of its extreme stability and resulting persistence and accumulation in the environment are two chapters dealing in turn with its degradation by micro-organisms and with its stability in foods and feeds and its degradation or removal from them during cooking and processing. The metabolism of DDT by micro-organisms, initially under anaerobic conditions, is seen as a major factor in its environmental degradation, although there is no evidence that microbes utilize DDT for growth. The third DDT chapter is a lengthy discussion on routes of environmental accumulation and on the widespread controversy and repercussions that have stemmed from the concern engendered by this particular example of bioconcentration. The author admits to raising questions as much as providing answers, but he also provides an enormous amount of data on DDT residues in soils, air, rainwater and surface waters, in plants and in animal tissues, including those of man.

The final chapters of this volume describe bioassay techniques for foliar herbicides (amitrole, arsonates, dalapon, diquat and paraquat, 2,4-D and picloram) and report on the metabolism of gossypol, the yellow pigment of cottonseed, in a variety of animal species and on the physiological and nutritional effects of this possible feed contaminant. The latter chapter presents another extensive view of relevant literature in the manner for which this series is well known.

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

In vitro studies have always played an important role in biochemical research but until a few years ago the cell homogenate, either as a whole or fractionated, was the principal means for studying the characteristics and function of the various cell components. No one would deny the enormous advances in cell biology that these methods have facilitated, but the results have always been open to the objection that they may not reflect adequately the situation *in vivo*. In the past some answer to this objection was attempted with the use of tissue slices, but these had the disadvantage of deteriorating rapidly, leaving again doubts about the relevance of the information so obtained. Recently, however, the development of techniques for keeping mammalian cells alive for prolonged periods in culture has opened yet another avenue for the biochemist.

Although separated from the rest of the organism, the cells are capable of independent existence and have been seen as an important link between tissue homogenates and the intact animal. The use of this type of cell culture is now being explored and the experiences and results of many prominent workers were reported at the FEBS Advanced Course No. 38 on the use of isolated liver cells and kidney tubules in metabolic studies, held in Luzarches, France, in July 1975, and at a symposium held in Paris at about the same time. The communications presented at these meeting have been combined to form the text of the book named above.

Significantly, most of the papers dealt with interrelationships and control mechanisms, which cannot be studied adequately using homogenates. Inter-relationships between mitochondria and cytosol in cell metabolism, and the regulation of glucose and glycogen metabolism and of ureogenesis comprised three major topics. These problems were formerly studied *in vivo*, but the influence of hormones and other homoeostatic factors in the intact organism did not permit clear interpretation of results. While the reports on the use of isolated cells in metabolic studies centred on endogenous compounds, the techniques described could form the basis for the study of xenobiotic compounds as well.

Although experience in maintaining liver and kidney cells in culture has been accumulating for several years, more background information is needed to enable biochemists to make good use of these cultures. Part of this need is met by a section concerned with the properties of hepatocytes when freshly isolated or maintained in culture.

Both biochemists and cell biologists should find a mine of useful information in this volume.

Progress in Liver Diseases. Vol. V. Edited by H Popper and F. Schaffner. Grune & Stratton, New York, 1976. pp. xvi + 733. £27.85.

The liver is a popular organ, attracting the attention of large numbers of biochemists and experimental pathologists. In fact, some cynics have pointed out that there are very few scientists in these fields who have not done some work with the rat or mouse liver at some stage of their careers. Much of the work of toxicologists and clinicians also centres on this organ, presumably because of the frequent occurrence of liver lesions in naturally occurring diseases and in poisoning by chemicals. Some 20 years ago, a survey of toxicity tests carried out in rats revealed that liver lesions were found in 25% of the studies reviewed.

The importance of the liver to all these groups of workers is well reflected in the series entitled "Progress in Liver Diseases" edited by two doyens of hepatology, Hans Popper and Fenton Schaffner. The first four volumes covered the field in an admirable way up to 1972. Volume V covers the three years from 1973 to 1975.

Most of the articles in this volume review progress made in the basic sciences. There are articles on the use of stereology in the study of biopsy specimens

of human liver and on scanning electron microscopy of the liver, a new technique which has produced pretty three-dimensional pictures but does not seem, so far, to be fulfilling the expectations of its enthusiastic protagonists. On the other hand, the well-established field of transmission electron microscopy is yielding a valuable harvest. The endoplasmic reticulum has been studied in great detail by means of cytochemical techniques, and the morphological patterns observed have been compared with conceptual models built over the years as a result of intensive biochemical and physico-chemical investigations. On a more practical plane, an effort has been made to relate to malfunction the changes in organelle structure that can be demonstrated by electron microscopy. Most of the data are derived from human disease, but a lot of information is also presented from animal experiments, particularly those concerned with chemically-induced damage. In many ways, this complements the human data, and in some instances it provides an insight into the meaning of the changes observed in man.

Two other chapters of particular interest to the toxicologist may be identified. One of these provides an excellent account of the methods available for isolating hepatocytes from liver tissue and culturing them. Emphasis is laid on methods for the identification of liver cells in culture, since they are often found to be mixed with fibroblasts. This is an extremely useful discussion, which deserves the attention of all who are engaged in this field of research. The second of these chapters is concerned with the significance of covalent binding in the production of tissue damage. This concept is not new, but the authors attempt to distinguish between the type of covalent binding that is apparently protective or at most harmless and the type that results in tissue damage. Their approach is that of biochemical pharmacologists but the conclusions they draw are clearly of importance to toxicologists.

The rest of the volume is devoted to relevant advances in clinical medicine. With the exception of one topic, the hepatitis B virus, little advance seems to have been made in most of the areas considered and the chapters have a familiar ring, as if one has already met the same views in earlier volumes in this series. Much the same comment can be made about the final chapter, on hepatocarcinogenesis by chemicals. Despite this, however, the book is a very useful addition to the series and seems likely to suffer the same fate as its predecessors. According to Dr. Franz J. Ingelfinger, who introduces the latest volume, the local medical library's copies of the earlier volumes in the series had their bindings broken, their pages dog-eared "and—the ultimate testament of value—many a page had been torn out".

Lysosomes in Biology and Pathology. Vol. 4. Edited by J. T. Dingle and R. T. Dean. North-Holland Publishing Company, Amsterdam, 1975. pp. xviii + 614, $74.95.

This volume continues the series which was started in 1969 with the simultaneous publication of volumes 1 and 2. Rather than up-dating the material that appeared in earlier volumes, the latest aims at covering new ground, especially that broken during the years 1972–1975.

The importance of the study of lysosomes in the fields of biology and pathology has been underlined by the 1975 award of a Nobel Prize to Christian de Duve, who pioneered the work and framed most of the concepts. This volume is dedicated to him.

The book is split into five parts, each of which has at least two contributors. Of the five chapters that make up part I, four describe lysosomes in animal tissues—skin, skeletal muscle, arterial wall and blood platelets—while the other describes the properties of lysosomes in the ciliate protozoan *Tetrahymena pyriformis*. All the 'tissues' pose difficulties, either in the isolation or in the *in situ* cytochemical staining of lysosomes.

A short part II, "Lysosomes in Pathology", comprises chapter 6 on lysosomes and radiation injury and chapter 7 on lysosomal enzymes in the heart. Both of these chapters are almost entirely biochemical. In complete contrast is chapter 8, which sets out to describe the historical, technological and methodological steps that led to the biochemical and cytochemical localization of tissue proteinases. Completing part III and stemming from the work of R. E. Smith described in chapter 8, are three short chapters, two dealing with the distribution of proteases and the catabolism of glycoproteins, and a third giving a brief account of the kinetics of intracellular protein turnover.

For nearly 20 years there has been argument over the question of whether all lysosomes in all tissues contain a full complement of all the hydrolases that have been discovered, or whether some lysosomes contain proteases, for example, while others carry esterases. The argument is considered in part IV. Chapter 12 examines the evidence on both sides but comes to no definite decision, while chapter 13 approaches the question from a different angle, that of the 'multiple forms' of enzymes, defined as "all proteins possessing the same enzyme activity and occurring naturally in the same species".

The fifth part of the volume deals with control mechanisms. In chapter 14, Clara Szego seems to have aimed at a *magnum opus* rather than a review. Running to 90 pages it is full of information, but also abounds with florid and pretentious phrases. Perhaps the author's mother tongue is not English: in any event she would do well to examine the writing style of Christian de Duve in volume 1, chapter 1. Chapter 15, half the length, ploughs a narrower furrow, that of the control of enzyme release from neutrophil leucocytes.

Much more esoteric, but none the less fascinating, is the final chapter, by Yolande Heslop-Harrison of the Royal Botanic Gardens, Kew. It deals with the carnivorous plants, of which there are over 360 species worldwide, and describes the many mechanisms they have evolved to allow release of hydrolytic enzymes onto their prey and to enable absorption of the digested material. It is refreshing to escape from animal limitations and consider the problems that sessile plants have managed to overcome in capturing fast-moving prey.

As we have come to expect from North-Holland/

Elsevier, the production of the book is beyond reproach; in its typography, layout, monochrome and colour reproduction and copious contents and index sections there are few flaws. The price is high, but not excessive in comparison with modern specialist journals. It is good news that volume 5 in this series on a rapidly expanding science was already in preparation when its predecessor appeared.

Lysosomes: A Survey. Cell Biology Monographs. Vol. 3. By E. Holtzman. Springer-Verlag, New York, 1976. pp. xi + 298. $53.30.

The appearance of a collection of volumes entitled "Lysosomes in Biology and Pathology" in the late 1960s and early 1970s marked a time of review and reflection on the first 15 years of research into this interesting organelle. It is not surprising, therefore, that this massive work should quickly be followed by a more concise volume, and Professor Holtzman has produced a book that more than adequately covers a wide and diverse area of research in a volume of reasonable size. Inevitably the research interests of the author influence the content of the work, the difficulties that arise in morphological classification being handled with great skill. Nevertheless the biochemical aspects of the subject have not been neglected and a successful union of the two branches of investigation has been achieved.

Although it is not always possible to distinguish clearly between normal and pathological functions, the first half of the book deals with the processes that are generally considered physiological rather than pathological. The book is at its best here, as pathological phenomena in which the lysosome plays an important role are less clear cut and the latter half of the text is therefore more prone to conjecture.

As in other monographs in this series, the chapters are well arranged and supplied with numerous subheadings. In general, the text must be criticized for the use of unnecessary jargon, so that material becomes "internalized" and controversies tend to be "ongoing", and also for some lack of clarity, some sentences being so unintelligible that the author has found it necessary to add a second explanatory one. In spite of these problems, the book is full of well-organized and useful information and should prove stimulating reading for those interested in lysosomes.

Biology of Cancer. Edited by E. J. Ambrose and F. J. C. Roe. 2nd Ed. Ellis Horwood Ltd., Chichester, 1975. pp. vii + 315. £15.00.

Many books on cancer attempt to encompass the entire subject and suffer in consequence from the need to over-simplify in order to cover all aspects of the problem. This book on the other hand is a collection of papers dealing in greater depth with topics selected to give insight into current research. It shows how varying approaches reveal different aspects of neoplastic change, all of them being important for a general understanding of the subject.

Since the first edition of this book appeared in 1966 (*Cited in F.C.T.* 1967, **5**, 221), considerable strides have been made towards an understanding of the mechanisms involved in neoplastic processes, and the speed of developments in this field is reflected in the considerable differences between the two editions. Four new chapters have been added to the current volume, adding further well-known names to those already established on the contents page, and a quick glance at the dates of the references listed at the end of each contribution clearly indicates the extensive revision to which the original chapters have been subjected.

A brief introduction dealing in general terms with the occurrence of cancer in human populations is followed by a consideration of chromosomal changes and the heredity of cancer. In the next two chapters the role of the cell surface in tumour development and the mechanism of invasion are discussed at length. A new chapter describing metastatic spread is followed by accounts of how cancer cells differ biochemically from normal cells and of how their function is distorted (the latter chapter being another new addition). The principles of epidemiology are then explained in a third new contribution, and three chapters on the various causative agents and their interaction with the target cells follow. The book concludes with revised contributions on antigenic changes and cancer chemotherapy. The arrangement of the chapters might have been improved by discussing the causative agents at the beginning of the book, thus avoiding the separation of the description of chromosomal changes from that of changes in DNA.

This book should prove particularly useful to those embarking on cancer research with some prior general knowledge of the subject; those without such knowledge may find certain passages difficult to comprehend, since there is no description of how cancer cells are recognized and classified.

Persons at High Risk of Cancer. An Approach to Cancer Etiology and Control. Edited by J. F. Fraumeni, Jr. Academic Press Inc., New York, 1975. pp. xvii + 544. $19.00.

Cancer is now second to heart disease as the major cause of death in the Western world. Although cancer research has attracted a major share of funds for medical research (the National Cancer Institute Budget for 1975 was $669 million), the lack of success is shown by the nearly static survival figures for common cancers. Consequently, there has been increasing recognition of the possibility that progress might be accelerated if emphasis were placed more on identifying the people susceptible to cancer than on relying on treatment once cancers had arisen. The identification of high-risk groups could lead to significant developments in the field of prevention and to the detection of cancer at an early stage, when cure is more likely.

This book contains the proceedings of a conference held by the National Cancer Institute and the American Cancer Society in December 1974. The conference considered the present state of knowledge on high-risk groups, and explored ways of applying such information to the ultimate reduction of cancer mortality.

The text is divided into six sections. The two major ones deal with the factors, either host or environmental, that are thought to increase cancer incidence. Family history, genetic or immune disease, radiation, occupation, diet, viruses and sexual factors comprise some of the topics covered in separate chapters. The third section looks at geographical and racial features that influence the distribution of risk factors, and section four illustrates the monitoring of high-risk groups and the application of preventive measures. A fifth section discusses the identification and possible interaction of various risk factors. Each section has a summary chapter and the book ends with a section on future prospects in cancer aetiology and control.

The standard of the presentations is generally very high. They are extensively referenced and provide a wealth of up-to-date information. It is manifestly apparent, however, that the issues involved are extremely complex ones, and an interdisciplinary approach is essential if progress in cancer control is to be made. Although suggestions for future work abound, we have, unfortunately, only just set our foot on the alternative path that cancer research is advised to travel.

BOOKS RECEIVED FOR REVIEW

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Residue Reviews: Residues of Pesticides and Other Contaminants in the Total Environment. Vol. 62. Edited by F. A. Gunther. Springer-Verlag, New York, 1976. pp. viii + 181. DM 41.00.

Progress in Liver Diseases. Vol. V. Edited by H. Popper and F. Schaffner. Grune & Stratton, New York, 1976. pp. xvi + 733. £27.85.

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

Pesticide Residues in Food. Report of the 1975 Joint Meeting of the FAO Working Party of Experts on Pesticide Residues and the WHO Expert Committee on Pesticide Residue, Geneva, 24 November–3 December 1975. Tech. Rep. Ser. Wld Hlth Org. 1976, 592. pp. 45. Sw. fr. 6.00 (available in the UK through HMSO).

A Dictionary of Life Sciences. Edited by E. A. Martin. Macmillan Press Ltd., London, 1976. pp. 374. £5.95.

Immunopharmacology. Edited by M. E. Rosenthale and H. C. Mansmann, Jr. John Wiley & Sons Ltd., Chichester, 1976. pp. vii + 332. £16.45.

Perinatal Pharmacology and Therapeutics. Edited by B. L. Mirkin. Academic Press Inc., (London) Ltd., 1976. pp. x + 455. £13.75.

Fd Cosmet. Toxicol. Vol. 15, pp. 347–356. Pergamon Press 1977. Printed in Great Britain

Information Section

ARTICLES OF GENERAL INTEREST

UNRAVELLING THE GLUTAMATE TANGLE

A flood of apparently conflicting reports leaves much to be established about the toxic effects of monosodium glutamate (MSG). A particular point of dispute has been a series of studies in neonatal animals, the results of which could be interpreted as demonstrating that infants and children frequently consume in animal and vegetable proteins quantities of aspartate and glutamate very close to the levels claimed to be toxic to the neonates (Olney & Ho, *Nature, Lond.* 1970, **227**, 609; Bigwood, *Fd Cosmet. Toxicol.* 1975, **13**, 300). This criticism has been countered (Olney, *ibid* 1975, **13**, 595) by the argument that there is a difference between ingesting amino acids bound to protein and ingesting free amino acids as food additives, since in the former instance absorption is inevitably more gradual and detoxication more effective.

Brain damage is probably the most frequently reported toxic effect of MSG. Studies of the apparently variable effect of the blood–brain barrier in protecting animals against circulating glutamate have been described (*Cited in F.C.T.* 1971, **9**, 717). More recent work on changes in the permeability of cerebral tissue to trypan blue during convulsions induced in immature rats has thrown further light on this factor (Nemeroff & Crisley, *Envir. Physiol.* 1975, **5**, 389). Trypan blue injected intra-arterially into rats younger than 10 days appeared in cerebral tissue when given simultaneously with an ip injection of 4 g MSG/kg, which induced seizures, but the dye failed to penetrate when injected either before or after the MSG-induced seizures. When the experiment was repeated using rats older than 42 days, MSG injection did not induce any substantial seizure activity, and trypan blue did not penetrate into the brain. These findings suggest that MSG-induced seizures are associated with a rapidly reversible alteration in the permeability of the cerebrovascular system to plasma proteins able to bind with the dye, and are completely dependent upon the age of the animal. Characteristic lesions of the arcuate nucleus were found only in animals up to the age of 10 days and thus were not correlated with seizure activity, which continued up to 42 days of age.

Olney *et al.* (*New Engl. J. Med.* 1973, **289**, 1374) found that guinea-pigs were susceptible to brain damage by 1 g MSG/kg injected sc. Guinea-pigs, like primates, have a well-developed nervous system at birth, and earlier work by the same group demonstrated brain lesions in infant monkeys (*Cited in F.C.T.* 1970, **8**, 590). In this species, large brain lesions were induced by high doses of MSG given sc and giving rise to blood-glutamate levels of 70–100 mg/100 ml, while relatively small lesions resulted from the oral ingestion of doses producing blood-glutamate levels of 20 mg/100 ml (Olney *et al. J. Neuropath. exp. Neurol.* 1972, **31**, 464). Lemkey-Johnston & Reynolds (*ibid* 1974, **33**, 74) gave doses of 4 g MSG/kg to neonatal mice by gastric intubation and found that oedema of astrocytic glia in the arcuate nucleus was detectable by electron microscopic examination after 15 minutes, while after 20 minutes lesions were also apparent by light microscopy. After 30 minutes the damaged area was filled with large dilated processes and some neurons appeared swollen, with vacuolated cytoplasm. Phagocytosis began 3–6 hours after administration of the dose, with increased numbers of pyknotic nuclei and the appearance of peripheral basophilic cells. After 24 hours the lesions appeared to be filled with phagocytes and nuclei in advanced stages of pyknosis. Ultrastructurally, astrocytic processes were shown to have proliferated and to contain numerous phagocytic inclusions. The early distribution of lesions (15–60 minutes after dosage) involved the arcuate and/or preoptic region of the hypothalamus, while later the lesions spread over a much wider area. In general, the progression of the lesion suggested an inflow of the toxic agent from the cerebrospinal fluid. These workers also found lesions of the arcuate nucleus in adult mice given doses as low as 2 g MSG/kg. Although the lesions were less extensive in the adults, this indicates that susceptibility to MSG toxicity is not restricted to neonates.

It has been demonstrated that the rodent placenta, unlike that of the primate, is permeable to MSG (Reynolds *et al.* IX International Congress of Nutrition, 1972). Semprini *et al.* (*Biomedicine* 1974, **21**, 398) failed to detect any lesions in the arcuate region of the hypothalamus in newborn and 15- and 30-day-old mice and rats from dams fed 1 or 2% MSG (equivalent to 1·5–6 g/kg brain in the mouse) from the start of pregnancy until the end of lactation. The neurons showed a normal pattern, without evidence of pyknosis and fragmentation. There was no discrepancy in cell numbers in the tissue between treated animals and controls. Semprini *et al.* (*loc. cit.*) suggest that earlier observations (*Cited in F.C.T.* 1969, **7**, 682; *ibid* 1970, **8**, 225 & 590) on mice and monkeys may have been artefacts or that findings with injected MSG should not be considered relevant to the continuous ingestion of small amounts of the compound, a point that we have made in the past (*Food and Cosmetics Toxicology* 1970, **8**, 590). Unfortunately their own results gave no indication of the blood-MSG levels of the neonates. Abraham *et al.* (*Expl mol. Path.* 1975, **23**, 203) have added to the criticism by examining neonatal rhesus monkeys killed 3 or 24 hours or 8,

15 or 30 days after a single intragastric or sc dose of 0·25, 1 or 4 g MSG/kg. They also examined monkeys given oral doses of 250 mg or 1 mg MSG/kg for as long as 30 days. These animals showed no hypothalamic lesions when the tissue was examined by light or electron microscopy. The arcuate nuclei and all the components of the median eminence were not significantly different from those of controls. In both control and treated monkeys there were a few damaged or necrotic neuronal cells and oligodendrocytes in the arcuate region of the hypothalamus.

Nevertheless, behavioural effects have been reported in rats fed 4 g MSG/kg daily for the first 10 days of life and suggest minor brain damage (Berry *et al. Devl Psychobiol.* 1974, **7**, 165). Rats given this MSG treatment were tested at 50 days of age in a swimming maze, where they were less able to learn the maze solution than littermates that had not received MSG. Test animals examined on day 10 of the course of MSG showed higher concentrations of aspartic and glutamic acids, taurine, urea and glutathione, but lower concentrations of tyrosine and histidine in brain, liver and blood, than did controls. Berry *et al.* (*loc. cit.*) suggest that these abnormalities may well have altered the structural development of the central nervous system and induced a lasting functional impairment of brain function at the time in the animals' development when enzyme and transport systems were immature.

A single injection of 4 g MSG/kg given to 5-day-old chicks killed 18% within 4–6 hours and a further 2% within 48 hours (Snapir *et al. Path. Eur.* 1973, **8**, 265). Hypothalamic damage, indicated by the development of a zone staining with thionine–Nissl stain after 2 hours and its persistence for the next 15 days, was located bilaterally near the midline, close to the third ventricle. During the first 6 hours, it extended dorsally to involve the subrotundus nuclei, laterally to involve the rotundus nuclei, and ventrally to the tractus opticus marginalis. There was a significant reduction in the number of neurons in the affected area, which was bordered by a zone of darkly stained condensed cells. Damage to the rotundus nuclei involved early neuronal swelling followed by a decrease in the number of nerve cells and, after 40 days, some vacuolation. Some birds given MSG also showed an abrupt diminution of nerve fibres and cells in the lateral forebrain bundles.

Injections of MSG in newborn mice have been found to cause female sterility (*Cited in F.C.T.* 1969, **7**, 682), although pregnant rats treated with MSG reproduced normally (*ibid* 1971, **9**, 718). More recently, other reproduction studies in mice, rats and hamsters have been reported. The survival of second-generation newborn mice was increased by feeding two successive generations on diets containing 1 or 2% MSG (1·3–6·2 g MSG/kg/day) together with a vitamin supplement at a level of 1 or 2% (Semprini *et al. Nutr. Metabol.* 1974, **16**, 276). The average weight of the treated animals at weaning was higher than that of controls, but no detectable alteration in central nervous system histology resulted from MSG feeding. Trentini *et al.* (*Fertil. Steril.* 1974, **25**, 478) treated rats with daily sc injections of MSG in doses that were increased gradually from 2·2 to 4·2 g/kg from day 2 to day 11. From the seventh injection, MSG induced brief seizures in 85% of the animals. Hair growth and eye opening were normal. In females, sexual development was premature but fertility was not impaired. Body weights and relative weights of pineal, pituitary, thyroid and adrenal glands were significantly reduced in treated females 105 days old, except in lactating animals. In the case of males, body weights and thyroid weights at 130 days were not significantly different from those of the controls, but pineal, pituitary, adrenal and testis weights were significantly decreased. No histological abnormalities of the endocrine organs or hypothalamus were observed in either sex.

Neonatal hamsters were given daily sc injections of 4 or 8 g MSG/kg on days 1–5, 6–10 or 1–10 of life, and were killed on day 60 (Lamperti & Blaha, *Biol. Reprod.* 1976, **14**, 362). Weights of the reproductive organs and of the adrenal and pituitary glands were significantly lower in animals treated on days 1–5 than in controls, and females on the higher dose had significantly fewer tubal ova. Lesions of the arcuate nucleus of the hypothalamus appeared only in hamsters given 8 g MSG/kg during days 6–10 or 1–10. Females of these groups had ovaries with small follicles and no corpora lutea; males had atrophic seminiferous tubules, and showed little $\Delta 5$-3β-steroid-dehydrogenase activity in their interstitial cells. Since treatment with chorionic gonadotrophin reversed the effect of MSG on testicular function and restored ovulation in hamsters, it seems evident that MSG affects that part of the hypothalamus that controls the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH).

Certainly most of the reports described above contain some sort of evidence of MSG toxicity. However, the critical influence of factors such as species variation, developmental age, route of administration, number of doses and the timing of brain-tissue examinations leaves us with a mass of inconclusive evidence. More feeding studies are still needed, but without some change of direction, the question of MSG toxicity will remain a recurrent controversy.

[P. Cooper—BIBRA]

ERUCIC ACID IN RAPESEED OIL

The lipids of many cruciferous seeds are known to contain variable amounts of the mono-unsaturated fatty acid, *cis*-13-docosenoic acid, commonly referred to as erucic acid (EA). In recent years, growing importance has been attached to the use of rapeseed as a valuable oilseed crop, but the oil contains relatively high quantities of EA and these have been held responsible for fatty deposits in heart muscle and associated myocardial lesions in a number of experimental animals (*Cited in F.C.T.* 1974, **12**, 255).

Although no research has as yet indicated similar effects on the human heart, several countries that

grow rapeseed on a large scale have considered it prudent to switch to the development and production of new rapeseed varieties containing low levels of the fatty acid. This move towards a reduction in the EA content of the oil has prompted a number of investigators to compare the effects induced by oils obtained from the conventional and from the novel cultivars of the rapeseed plant. A recent review describing several of these early studies (*ibid* 1975, **13,** 130) implied that EA was the sole cause of the adverse nutritional effects. Although one or two of the more recent studies substantiate this hypothesis, the majority support alternative suggestions for the effects.

Engfeldt & Brunius (*Acta med. scand.* 1975, **198,** Suppl. 585, p. 15) suggested that other long-chain fatty acids as well as EA might be responsible for the observed myocardial effects. In short-term studies in rats, they fed rapeseed oils in the diet at concentrations providing EA levels of 0·1–10·3% (w/v) for 10 days. The level found to cause pathological fatty accumulation was approximately 2%, whilst the myocardium was normal in rats fed 1%. When similar diets were fed for a longer period of 160 days, myocardial fatty accumulation was observed throughout the feeding period in rats fed conventional rapeseed oil at a level providing 10% EA in the diet, but the number of fat droplets decreased with time (*idem, ibid* 1975, **198,** Suppl. 585, p. 27). Focal lesions were widespread in the myocardium of these rats after 40 days and consisted of histiocytic infiltration, lipid-loaded macrophages, myolysis, proliferation of fibroblasts and scarring. Experimental groups given a 0·06%-EA diet containing rapeseed oil from a low-EA (0·3%) cultivar (Oro) did not exhibit histiocytic foci or scarring. Controls fed arachis oil exhibited no fatty accumulation, but small myocardial lesions were found occasionally and the investigators concluded that this form of lesion was a 'normal' finding. The number and size of the foci observed in animals fed the conventional oil indicated, however, that under these circumstances they had to be considered a pathological effect.

Beare-Rogers *et al.* (*Nutr. Metabol.* 1974, **17,** 213) argued that the incidence of lesions was related to the level of the test oil rather than to its EA content. In their studies, both high-EA (38·1%) and low-EA (2·9%) rapeseed oils gave rise to cardiac necrosis and fibrosis in rats. When oils from two of the low-EA varieties of rapeseed were fed for 16 weeks, a dose response in the incidence of lesions was demonstrated with dietary levels of Span oil (2·7% EA) between 5 and 20%, whereas with Oro oil an increased incidence of lesions occurred only at the top levels (15 and 20% oil). Although it was possible that background lesions observed in these experiments predisposed the myocardium to effects from even low levels of EA, these authors postulated that an alternative factor in the oil was contributing to the cardiomyopathy.

This elusive factor may be the total amount of mono-unsaturated acids present or the ratio of mono-unsaturated to saturated fatty acids in the rapeseed oil. Evidence to corroborate this idea has been put forward by Vogtmann *et al.* (*Int. J. Vitam. Nutr. Res.* 1975, **45,** 221). Their results strongly indicated a correlation between the incidence of multiple extensive lesions in rats and the ratio of mono-unsaturated to saturated fatty acids in the diet, the incidence being 7, 7, 3, 4 and 1 for ratios of 18·2 (high-EA rapeseed oil), 17·0 (low-EA rapeseed oil, Span cultivar), 3·6 (commercially hydrogenated Span oil), 7·9 (low-EA rapeseed oil, Zephyr cultivar) and 1·6 (soya-bean oil).

Further confirmation has come from investigations undertaken by Hulan *et al.* (*Lipids* 1976, **11,** 9). Rendered pig fat containing 5·6% EA was fed to male rats in three separate experiments at 20% (w/w) of the diet for 16 weeks. In experiment I the effects of rendered pig fat were compared with those of Span oil (containing 4·8% EA), whilst in experiments II and III rendered pig fat was compared to commercial lard (containing 0·2% EA), to commercial lard to which 5·4% free EA had been added and to Span oil. No significant difference was observed in the levels of EA present in the hearts of rats fed diets of rendered pig fat, Span oil or commercial lard plus EA. However, both the incidence and severity of cardiac lesions were significantly higher in rats fed Span rapeseed oil than in rats fed control diets. The authors suggest that the imbalanced fatty acid composition of rapeseed oil was responsible for the myocardial lesions and, in fact, their results indicate that a certain level of saturates in the diet may actually prevent myocardial necrosis. When the various oils containing approximately 5% EA were fed to male rats, the incidence of myocardial lesions was seen to decrease as the level of saturated fatty acids in the dietary oils increased.

This hypothesis is not without its opponents. Vles *et al.* (*Fette Seifen AnstrMittel* 1976, **78,** 128) could find no indication that the ratio of mono-unsaturated fatty acids to saturated fatty acids in experimental diets had a substantial influence on the cardiopathogenicity of rapeseed oil in rats. On the contrary, their results indicated that by decreasing the EA content of the diet it was possible to normalize the growth performance, food intake, haematological picture and weight of the heart and other major organs, as well as to lower considerably the incidence and severity of the cardiac effects.

Vles *et al.* (*loc. cit.*) introduced a further issue into the debate. They remarked that male rats, particularly those of the Sprague–Dawley strain, appeared to be highly susceptible to the cardiopathogenic effects of relatively low dietary levels of EA. Certainly, it has already been shown that the male rat was more susceptible than the female to the effects of EA. Vogtmann *et al.* (*loc. cit.*) had observed this feature when fully refined rapeseed oils of high (20·6%) and low (0·8–3·7%) EA content and soya-bean oil were fed to male and female Sprague–Dawley rats at a level of 15% (w/w) in the diet. Focal lesions were found in the cardiac tissue of the rats but not in liver and spleen tissue, and the incidence of lesions was similar with all feeds. There was, however, a significantly ($P < 0·01$) higher incidence of cardiac lesions in males than in females.

A similar phenomenon was noted by Charlton *et al.* (*Can. J. comp. Med.* 1975, **39,** 261), again with the Sprague–Dawley strain of rat. Fully refined, oxidized or unoxidized rapeseed oils containing varying amounts of EA were fed, at 20% (w/w) of the diet, to weanling male and female rats for periods up to 112 days. A transient myocardial lipidosis characterized by an accumulation of fat droplets in myocardial

fibres was marked in male and female rats fed rapeseed oils containing 22·3% EA, moderate in those fed oils containing 4·3% EA and very slight in those fed oils containing 1·6% EA. A peak in the intensity of myocardial lipidosis occurred on days 3–7, an effect which has been described by several workers (Engfeldt & Brunius, *loc. cit.* p. 27; Hulan *et al. loc. cit.*; Kramer *et al. J. Nutr.* 1973, **103,** 1696). More significantly, the male rats had distinct foci of necrosis and fibrosis in the myocardium, whereas this form of lesion was rarely observed in female rats. Using a similar dietary regime, Kramer *et al.* (*loc. cit.*) reported an absence of myocardial necrosis and fibrosis in female rats. Any explanation of the pathogenesis of myocardial necrosis must therefore take into account the predominance of lesions in male rats and the suggested strain differences. Vles *et al.* (*loc. cit.*) based their conclusions on studies undertaken with the Wistar strain and mooted that differences in growth rate, body weight, food intake and heart-to-body weight ratios might be of importance in explaining these sex and strain peculiarities.

In addition to the development of new cultivars, a certain degree of protection may be effected by the partial hydrogenation of rapeseed oil. Beare-Rogers *et al.* (*loc. cit.*) found in a 16-week study that partial hydrogenation of Zephyr oil (0.3–0.7% EA) reduced its cardiopathogenic properties when compared with the unhydrogenated oil administered under similar conditions and intimated that a factor might be removed or destroyed during the processing.

Using alternative methods to reduce Span rapeseed oil toxicity by subjecting the oil to exhaustive molecular distillation or adsorption chromatography, Kramer *et al.* (*Lipids* 1975, **10,** 505) were unable either to demonstrate the presence of a distillable toxic component or to show that the highly purified triglycerides were less cardiotoxic than the original oil.

Lipidosis of the heart muscle caused by feeding rapeseed oil has been observed not only in rats but in a number of other animal species (Abdellatif, *Nutr. Rev.* 1972, **30,** 2). Abdellatif & Vles (*Poult. Sci.* 1973, **52,** 1932) compared the pathogenic effects in ducklings following the administration of a rapeseed oil containing 50% EA and one containing 8·5%. The former caused growth retardation, severe mortality, hydropericardium and pathological changes in the heart, liver, skeletal muscles and spleen after administration for only 2 weeks. After 3 months cardiac fibrosis was observed. In animals fed the low-EA rapeseed oil, growth was normal and no deaths were recorded, but here, too, vacuolar changes of the heart were apparent after 2 weeks although hydropericardium was absent. At the 3-month stage the lesions had largely disappeared, leaving no permanent tissue change and indicating that an EA intake equivalent to approximately 4% of total calories was well tolerated by the duckling. This animal is known to be particularly susceptible to dietary rapeseed oil, showing a high death rate at levels causing no deaths in other species. This is one of the reasons why the duck is unlikely to be a popular species for investigations of the long-term effects of rapeseed oil.

Studies in the pig are generally considered to be more likely to yield results that can usefully be extrapolated to provide an indication of potential effects in man, because there are many physiological similarities between the two species. Unfortunately, recent experimental results for swine are not in agreement. Aherne *et al.* (*Can. J. Anim. Sci.* 1975, **55,** 77) examined histological changes in the tissues of pigs fed high (20·6%) or low (4·0%) EA rapeseed oils or soya-bean oil at a level of 15% in the diet for about 12 weeks. Focal areas of interstitial myocarditis with eosinophils, lymphocytes and a number of plasma cells were present in 35·8% of all the hearts examined, but no histological differences were observed between the pigs fed diets containing 15% rapeseed oil or the same amount of soya-bean oil. The presence of vacuoles in the cardiac muscle fibres was also reported, but no fat was demonstrated within these structures.

In contrast, Friend *et al.* (*ibid* 1975, **55,** 49) described traces of myocardial fat accumulation in pigs fed Span rapeseed oil (4·3% EA) at 20% of the diet for 16 weeks. This fat infiltration, while mild and far less marked than had been reported in the rat, occurred more frequently in both male and female pigs fed Span rapeseed oil than in those fed soya-bean oil. It is evident that further studies in the pig are needed to clarify the situation.

Similar observations have been made under certain conditions in man. Borg (*Acta med. scand.* 1975, **198,** Suppl. 585, p. 5) refers to an examination of hearts from 97 consecutive autopsies at Sabbatsbergs Hospital in Stockholm. In twelve individuals, fatty droplets were observed in small areas of the fibres of the left ventricular muscle and this fatty infiltration was similar in nature and extent to that in rats fed rapeseed oil. The diagnosis for the 97 cases varied and the average age was high, but unfortunately nothing was known about the eating habits of the individuals concerned.

Work has also continued on the biochemical mechanisms underlying the pathological effects of rapeseed oil and EA. Gatti & Michalek (*Arzneimittel-Forsch.* 1975, **25,** 1639) administered intragastrically to young female mice 50% of the daily calorie requirement in the form of rapeseed oil (containing 52% EA and 7·4% gadoleic acid, C20:1). After 3 days of this treatment it was evident that the deposition of these two fatty acids was greatest in the heart and liver, where they accounted for approximately 10 and 9% of the total fatty acids respectively. The EA to gadoleic acid ratio was approximately 3:1 in the heart and liver, whereas in the rapeseed oil supplied to the animals it was of the order of 7:1. It is possible, therefore, that the gadoleic acid found in the tissues is derived only partly from the diet, other possible key sources being the β-oxidation of EA and, to a lesser extent, synthesis by elongation of oleic acid. Gadoleic acid was in fact present in small amounts in all analysed tissues of control animals, although erucic acid was absent.

Hulan *et al.* (*loc. cit.*) attempted to investigate the changes in myocardial fatty acids over a long-term period when they examined the composition of the cardiac lipids in rats fed the experimental diets for 3 days and 1, 8 and 16 weeks. In general, the relative concentration of the saturated fatty acids (C 14:0, C 16:0 and C 18:0) in the heart remained constant throughout the feeding trial and there were no signifi-

cant differences due to diet. Similarly, the effect of diet and length of feeding period on the relative concentration of the shorter chain mono-unsaturated fatty acids C16:1 and C18:1 was not significant. However, considerable differences were observed in the relative concentrations of the long-chain fatty acids, gadoleic acid and EA. Gadoleic acid accumulated in the hearts within the first week of the feeding trial but had decreased by weeks 8 and 16. In rats fed commercial lard containing 5·4% added EA, the levels of gadoleic acid were higher than those in rats fed the ordinary lard diet, presumably because of β-oxidation of EA to gadoleic acid. EA levels increased in rats fed diets containing Span rapeseed oil, rendered pig fat (containing 5·6% EA) or lard plus 5·4% EA; during the first week the levels rose to approximately 4–5% and subsequently declined to 1% by week 16, an observation reported by a number of investigators.

Kramer *et al.* (*J. Nutr.* 1973, **103,** 1696) recognized comparable features in their studies. An early temporary increase in cardiac lipids was observed with diets containing Span (low-EA) oil, conventional rapeseed oil and, to a lesser extent, Oro (low-EA) oil. The tissue fatty acids clearly reflected levels of these compounds in the diet. In rats fed corn oil, C18:2 was high in the heart and liver, whereas in those fed Oro, Span or RSO diets C18:1 was high and there was pronounced accumulation of gadoleic acid and EA in the heart.

A similar situation may also exist in the chicken, although the picture is not entirely clear. Vogtmann *et al.* (*Nutr. Metabol.* 1974, **17,** 136) have reported that the average lipid levels in the hearts of chickens fed diets containing conventional rapeseed oil or Canbra (low-EA) oil were significantly lower than those in chickens fed diets containing Span (low-EA) oil, soya-bean oil or lard. These findings suggest that at the age of 4 weeks, chickens are capable of metabolizing fatty acids in Canadian-type rapeseed oil as efficiently as the fatty acids of other oils present in the diet. However, this does not rule out the possibility that during the first week of rapeseed-oil ingestion, a peak of lipid deposition in cardiac tissue might occur, as in the rat. This team also described a striking increase in the concentration of gadoleic acid in heart, liver, spleen and breast-muscle lipids in relation to the amount of this fatty acid present in the feed. It may well be that the 4-week-old chicken is able to metabolize EA to the same extent as other fatty acids, and apparently degradation of EA to gadoleic acid and oleic acid occurs to a considerable degree.

Once again results of studies in swine may provide a more rewarding insight into the sequence of events in man. Friend *et al.* (*loc. cit.*) fed two groups of weaner boars on a control diet or on one containing 10% Span rapeseed oil. Two pigs were removed from each group and killed on days 0, 4, 8, 12, 16 and 20 of the study to provide cardiac tissue for fatty acid analysis. Although the proportions of the major fatty acids were similar between diets and for the duration of feeding, there were certain differences in a number of the minor acids. EA was present in the cardiac lipids of all control animals, but to a significantly lesser degree than in those of the pigs fed the Span rapeseed oil. In contrast to studies in other species, the concentration of EA showed an increase up to day 16, with a subsequent decrease by day 20 of the experimental feeding. This could have been a reflection of a slower accumulation of this fatty acid in the cardiac tissue of the pig, with a peak occurring at weeks 2–3.

Biochemical observations in man are somewhat limited. As mentioned previously, it is known that gadoleic acid in the tissues is derived mainly from dietary rapeseed oil, both directly as a product of β-oxidation of EA and to a lesser extent by synthesis. Hence, with a certain degree of approximation, these two fatty acids may be considered as an index of rapeseed-oil exposure. Bearing this in mind, Gatti *et al.* (*loc. cit.*) collected data on the presence of EA and gadoleic acid in human adipose tissue and myocardium. The two acids were located in the adipose tissue of each of 16 individuals examined and together accounted, on average, for approximately 4·8% of total fatty acid, although they exceeded 10% in several cases. In the myocardium, EA and gadoleic acid were measurable in 13 and nine subjects, respectively, out of 14 analysed. In this tissue, the two acids accounted for about 1·3% as a mean value, reaching values of 3·2% in some samples, but the levels of the two fatty acids were always considerably lower in the myocardium than in the adipose tissue.

The rise in tissue levels of EA in animals fed rapeseed oil is presumably not due to a greater intestinal absorption of EA than of other fatty acids, as the digestibility of EA-containing rapeseed oil is known to be lower than that of other vegetable oils (Sergiel & Rocquelin, *Annls Biol. Anim. Biochim. Biophys.* 1975, **15,** 103). One might deduce, therefore, that fat accumulation is due either to increased cellular uptake, to decreased triglyceride utilization or to decreased oxidation.

It has been shown (Houtsmiller *et al. Biochim. biophys. Acta*, 1970, **218,** 564) that the accumulation of fat in the heart is accompanied by a decrease in mitochondrial respiration. In an attempt to investigate this effect further, Heijkenskjöld & Ernster (*Acta med. scand.* 1975, **198,** Suppl. 585, p. 75) monitored the metabolic activities of heart mitochondria isolated from rats fed 1·4 or 2·6% EA for 1–8 weeks. They reported a diminished ability of the mitochondria to oxidize various substrates. This was most pronounced with palmitylcarnitine as substrate, the rate of oxidation being decreased by more than 50% following ingestion of 1·4% EA in the diet for 2–4 weeks. The oxidation of palmitylcarnitine was also found to be inhibited following the addition of erucylcarnitine to isolated heart mitochondria from control animals. Inhibition was accompanied by a decrease in the rate and extent of reduction of mitochondrial flavoprotein. A similar inhibition of flavoprotein reduction as well as of nicotinamide nucleotide reduction was encountered when erucate was added to the perfusing medium of a rat heart respiring with oleate as substrate. These results thus support earlier ideas that EA interferes with the enzyme system responsible for the mitochondrial oxidation of long-chain fatty acids, probably at the level of acyl-CoA dehydrogenase.

Conflicting results have been reported by other workers, however. Kramer *et al.* (1973, *loc. cit.*) examined the enzymes of fatty acid oxidation by incubating

isolated heart mitochondria with appropriate substrates. Both palmitylcarnitine and erucylcarnitine were readily oxidized by heart mitochondria isolated from the rats of the experimental groups. A decreased oxidation of palmitylcarnitine was revealed at week 4 for the heart mitochondria from rats fed conventional rapeseed oil, but in no case was there a marked depression in respiratory activity comparable to that described previously (Houtsmiller *et al.*, *loc. cit.*).

Of greater significance was the observation by Kramer *et al.* (1973, *loc. cit.*) of considerable differences in the ability of enzymes to metabolize palmitate, oleate and erucate. Erucate activation to its CoA ester by rat-heart thiokinase represented only about 2% of the activity exhibited by the same enzyme with palmitate as the substrate. Blond *et al.* (*Biochimie* 1975, **57**, 361) suggested that the enzymes involved in fatty-acid activation and oxidation had a greatly reduced affinity for EA and that fat accumulation associated with the feeding of rapeseed oils rich in EA was due to a low activity of triglyceride lipase against EA, reduced rates of β-oxidation of this fatty acid and an inhibitory effect of EA on the metabolism of other long-chain fatty acids. The observed reduction in tissue-lipid levels after prolonged administration of rapeseed oil could be related to increased activities of the fat-metabolizing enzymes.

Subsequent work appears to have confirmed these ideas. In further studies on rat-heart preparations, erucic acid was metabolized at a slower rate than palmitic acid (Cheng & Pande, *Lipids* 1975, **10**, 335) and the rate-limiting reaction in the overall metabolism of erucate was thought to be its activation. The presence of erucyl-CoA of erucylcarnitine reduced the rate of mitochondrial oxidation of palmityl groups, presumably by competition for the same enzyme system.

In an attempt to identify similar features in mitochondria from the human heart, Clouet *et al.* (*C.r. hebd. Séanc. Acad. Sci., Paris* 1974, **279D**, 1003) subjected the isolated mitochondria to [14-^{14}C]EA or [10-^{14}C]oleic acid. They reported a slower rate of oxidation for EA, as measured by the formation of $^{14}CO_2$ and the appearance of radioactivity in the acid-soluble phase following filtration.

Mitochondria from the livers of rats and other animals fed on rapeseed oil do not seem to be affected, but conflicting accounts have been published of the effects of such treatment on the hepatic endoplasmic reticulum. In young Wistar rats fed either a 3 or a 20% rapeseed-oil diet for 6 or 8 days or 16 weeks (Collomb *et al.* *Enzyme* 1974, **18**, 300), the only observed change was a reduction in the activity of glucose-6-phosphatase. In contrast, Gaillard *et al.* (*Annls Nutr. Aliment.* 1974, **28**, 17) reported the induction of several microsomal enzymes, including those effecting aniline hydroxylation and the *N*-demethylation of *N*-methylaniline, following the feeding of 20% rapeseed oil in the diet of rats for 8 weeks.

The importance of the multiplicity of EA effects in animals is unquestionable. Several animal species are known to react to rapeseed oil in a similar way and the implications for its incorporation into animal feedstuffs are apparent. For man, the situation remains far from clear and it is evident that further studies are required along the lines of those already undertaken in Italy to assess the exposure levels of the population to rapeseed-oil ingestion. In a sample of 70 apparently healthy Italian railway workers, 40 had measurable EA in the serum in quantities ranging from 0·3 to 3·8% of the total fatty acids (Del Carmine *et al. Proc. Eur. Soc. Toxicol.* 1975, **16**, 209). The presence of behenic acid (C22:0), a possible metabolite of EA in man, was also reported. Previous investigations in 1964 (Cali *et al. Boll. Soc. Ital. Biol. sper.* 1965, **41**, 625) and 1968 (Maranesi *et al. ibid* 1972, **48**, 1205) had indicated an absence of the two compounds, although the latter paper reported their detection in 1972. The difference in these results suggests that EA and behenic acid only became components of serum fatty acids in Italians after 1968. It is interesting to note that during the late 1960s rapeseed oil with a high EA content (up to 55%) was introduced into mixtures of seed oils because of its low cost. Prior to that time, olive oil had been the main source of edible fat. In 1974, as the pathogenicity of EA in man had not been ruled out, the Italian authorities introduced a limitation of 15% EA in seed-oil mixtures (Gatti & Michalek, *loc. cit.*).

The paucity of human data was stressed in a recent EEC proposal for a Council Directive to limit the maximum level of EA in edible fats and oils (*Off. J. Europ. Commun.* 1975. **18**, (C150), 5). The Council indicated that although undesirable effects had been observed in experimental animals, the applicability of these observations to man had not been validated. As further research was in progress, a proposed restriction on the level of EA was fixed at 15% (calculated on the total level of fatty acids in the fat component) for the period 1 July 1976 to 30 June 1977. At the end of this period a proposed maximum limit of 10% would come into operation.

Early last year, the final Directive was promulgated (*ibid* 1976, **19** (L202), 35) with a modified maximum limit set at 5% following a resolution passed by the European Parliament (*ibid* 1975, **18** (C280), 15). This limit will take effect from 1 July 1979, with an interim limit of 10% set for the period 1 July 1977 to 30 June 1979.

Despite repeated calls over the past few years for the provision of data from exposed human populations, little information has been forthcoming and only recently have recommended outlines for further research been presented (Scientific Committee for Food, Commission of the European Communities, Report 1585/VI/76/E). The research outline is not restricted to EA but extends to other long-chain fatty acids and oils and fats used in food. The studies requested include an investigation of the absorption, distribution, excretion and kinetics of distribution following oral administration of these compounds. The report recommends the use of isotopically labelled material in these studies.

In addition, the Committee recommends research aimed at correlating observed biological effects with blood and tissue levels of the fatty acids or their triglycerides. Of primary importance would be the establishment of a no-effect level and an identification of any reversibility of adverse effects that might occur. Epidemiological studies of exposed populations should also prove rewarding, with investigations on

blood and tissue levels of individual long-chain fatty acids as well as effects on human-tissue cultures or preparations of human mitochondria. Certainly whilst further research is being undertaken, levels of the order recommended by the European Parliament should be maintained until substantial evidence of any adverse effects in man is available.

[S. P. Johnson—BIBRA]

A HEXACHLOROPHENE MISCELLANY

In 1971 the FDA published a study indicating that hexachlorophene (HCP) could induce central nervous system (CNS) degeneration in rats when fed over a short period (*Cited in F.C.T.* 1972, **10,** 275). About the same time it was reported that significant concentrations of HCP had been found in the blood of infants bathed daily with a detergent preparation containing 3% HCP (*ibid* 1972, **10,** 114). Such reports led to restrictions on the use of HCP in the USA, UK and elsewhere. We have previously reviewed various aspects of HCP toxicity (*ibid* 1974, **12,** 563; *ibid* 1976, **14,** 642), but have given little consideration to the role of HCP as an environmental contaminant, a problem demonstrated in a recent American study concerned with the dispersion of HCP into inland waters.

An examination of the drainage area of the Upper Haw River in North Carolina (Sims & Pfaender, *Bull. env. contam. & Toxicol.* (*U.S.*) 1975, **14,** 214) showed the presence of 16·4–44·3 ppb (b = 10^9) HCP in the surface water and 9·3–3·77 ppb in the sediments of a small stream deriving up to 90% of its flow from the effluent of a town's sewage-treatment plant. While this and another wastewater-treatment plant were the major sources of HCP in the waters of this area, other sources of contamination were the untreated water from residential areas, effluent from industries using HCP-based cleaning solutions and two hospitals, in which 3% HCP preparations were used for cleaning. HCP concentrations at individual sites varied greatly on different sampling dates, but there was some indication of a reduction in levels following the introduction of restrictions on HCP use. Organisms collected from the streams all showed levels of HCP higher than those of water or sediment and concentrations increased at the higher trophic levels. The amounts of HCP detected in the water and in stream sediments did not constitute a direct toxic hazard to human health or to micro-organisms, but the concentration of HCP through food chains may be a hazard.

Nevertheless, absorption through the skin following the direct application of HCP in soaps and cosmetics must still be considered the major problem presented by this compound. Percutaneous absorption studies in rats given skin applications of ^{14}C-labelled HCP to shaved areas for up to 24 hours have shown that the age of the animal does not consistently affect absorption, but that the nature of the solvent may do so (Nakaue & Buhler, *Toxic. appl. Pharmac.* 1976, **35,** 381). Up to 53% of applied radioactivity disappeared from the skin within 24 hours, and absorption was slightly slower in females than in males. No alteration of HCP absorption with the age of the animal, like that reported for the human infant, could be detected, and in fact absorption through the skin of newborn rats was somewhat less than that in mature animals. Maximal penetration occurred when dimethylsulphoxide was used as solvent, and penetration was minimal with 1% aqueous sodium lauryl sulphate. Acetone promoted rapid absorption of HCP, but about 27% of the applied activity remained bound to the skin at 4 and 24 hours and was not removed by washing with acetone. The absorption rate was directly dependent upon the original concentration applied, and peak blood concentrations of HCP were reached 12 hours after application in acetone. Most of the absorbed radioactivity subsequently appeared in the faeces, the 24-hour faecal sample accounting for 14·8 and 35·5% of the applied dose in adult and weanling males, respectively, with the 24-hour urine containing only 2·6 and 4·3% in these two age groups. The highest body levels of HCP appeared in liver, kidney and plasma, and in the intestine and its contents. The presence of some HCP in the stomach may have been due to coprophagy.

The effects of factors such as post-exposure time, number of exposures, route, previous exposure and age on blood-HCP concentrations in rats were studied by Kennedy *et al.* (*ibid* 1976, **37,** 425). Highest blood concentrations were found approximately 4 hours after administration of the last five oral doses of 6·5 mg HCP/kg/day, but no such peak was found at the higher dosage of 40 mg/kg/day. Dermal application of 40 mg HCP/kg/day resulted in a peak HCP concentration 24 hours after the last of five exposures; the mean blood concentration 4 hours after the last exposure was slightly lower than that resulting from oral administration of the same daily dose. Repeated oral dosing with HCP (6·5 mg/kg/day) did not increase blood-HCP concentrations over a 42-day period. Little HCP was detected in the blood 4–7 days after exposure by either route. Previous ingestion of HCP either for 14 days or over three generations did not alter the blood-HCP level resulting from administration of a single dose of 40 mg HCP/kg. Blood-HCP concentrations were found to be similar in rats ranging in age from 21 to 200 days, following either oral or dermal administration, a finding which supports the results for dermal application reported by Nakaue & Buhler (*loc. cit.*).

The CNS is the main target of HCP toxicity. Kennedy *et al.* (*Toxic. appl. Pharmac.* 1976, **35,** 137) have reported reversible toxic effects of HCP on the CNS of the rat. The animals were given 10, 20 or 40 mg HCP/kg/day by gavage for 4 weeks, after which the 10-mg/kg dose was increased to 60 mg/kg for a further 2 weeks, the other dose levels remaining unchanged. All animals were killed after 6 weeks. Growth was retarded by 40 mg HCP/kg/day or more, and with 60 mg/kg/day, eight of 20 animals died after periods of ataxia and hypoactivity. HCP had no effects on haematology or clinical chemistry, but his-

tological examination of the white tracts on the CNS showed focal vacuolization, the intensity of which was dose dependent. The lesion was most severe in the cerebellum, less so in the spinal cord, and mildest in the brain stem.

When rats were given a diet containing 20, 65 or 200 ppm HCP (approximately 2, 6·5 and 20 mg/kg body weight, respectively) for 90 days, no CNS disturbances were seen, but examination of the brain and spinal cord of rats fed 200 ppm showed moderate vacuolization in two of the 20 animals. Young adults given 40 mg HCP/kg/day by gavage for 42 days showed retarded growth, lethargy, diarrhoea and hind-limb paralysis, and seven of 70 died (Kennedy *et al. ibid* 1976, **35,** 137). After withdrawal of HCP for 3–5 days, the hind-limb paralysis receded completely. All white tracts of the CNS in rats examined 6 hours after the last dose of HCP showed vacuolization, but after a recovery period of 7 days vacuolization was less severe and subsequently damage continued to diminish, no lesions being detectable after a recovery period of 168 days. These observations were in general agreement with the results of a study reported earlier (*Cited in F.C.T.* 1972, **10,** 275).

Newborn swine given 0·1–45 mg HCP/kg/day orally in corn oil for 36 days showed varying degrees of CNS dysfunction depending on dose level (Robinson *et al. Am. J. vet. Res.* 1975, **36,** 1615). Those given 0·1 mg HCP/kg/day were normal throughout, while four of seven receiving 1 or 2 mg/kg/day developed signs of toxicity and one of these died. All seven pigs given 4 or 5 mg HCP/kg/day showed signs of CNS impairment or lesions and four died, and all four on the highest dosage levels (15 or 45 mg/kg/day) died within 24 hours of the first treatment. All tissues in the treated pigs contained HCP residues, the highest concentrations being usually in the kidney, but sometimes in the liver. Washing pigs with up to 45 mg HCP/kg/day as a 3% HCP/detergent preparation induced signs of CNS dysfunction but no histological lesions.

Three pigs (including one control) died after topical treatment, signs of poisoning being apparent in the one exposed to 11·25 mg/kg/day but not in the one on the 45-mg/kg doses (Robinson *et al. loc. cit.*). Blood concentrations of HCP after topical application were much lower than those after oral administration. The effects of exposure to the 3% HCP shampoo for 92 days were no more pronounced than those of exposure for 36 days and little difference was found in the tissue concentrations of HCP after these periods. As in rats, HCP intoxication in swine was associated with vacuolation of the central nervous tissue. The data obtained in these experiments are considered to indicate that pigs have only a limited value as a model for studying HCP toxicity in man, offering no advantages over rats and monkeys.

The relationship between HCP-induced paralysis in the cat and changes in cerebrospinal-fluid (CSF) pressure has been studied by Hanig *et al.* (*Proc. Soc. exp. Biol. Med.* 1976, **152,** 165), who gave the animals 20 mg HCP/kg/day until complete paralysis ensued. Early signs of HCP intoxication, notably stiffness of the hind limbs and swinging movements of the pelvic girdle, developed after 2–12 days and progressed to severe intoxication and muscular paralysis after a further 1–4 days. Cats in complete paralysis had a mean CSF pressure of 174 mm saline, compared with a control value of 19 mm. CSF pressure fell after an iv infusion of 2 g urea/kg (30% urea in 10% invert sugar solution), probably as a result of removal of fluid from the brain mass, but it was not affected by acetazolamide or prednisolone. White matter in the brain and spinal cord of intoxicated cats showed vacuolation, but there was no evidence of damage to the cerebral ventricles. The ineffectiveness of acetazolamide and prednisolone in reducing CSF pressure indicated that the HCP-induced lesion was not inflammatory and involved neither ventricular obstruction nor overproduction of CSF.

Recently it has been proposed that HCP could alter the permeability properties of the cellular membrane. In a study of some biochemical aspects of HCP activity, Mavier *et al.* (*Biochem. Pharmac.* 1976, **25,** 305) found that adenylate cyclase activity in a purified plasma membrane prepared from rat liver was inhibited *in vitro* by concentrations of HCP as low as 1·0 μM–0·1 mM. The inhibition was dose related and was complete with 1·0 mM-HCP. It was not modified by addition of 10 μM-guanosine 5′-triphosphate, 0·1 μM-glucagon or 10 mM-sodium fluoride, nor by the presence of an ATP-regenerating system in the medium. Increasing the concentration of the substrate (in the form of an ATP–Mg complex) did not reverse this HCP-induced effect, which was immediate and did not reverse spontaneously. Demonstration of a lack of effect of 2-mercaptoethanol on the inhibition suggested that no reactive thiol groups were involved. HCP also inhibited ATPase, and the authors suggest that some of the clinical features of HCP intoxication may be directly attributable to changes in the permeability of the liver plasma membrane brought about by the powerful inhibition of these two enzyme systems of the membrane.

While much effort has been expended on studies relating to the possible effects of applying HCP dermally to neonates, fewer reports have been concerned with its effects on reproduction and foetal development. In one earlier study (*Cited in F.C.T.* 1974, **12,** 567), feeding HCP to rats at a dietary level of 100 ppm was found to cause slight reductions in offspring survival in the F_1 generation and in the size of F_2 litters, but CNS vacuolation decreased in successive generations. A dietary level of 20 ppm had no effect on reproduction. In a more recent study, feeding albino rats with 12·5, 25 or 50 ppm HCP over three generations had no significant effect on mating, fertility, length of gestation or litter size (Kennedy *et al. J. agric. Fd Chem.* 1975, **23,** 866). Neonatal survival and weight at weaning were not altered, no gross abnormalities or behavioural changes were apparent, and parents from each generation and weanlings of the F_{3b} generation showed no histological lesions that could be attributed to HCP.

In a teratological study in rats (*idem, Teratology* 1975, **12,** 83), the same investigators gave the animals 15 or 30 mg HCP/kg/day by intubation on days 6–15 of gestation. In addition rabbits were given 3 or 6 mg/kg/day on days 6–18. Animals of both species given the higher doses showed weight reductions but no increase in foetal deaths. The lower doses had no teratological effect, but in foetuses from rats exposed to

30 mg HCP/kg there were low incidences (1·8 and 4·3%, respectively) of defects in eye development (microphthalmia or anophthalmia) and of angulated ribs. Of the 175 foetuses from rabbits exposed to 6 mg HCP/kg, three (1·7%) had curved, fused or forked ribs. These types of malformation were among those seen, in an earlier study (*Cited in F.C.T.* 1974, **12,** 567), in a high proportion of foetuses produced by rats given a large intravaginal dose of HCP on days 7–10 of gestation.

[P. Cooper—BIBRA]

BIOACTIVATION, TOXICITY AND SAFETY

Metabolic conversion of chemicals by living organisms has attracted considerable attention for many years, particularly in relation to the formation of biologically active molecules (bioactivation) and the conversion of active molecules to harmless ones (detoxication). While the pharmacologist looks favourably on bioactivation as the basis of the pharmacological activity of many compounds, the toxicologist sees it as the initial stage of a toxic sequence leading to tissue damage and perhaps to death. Both groups, however, view detoxication mechanisms as a bulwark against injury by foreign chemicals.

This whole question received a valuable airing at a recent symposium sponsored by The Swedish Academy of Pharmaceutical Sciences (Symposium on Bioactivation, Toxicity and Safety Evaluation, Stockholm, 24–26 November 1976).

Much current work is concerned with the biological mechanisms available for counteracting the potential effects of active metabolites, and particularly with the structure of the active compounds and their binding with biological macromolecules. Pharmacologists and biochemists are paying much attention to the biological consequences of such binding, particularly in relation to drugs, and their approach to the problem is of potential value to toxicologists.

Autoradiography has proved to be an increasingly valuable means of demonstrating the organ distribution of foreign compounds in test animals. Modification of the technique now enables a distinction to be made between mere accumulation, due to a non-specific process of concentration, and accumulation of a reactive metabolite bound covalently to the tissues. This is a particularly useful development in cases where the site of maximum accumulation does not coincide with that of the major biological effects, and is one likely to have important repercussions on our understanding of chemical toxicity.

Also of importance in the development of toxic effects are the rate of deposition of a compound in an organ and the balance between its rate of uptake and the formation of covalently bound species and detoxication products. Efforts are being directed towards relating these properties qualitatively and quantitatively to pharmacological action or tissue damage. Development of this kinetic approach offers some explanation for individual variations in susceptibility to the lesion-inducing effects of various chemicals, but it may also have wider implications. Another concept to attract some attention is the idea that reactive intermediates may be looked upon as binding with informational and/or structural macromolecules. Unless repair takes place, damage to the former macromolecules would lead to mutagenic effects and cancer, while damage to the latter would lead to cell death. This hypothetical division has many points to recommend it, but it is hardly likely that biological macromolecules would fall neatly into these two categories.

Underlying these lines of thinking is the need to establish at molecular levels the dose range in which a particular compound is safe and that in which it will present a hazard, a quest that has given rise to the concept of non-immune defence mechanisms. Apart from the accepted detoxication pathways, glutathione is currently being studied as a means of trapping the reactive metabolites of such compounds as paracetamol, acetaminophen, phenacetin and acetanilide. At therapeutic dose levels, these compounds are safe because their electrophilic metabolites are normally detoxified in the liver by preferential conjugation with glutathione; when the available glutathione is exhausted by excessive doses of the drug, the metabolites are left free to react with vital nucleophilic macromolecules in the hepatocytes and thus cause hepatic necrosis. The knowledge that many compounds interact with sulphydryl groups is not new, but the importance of such reactions in detoxication processes was recognized more slowly. Not many years ago this reaction was thought by some to be a major factor in transforming a normal cell to a neoplastic one, but it now seems to be of paramount importance in trapping at least some active carcinogenic intermediates, provided they are present in small doses.

Certain other substances in addition to glutathione have been implicated in non-immune defence mechanisms, but the experimental evidence supporting their existence or their defensive role rests on somewhat shaky foundations. These substances include chalones, peroxides and reducing molecules. It is of some interest that certain xenobiotic reducing substances, such as butylated hydroxytoluene and butylated hydroxyanisole, are thought to participate in this type of defence.

Perhaps the most important non-immune defence mechanism, particularly in relation to mutagenesis and cancer, is that which permits the repair of damaged DNA. The current view is that the active form of a carcinogenic chemical binds covalently at the 6(O) position of the guanine base, the alkylated molecule being removed more rapidly from some organs than from others. In general, slow removal seems to correlate with the emergence of cancer, but some organs, such as the liver, have a capacity for repairing this type of damage very quickly and yet are the targets for a host of carcinogens. Obviously much more study will be required before the critical sites of DNA damage can be mapped with any accuracy.

It is perhaps a reflection of our time that so much emphasis is now placed on understanding the mechanisms that determine what constitutes a safe dose of a therapeutic compound. Herein lies the field in which pharmacology seems to have most to offer to toxicology, and these approaches, combined with the availability of sophisticated laboratory tools, are likely to result in considerable achievements in the next few years. One may be sure, however, that the unexpected development will always be at hand to demand the exercise of ever greater effort and skills.

[P. Grasso—BIBRA]

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TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS

ANTIOXIDANTS

3166. Polymeric BHA

Furia, T. E. & Bellanca, N. (1976). The development of new, non-absorbable polymeric antioxidants for use in foods. *J. Am. Oil Chem. Soc.* **53**, 136.

A final toxicological judgement on a number of permitted antioxidants, particularly butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), is still awaited after trials spanning several decades. The development of an efficient, uncontroversial antioxidant is therefore an attractive prospect, and the paper cited above describes a new class of polymeric antioxidants which technically may be capable of displacing BHA and BHT. The compounds, designated Poly-AO, are a series of phenols or substituted phenols covalently bonded by hydrocarbon-linking units.

When added to vegetable oils at a level of 200 ppm in the presence of 200 ppm citric acid, three of these polymeric antioxidants (Poly-AO I, II and III) were superior to BHT/BHA in antioxidant performance on a weight-for-weight basis; moreover small increases in aromatic hydroxyl content significantly improved the antioxidant properties of the polymers. Although direct addition of the polymer to oil–water emulsions was impossible, owing to coacervation effects, this problem could be circumvented by dissolving the Poly-AO in the oil phase and then forming the emulsion. Like the treated vegetable oils, the stabilized emulsion was more resistant to oxidation than analogous formulations protected by monomeric antioxidants.

Depolymerization, which would limit the upper working temperatures of Poly-AO, occurred at about 300°C, a marked improvement over BHT with a maximum working temperature of about 170°C. In addition, the polymeric additive was relatively non-volatile under deep-frying conditions: losses of Poly-AO as a result of steam distillation were negligible after repeated frying, and carry-through into the food was almost quantitative, whereas with BHA and BHT losses under similar conditions can be in excess of 15%.

Studies in rats indicated that Poly-AO was poorly absorbed from the gastro-intestinal tract following oral administration. Only 0·44% of the radioactivity from an oral dose of a ^{14}C-labelled Poly-AO fraction (mol wt 7300) was detected in the urine and tissues of the rat, the remaining radioactivity being recovered in the faeces.

[It remains to be seen whether future work will confirm the expectations of these authors that polymeric food additives in general, and polymeric antioxidants in particular, will offer toxicological and technological advantages over currently permitted materials. The absorption data presented here are encouraging from a safety-in-use viewpoint and it is to be hoped that they will be substantiated by the results of feeding studies.]

MISCELLANEOUS DIRECT ADDITIVES

3167. The Feingold debate

Salzman, L. K. (1976). Allergy testing, psychological assessment and dietary treatment of the hyperactive child syndrome. *Med. J. Aust.* **2**, 248.

Cook, P. S. & Woodhill, J. M. (1976). The Feingold dietary treatment of the hyperkinetic syndrome. *Med. J. Aust.* **2**, 85.

Werry, J. S. (1976). Food additives and hyperactivity. *Med. J. Aust.* **2**, 281.

Dr. Ben Feingold has achieved some degree of popular and scientific fame over the past few years after publicizing his views on hyperkinesis in children. Briefly, his principal hypotheses are that the hyperactive disturbance is non-immunological, that it is an inborn chemical reaction affecting the nervous system of certain genetically predisposed individuals, and that the causative agents are volatile and of low molecular weight. The salicylates (natural or synthetic) and artificial colourings and flavourings have been selected as possible causative agents and their elimination from the diet forms the basis of the Feingold treatment of hyperkinesis.

This theory has received some attention outside the USA and in two preliminary studies, cited above, small samples of Australian children with behavioural problems were treated with the Feingold diet. Prior to treatment, the children included in the Salzman study were screened with the Hawley–Buckley allergy test, which entailed observing the acute effect of the sublingual administration of artificial colourings. Of the 31 children tested, 18 exhibited a positive response, and 15 of these were given the Feingold diet. In both experiments a marked improvement in behaviour was judged by parents and teachers to have followed the introduction of the restricted diet. In the Cook & Woodhill study, 13 of the 15 subjects (approximately 87%) showed improvement after treatment for 9 months, while Salzman claimed that 14 of his 15 children (93%) responded favourably within a few months. Prior to receiving the Feingold treat-

ment, many of the hyperkinetic children were being controlled by drugs, usually amphetamines, but the subjective improvement noted in their behaviour following dietary control was considered to justify a cautious reduction in medication or even its termination. When the children broke the diet, a complete or partial return to the hyperkinetic condition was reported.

The conclusions of these investigators regarding the benefits of the Feingold therapy were criticized vehemently in an editorial by Werry, also cited above. He pointed out that no attempt was made to conduct the experiments on sound scientific lines by using a double-blind technique so that neither the subjects nor the immediate investigators would know when the diet was being administered, by introducing scientific and objective assessments of behaviour, and by maintaining appropriate control groups. The importance of these aspects of experimental design were stressed in the recommendations of the Interagency Collaborative Group on Hyperkinesis (*Food Chemical News*, 1976, **17**, (43) 41. Werry (*loc. cit.*) considered the absence of control groups to be particularly important, as hyperkinetic children have a strong tendency towards spontaneous recovery. He also considered the preliminary results of a further Feingold-type study, conducted by Dr. J. P. Harley of the University of Wisconsin, who used a double-blind technique and assessed behaviour by established rating scales. Few changes of any kind were observed in the treated individuals in this study, although there was a suggestion of slight subjective improvements in the behaviour of pre-school children.

3168. Citric acid and rodent lifespan

Wright, E. & Hughes, R. E. (1976). The influence of a dietary citric acid supplement on the reproduction and survival time of mice and rats. *Nutr. Rep. Int.* **13**, 563.

Man's total daily consumption of citric acid from 'natural' and additive sources may exceed 0·4 g/kg body weight. Citric acid is generally considered to be largely innocuous and recent studies confirmed that high dietary levels did not affect the growth of experimental animals unless they were on a low-calcium diet, in which case they showed a reduction in weight gain (Wright & Hughes, *Fd Cosmet. Toxicol.* 1976, **14**, 561). This work also indicated that a reduced packed cell volume resulted from the administration of 5% dietary citric acid. The paper cited above attempts to characterize further the physiological effects of this acid.

The experiments described were designed to examine the effect of citric acid on the survival time of immature and sexually mature male mice and on the survival time and reproductive capacity of rats and mice. In each experiment a control group was compared with a group given a 5% dietary supplement of citric acid. Although food intake was unaffected, 5% citric acid depressed the body weight and reduced the survival time of mice, the effects being slightly more marked in mice that had reached sexual maturity before being exposed to dietary citric acid. However, there was no evidence that the treatment influenced the number of young born to rats or mice, or their survival up to weaning.

The authors think it unlikely that the shortened lifespan and reduced weight gain were a result of a direct influence of citric acid on cellular metabolism. There is evidence that dietary citric acid may reduce the physiological availability of both calcium and iron, presumably by reducing their gastro-intestinal absorption, and the chelating activity of citric acid may reduce the availability of one or more trace elements to deficiency levels that would result in the type of reduction in lifespan demonstrated by these experiments without producing immediate or short-term effects.

[The above results indicate a need for further work on the long-term effects of citric acid and particularly on its effects on the uptake of trace elements. It might be useful to attempt to relate the suspected interaction of citric acid and essential elements to the previously demonstrated reduction in packed cell volume.]

3169. The virtues of dietary fibre

Ershoff, B. H. (1976). Synergistic toxicity of food additives in rats fed a diet low in dietary fibre. *J. Fd Sci.* **41**, 949.

Ershoff, B. H. (1976). Protective effects of cholestyramine in rats fed a low-fibre diet containing toxic doses of sodium cyclamate or amaranth. *Proc. Soc. exp. Biol. Med.* **152**, 253.

These papers confirm previous indications that plant fibres present in the diet protect the consumer against the toxicity of certain food additives (*Cited in F.C.T.* 1976, **14**, 365), an effect attributed to the physico-chemical properties of the fibres.

In the first study reported, supplements of 2% sodium cyclamate, 2% amaranth (FD & C Red No. 2) and 4% polyoxyethylene (20) sorbitan monostearate (Tween 60), used singly or in conjunction, were fed to immature rats in a low-fibre diet for 14 days. No significant adverse effect appeared with any one of the additives alone. With sodium cyclamate plus amaranth, weight gain was significantly retarded, fur took on an unthrifty appearance associated with alopecia, and watery diarrhoea occurred; three of the 12 treated rats died during wk 2 of feeding. There were similar but less extensive effects in rats fed a low-fibre diet with sodium cyclamate and Tween 60. However, the combination of amaranth and Tween 60 had no significant effect. When all three additives were fed simultaneously, the whole group died within 14 days after showing symptoms similar to these listed above. Concurrent feeding of dietary fibre, particularly blond-psyllium-seed powder at the 2·5% level or more, and to a lesser extent cellulose at the 5 or 10% level, offered protection against the additives, although 2·5% cellulose offered no protection. Since the amount of crude fibre in supplements of 10% carrot-root powder, cabbage powder or psyllium-seed powder is less than that provided by a 2·5% cellulose supplement, the protective effect was clearly not due to cellulose alone.

The dose of sodium cyclamate used in the study could be reached by human consumption of large volumes of soft drinks, but the dosage of the other additives was exaggerated beyond possible human consumption. Nevertheless, further attention should evidently be paid to the possibility of synergistic relationships between food additives.

The second study was concerned with the effect on male rats of feeding a low-fibre diet with 5% sodium cyclamate or 5% amaranth for 14 days. In those given 5% cyclamate, weight gain was significantly retarded, grooming was neglected, alopecia and watery diarrhoea occurred and five of six rats died before the end of the feeding period. Replacement of some of the sucrose in the diet by psyllium-seed powder offered marked protection, particularly at the 5% level. Cholestyramine, an anion-exchange resin, included at 1·25 or 2·5% was also protective, the higher level providing as much protection as 5% psyllium. In tests with other resins, protection against cyclamate was given by a strongly basic ion-exchange resin (Amberlite IRA-400) at 5%, and moderate protection by a weakly basic resin (Amberlite IR-45), while acidic resins had no beneficial effect. Amaranth added to the low-fibre diet caused cessation of growth and unthrifty fur in rats, with death within 14 days. This toxicity was largely counteracted by 2·5% cholestyramine or 2·5% psyllium, and 5% psyllium was even more effective.

The findings suggest that the protective influence of dietary fibre may derive from its anion-exchange properties. Further work should be directed towards the concentration, isolation and identification of the active factor or factors in the fibre-containing materials and towards identifying the mechanism of this protective effect.

AGRICULTURAL CHEMICALS

3170. Another instalment in the DDT story

Mahr, U. & Miltenburger, H. G. (1976). The effect of insecticides on Chinese hamster cell cultures. *Mutation Res.* **40,** 107.

The world-wide accumulation of persistent organochlorine insecticides in the organs of many animal species has prompted much research into the effects of DDT and its metabolites. Conflicting results have been found in attempts to detect chromosome mutations in mice and men exposed to DDT (*Cited in F.C.T.* 1974, **12,** 272; *ibid* 1976, **14,** 68 & 155) and further contributions to the evidence may be helpful.

In the study cited above, cultures of Chinese hamster cells were incubated with the *p,p'*-isomers of DDT (12–81 ppm), DDD (11–75 ppm), DDE (11–88 ppm) and DDA (20–100 ppm) in two separate series of experiments. In the first, the exposure period was only 4 hr so that the cells but not their progeny were treated; in the second, pesticide was present in the medium for 4 days, affecting both cells and their progeny. The 4-hr treatments with 75, 45 and 22 ppm DDD produced a dose-dependent inhibition of cell growth. DDT at 81 ppm and DDE at 88 ppm had less effect on growth, while DDA at 100 ppm was almost devoid of effect. DDD, evidently the most toxic of the compounds tested, killed 96% of cells within 4 days when added at 75 ppm in the longer treatment. No measurable chromosome damage was produced by DDT at 12 and 24 ppm, but at 41 ppm DDT enhanced the gap rate and at 81 ppm both the gap and the break rate. DDD at 45 ppm enhanced the gap rate and at 75 ppm induced higher gap and break rates. The effects of DDE were more moderate, while DDA at 100 ppm increased only the gap rate, and this only relatively slightly. The cell damage produced by high concentrations of DDT or DDD during exposure for longer than 4 hr was potentially lethal. However, chronic treatment with DDT at 8 ppm for 3 months failed to alter the cell proliferation rate, the sensitivity of the culture to acute treatment with higher concentrations of DDT, or the chromosomal aberration rate.

[The latter result is probably most relevant to the levels of DDT normally encountered by man, but 3 months is not long enough to assess long-term effects.]

3171. Processing beef to reduce DDT levels

Hearnsberger, A. P., Kilgore, L. T. & Rogers, R. W. (1976). Degradation of 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) in beef by canning and cooking. *J. agric. Fd Chem.* **24,** 677.

It has been reported (Carter *et al.* Science, N.Y. 1948, **107,** 347) that cooking beef by various methods caused no material decomposition or loss of DDT. In a more general study (Mcgill & Robinson, *Fd Cosmet. Toxicol.* 1968, **6,** 45), cooking was again found to have little effect on the chlorinated-insecticide content of the diet. However, other workers (Liska *et al. Fd Technol., Champaign* 1967, **21,** 117A; Ritchey *et al. J. Fd Sci.* 1967, **32,** 238; *idem, ibid* 1969, **34,** 569) have demonstrated some loss of DDT from poultry during cooking. It was decided that the increasing sensitivity of methods for determining DDT and the availability of naturally contaminated beef warranted further study of the beef as it would be eaten.

Naturally contaminated beef samples containing 5 and 8 ppm total DDT (DDE, DDD and DDT) were canned using two different sets of processing conditions (104°C for 137 min or 126·7°C for 66 min). Both processing methods reduced the total DDT compounds in the fat of the beef. Most of the loss was DDT itself, there being little change in DDE and an increase in DDD content, results in agreement with those of other processing studies. Mean overall losses were, respectively, 20 and 10% in the 5- and 8-ppm beef samples. Subsequent cooking in a conventional oven or microwave oven produced a further mean loss of 17% in total DDD, DDE and DDT in the fat.

Even after cooking, however, the DDT level in the high-residue fat was still above the allowed level of

5 ppm (CFR Sec. 180.147). These processing methods, therefore, would not provide a means of making naturally highly-contaminated meat acceptable for consumption.

3172. Cooking the carbaryl

Pretanik, J. S. & Childs, E. A. (1976). Degradation of carbaryl following thermal processing. *J. agric. Fd Chem.* **24,** 779.

Long-standing assumptions regarding the low toxicity of the pesticide carbaryl to mammals and the rapidity of its degradation in the environment have been somewhat shaken in recent years by reports of its tumorigenicity (Shimkin *et al.* Cancer Res. 1969, **29,** 2184) and ability to react with nitrite to form nitrosocarbaryl (Eisenbrand *et al. Fd Cosmet. Toxicol.* 1975, **13,** 365; Elespuru & Lijinsky, *ibid* 1973, **11,** 807), its apparent effects on reproduction and foetal development in some species (*Cited in F.C.T.* 1973, **11,** 331; *ibid* 1975, **13,** 475) and its persistence in soil (Caro *et al. J. agric. Fd Chem.* 1974, **21,** 1065). There appears to be little information about the persistence of carbaryl residues on field crops, and even less on the effects of commercial and domestic processing on the degradation or removal of these residues.

The authors named above therefore studied the degradation of carbaryl in green (French) beans during thermal processing under various conditions. The cut beans contained about 20,000 ppm carbaryl added as a methylene chloride suspension. After this treatment the beans were allowed to dry for 30 min prior to addition of distilled water or 2% brine to the containers (enamel-lined cans or glass jars), which were then exhausted, sealed and heated at 116°C, the cans for 25 min in a still retort and the jars for 20 min in a home pressure canner. Parallel procedures were followed with cans and jars containing only carbaryl and the liquid matrix. The samples were stored at ambient temperature, and unchanged carbaryl was determined by thin-layer chromatography and spectrodensitometry on days 0, 1, 7 and 14 as well as 1 and 3 months after processing.

In all the experimental systems, 96–98% of the carbaryl was degraded during the heat-treatment, the results being unaffected by the type of container, the use of water or brine or the presence of beans. During subsequent storage, however, a further significant decrease in extractable carbaryl occurred only with the combined use of enamel-lined cans and 2% brine in the presence or absence of beans. In these samples, degradation was virtually complete by the end of 1 wk. It appears therefore that industrial processing is more effective than home canning in breaking down carbaryl. Nevertheless acceptable levels of carbaryl in processed vegetables can be ensured only by controlling the levels in the crops.

3173. Toxaphene and the foetus

Chernoff, N. & Carver, B. D. (1976). Fetal toxicity of toxaphene in rats and mice. *Bull. env. contam. & Toxicol. (U.S.)* **15,** 660.

The toxicity of the chlorinated hydrocarbon insecticide toxaphene has previously been investigated in rats in respect of both subacute (Ortega *et al. A.M.A. Archs Path.* 1951, **64,** 614) and chronic effects (Fitzhugh & Nelson, *Fedn Proc. Fedn Am. Socs exp. Biol.* 1951, **10,** 295). Fatty vacuolization of the liver was observed in each of these studies. More recently, a reproduction study (*Cited in F.C.T.* 1974, **12,** 570) failed to demonstrate any changes in litter size, neonatal viability or weanling body weights following the feeding of 25 or 200 ppm toxaphene to rats. Similarly, no effects on fertility or neonatal viability were observed through two generations of mice given 25 ppm dietary toxaphene (Keplinger *et al. Inter-Am. Conf. Toxicol. occup. Med.* 1970, **6,** 125). The study now reported investigates further the foetotoxic effects of this insecticide.

Toxaphene was administered to random-bred albino mice and rats by gastric intubation at levels of 15, 25 and 35 mg/kg/day on days 7–16 of gestation, the period of organogenesis. The mice and rats were killed on days 18 and 21 of gestation, respectively, and uteri were removed and weighed. Live foetuses were weighed and the litters were either placed in Bouin's solution prior to autopsy or were stained with Alizarin Red S to facilitate skeletal examination. All foetuses were examined for external defects.

Rats receiving 35 mg toxaphene/kg/day exhibited a 31% maternal death rate. Toxaphene treatment also caused reductions in the weight gain of treated animals, in foetal weights and in the average number of sternal and caudal ossification centres, each of these effects being dose related. There were no dose-related effects on the number of foetal deaths or on the occurrence of structural anomalies.

Doses of 35 mg toxaphene/kg/day resulted in an 8% maternal death rate in the mice. Average maternal weight gain decreased with increasing dosage of toxaphene and the liver-to-body weight ratio increased. There were no significant dose-related responses in foetal mortality or weight or in the number of caudal or sternal ossification centres. Five litters of animals receiving the high dose of toxaphene included one or more individuals with encephaloceles.

These studies demonstrated a difference in the susceptibility of the rat and mouse to the foetotoxic effects of toxaphene given in doses toxic to the adults. At dose levels sufficient to cause a high degree of maternal toxicity in rats (35 mg/kg/day), there was a reduction in foetal weight and a decrease in the degree of skeletal ossification. In contrast, the only significant foetal effect noted in the mouse was the occurrence of encephaloceles at the highest dose-rate (35 mg/kg/day); this dose also increased maternal deaths and reduced maternal weight gain.

3174. Dieldrin carcinogenicity re-evaluated

Stevenson, D. E., Thorpe, E., Hunt, P. F. & Walker, A. I. T. (1976). The toxic effects of dieldrin in rats: a reevaluation of data obtained in a two-year feeding study. *Toxic. appl. Pharmac.* **36,** 247.

When rats were fed dieldrin at dietary levels of 0·1, 1·0 or 10·0 ppm for 2 yr, statistical analysis of tumour incidence revealed no treatment-related effects (*Cited in F.C.T.* 1970, **8,** 567). However, more tumours were found in certain groups, particularly in the females

fed 0·1 ppm, and differences in survival rates between groups could have affected the apparent tumour incidence. A re-analysis of the data obtained in this study has now been performed, using an actuarial method which makes allowance for differential survival (Peto, *Br. J. Cancer* 1974, **29,** 101), and tumour histology has also been re-evaluated.

The latter process increased the total tumours by three in the females and three in the males fed 0·1 ppm, two in the females fed 1·0 ppm and one in each sex fed 10 ppm, although in controls the total was reduced by one. It was assumed (in view of inadequate evidence to the contrary) that in all animals dying with tumours, the tumour had been responsible for death, and the probability of tumour death at a particular time was calculated from data for the control animals. Taking into account surviving group sizes at different time intervals, the ratio between observed and expected tumours was then calculated separately for each sex at each treatment level for the commonest tumour types (thyroid tumours in both sexes and mammary tumours in females), for animals with at least one tumour and for those with at least one malignant tumour.

In males there was good agreement between observed and expected values, except at the highest dose level, but no evidence of a consistent increase with dose was found, and results fell well short of statistical significance. In females, although there was a slight excess of observed over expected total tumours, this was neither dose-related nor statistically significant, and the differences in respect of thyroid and mammary tumours were less than those for tumours as a whole. Findings thus supported the previous conclusion that there was no treatment-related increase in tumour incidence.

3175. Teratogenicity of ethylene thiourea and related compounds

Ruddick, J. A., Newsome, W. H. & Nash, L. (1976). Correlation of teratogenicity and molecular structure: ethylenethiourea and related compounds. *Teratology* **13,** 263.

The dithiocarbamates, which include maneb and zineb, are widely used as biocides in agricultural applications. Ethylene thiourea (ETU), one of the many decomposition products of these materials, is known to be mutagenic to bacteria (*Cited in F.C.T.* 1975, **13,** 398), goitrogenic in rats (*ibid* 1973, **11,** 702) and able to induce foetal malformations in both rats and rabbits (*ibid* 1974, **12,** 282). More recently, metabolic studies have indicated that the teratogenic action of ETU is initiated by ETU *per se*, rather than by its degradation products (*ibid* 1977, **15,** 80). In the study cited above, sixteen compounds related to ETU and to its parent compound, ethylenebisdithiocarbamate, were investigated to determine the structural requirement for teratogenicity.

The following compounds were administered individually by stomach intubation to female Wistar rats on day 12 or 13 of gestation at the doses (mg/kg) indicated in parentheses: allylthiourea (240); allyl isothiocyanate (60); ethylenebisisothiocyanate (25); ethylenethiuram monosulphide (60); ethyleneurea (240); *N,N'*-dimethylthiourea (240); imidazole (240); 3-(2-imidazoline-2-yl)-2-imidazolidinethione (200); 2-mercaptobenzimidazole (120); 2-mercapto-1-methylimidazole (240); 2-mercaptothiazoline (240); 4-methylethylenethiourea (240); *N*-methylethylenethiourea (240); 3,4,5,6-tetrahydro-2-pyrimidinethiol (240); 1,1,3,3-tetramethyl-2-thiourea (240); thiourea (240).

Of the compounds tested, only 4-methylethylenethiourea (4-METU) produced anomalies that were indistinguishable in type and frequency from those produced by ETU. None of the remaining 15 compounds exhibited any teratogenic potential at the maternally tolerated dosages and all anomalies in foetuses exposed to these chemicals were minor, with the exception of a spina bifida in a foetus exposed to thiourea. Malformations in foetuses exposed to 4-METU included hydrocephaly, exencephaly, micromelia and talipes.

The imidazolidine ring appeared to be essential for producing teratogenic effects, as demonstrated by the lack of effects produced by dimethylthiourea and 3,4,5,6-tetrahydro-2-pyrimidinethiol. Dimethylthiourea differs from ETU only in having no ring structure and 3,4,5,6-tetrahydro-2-pyrimidinethiol differs only in having a 6-membered ring instead of the 5-membered ring of ETU. 2-Mercaptothiazoline has a similar ring structure to ETU but the exchange of an amide nitrogen for sulphur was associated with an absence of teratogenic effects. The specific location of the sulphur atom also seemed to be important in determining the teratogenic potential. The absence of sulphur from the 2-position resulted in a lack of teratogenic effects. The addition of a methyl group to the 4-position was the only structural change that did not alter the teratogenic potential although neither a methyl group at the 1-position nor a benzene ring at the 4,5-position resulted in teratogenic compounds.

The authors conclude that teratogenicity may be closely linked to molecular structure but that a compound's teratogenic activity can be determined only by appropriate tests and not by comparing its molecular structure with that of known teratogens.

THE CHEMICAL ENVIRONMENT

3176. Iron and the gastro-intestinal tract

Nayfield, S. G., Kent, T. H. & Rodman, N. F. (1976). Gastrointestinal effects of acute ferrous sulfate poisoning in rats. *Archs Path.* **100,** 325.

Acute poisoning resulting from ingestion of inorganic iron salts used in the treatment of iron-deficiency anaemia is common in children. The most prominent and consistent finding at autopsy is haemorrhagic necrosis of the proximal part of the gastro-

intestinal tract. Previous investigations have examined acute ferrous sulphate hepatotoxicity in rats (*Cited in F.C.T.* 1975, **13,** 149). The study cited above reports on the gastro-intestinal effects of this compound.

For mortality studies, rats were dosed with 1 ml ferrous sulphate solution by gastric intubation at levels of approximately 40, 80, 160 and 320 mg elemental iron/kg body weight. In the morphology studies, doses of 80, 160 and 320 mg elemental iron/kg body weight were administered. Death was restricted to four of ten rats given 320 mg/kg. Prostration was pronounced in groups given 320 or 160 mg/kg but improvement was apparent after 24 hr. Animals given 80 mg/kg became lethargic but were normal after 24 hr. No signs of poisoning were observed in rats given 40 mg/kg.

Microscopic examination of stomach sections revealed patchy mucosal necrosis with heavy iron deposition in the damaged tissue. Lesions were of mild to moderate severity in the central part of the stomach, particularly at the lesser curvature, especially the antrum. The squamous epithelial lining of the forestomach was not affected. A faint iron-positive reaction of the endothelium of submucosal vessels was demonstrated at the junction of the forestomach and gastric body, but there was no other evidence of iron absorption in gastric sections. In the small intestine, lesions were diffuse and varied from mild erosion of epithelial cells at the villus tips to severe necrosis involving the crypts. Haemorrhage was common and staining of the endothelium of submucosal and serosal blood vessels was iron-positive. Sections of infarcted bowel segments showed moderate to severe haemorrhagic necrosis and thrombi were identified in mesenteric vessels near these areas. Electron microscopy revealed iron deposits in the basement membranes of the vessels.

In general, the severity and siting of effects was dose related, and fatal doses were always associated with intestinal injury. The most severely affected site in rats given the highest dose was the proximal segment of the small bowel, although three of these six animals also developed infarcts of the terminal segment of the small bowel.

The lesions described were similar in morphology and location to those described in fatal cases of acute ferrous sulphate poisoning in children. The state of shock occurring in these cases has been attributed to the direct action of absorbed iron on plasma proteins and vasculature to produce venous pooling and increased vascular permeability. Vascular changes remote from the corrosive lesions support a theory of systemic toxicity, and the time of death points to iron absorption as the causative factor, since infarcts of the small intestine would have had a less rapid effect.

3177. Sniffing out nickel carcinogenesis

Torjussen, W. & Solberg, L. A. (1976). Histological findings in the nasal mucosa of nickel workers. A preliminary report. *Acta otolar.* **82,** 266.

The increased risk of nasal cancer in workers in the nickel industry is widely documented (*Cited in F.C.T.* 1972, **10,** 113; *ibid* 1974, **12,** 428). In the study cited above, nasal biopsy specimens of workers in a nickel refinery were examined in an attempt to identify precancerous histological changes.

Nasal biopsy specimens were taken from the mucosa of the middle turbinate of a randomly selected group of 92 nickel-exposed workers without known nasal diseases. There were 37 controls with no known previous exposure to nickel compounds. Sections were prepared and stained before being examined by a pathologist who had no knowledge of the backgrounds of individuals. All the biopsy samples showed inflammatory changes, more severe in the exposed group than in the controls. The main changes were leucocyte infiltration in the mucosa and squamous metaplasia in the epithelium. There was more pronounced epithelial keratinization in the nickel-exposed group. Atypical epithelial changes occurred in 17% of the exposed group but in none of the controls. All individuals with atypical epithelial changes worked in departments where there was maximal exposure to nickel and had worked in the nickel industry for more than 10 years. The histological changes were not related to age or smoking habits.

[It was observed in one study that susceptibility to nasal cancer increased with age at first exposure to nickel (*Cited in F.C.T.* 1972, **10,** 113) and it would be interesting to determine whether the histological changes now reported were similarly related to age at first exposure. Although no conclusions can yet be drawn, the changes observed may be related to the induction of nasal cancer and could possibly indicate workers at greatest risk.]

3178. Chelating agents and the foetus

Fisher, D. R., Mays, C. W. & Taylor, G. N. (1975). Ca-DTPA toxicity in the mouse fetus. *Hlth Phys.* **29,** 780.

Mays, C. W., Taylor, G. N. & Fisher, D. R. (1976). Estimated toxicity of Ca-DTPA to the human fetus. *Hlth Phys.* **30,** 247.

Diethylenetriaminepentaacetic acid (DTPA) has been shown to be effective in removing plutonium, lanthanum and yttrium from the body (*Cited in F.C.T.* 1964, **2,** 93), but one of the obstacles to its use for chelating toxic metals has been its tendency also to chelate essential trace metals such as calcium (Ca) and zinc (Zn). In rats, the cobalt and Zn chelates of DTPA have shown considerably lower toxicity than the Ca chelate (*ibid* 1964, **2,** 497), and the two papers cited above go further in demonstrating the relative safety of the Zn chelate.

The first paper describes an experiment in which female mice were given daily sc injections of 2·9 or 0·36 mmol Ca DTPA or Zn DTPA/kg body weight from 4 days after they were put with male mice until their pups were 13 days old. The animals given the higher dose of Ca DTPA produced no viable offspring, although the gross appearance of the one dead foetus seen was normal. There was no permanent effect on the reproductive systems of this group, since all of the dams subsequently produced normal litters.

With the lower dose of Ca DTPA, fertility, foetal development and growth rates during the period of lactation were almost normal. The potential toxicity of the lower dose of Ca DTPA may have been offset by a dietary level of 0·006% Zn. Both dose levels of Zn DTPA appeared to be completely harmless to dams and pups.

The authors warn that these results should not be interpreted as evidence for a tenfold safety factor for humans given a recommended daily dose of 0·036 mmol Ca DTPA/kg by iv drip or closely spaced fractionated injections for three reasons: small rodents eliminate DTPA more quickly than large mammals, a given daily dose of Ca DTPA is more toxic when given in divided fractions throughout the day than when given as a single daily injection, and finally the daily Zn intake per unit of body weight is 35 times lower in humans than in pregnant mice.

The second paper is concerned with estimating the toxic level of Ca DTPA in a pregnant woman. In such women, the daily dose of Ca DTPA predicted to carry a 50% risk of inducing foetal death lies between 10 and 80 μmol/kg on the basis of data from the work on mice or 13–26 μmol/kg on the basis of data from earlier studies using rats. The dose of Ca DTPA previously recommended is 29–36 μmol/kg daily, which is well within the estimated toxic range. It is urged, therefore, that the relatively safe Zn DTPA be used instead of Ca DTPA to reduce this hazard, particularly in women who may be pregnant.

3179. An industrial view of ETU

Smith, D. (1976). Ethylene thiourea—a study of possible teratogenicity and thyroid carcinogenicity. *J. Soc. occup. Med.* **26,** 92.

Ethylene thiourea (ETU), a degradation product of the ethylenebis(dithiocarbamate) fungicides, has been associated with mutagenic effects in bacteria, with the induction of hyperplasia and thyroid tumours in rats and with the development of foetal malformations in rats and rabbits (*Cited in F.C.T.* 1977, **15,** 157). In addition to its fungicidal connexions, ETU is important as an accelerator used in the compounding of rubber, and this industrial use has allowed an attempt to be made to establish the relevance of the experimental findings on this compound to man.

An epidemiological study was carried out among industrial users of ETU to determine whether there was any evidence of an increase in foetal malformations among the offspring of women exposed to the compound during early pregnancy and whether the incidence of thyroid cancer was increased in exposed workers of either sex. In the first case, a retrospective study of women of child-bearing age was carried out at a Birmingham factory manufacturing rubber products containing ETU. Of the women who had left this employment before the use of ETU was stopped in 1972, 255 were identified as having given birth to a total of 420 children. No excessive incidence of malformations was found in this group, compared with the anticipated incidence in the area. The number of women who were actually working in the factory during early pregnancy was small (only 59), but none of this group produced an abnormal child. Similarly, there were no records of congenital anomalies in children born to a group of women working at a large rubber-processing plant in a different area (H. Parkes, unpublished data 1973).

The expected incidence of thyroid cancer in the general population is 2·6/100,000 (0·6 in males and 2·0 in females, with an incidence of 1·45/100,000 in females aged 15–54 yr). None of 1929 employees of several large manufacturing firms using or producing ETU in the Birmingham region appeared in the thyroid cancer list of the Birmingham Cancer Registry for 1957–71. Another small survey (R. A. Randell, unpublished data 1973) revealed that 49 thyroid cancers were reported between 1957 and 1971 in a town in the Midlands, but none of these occurred in workers at a local rubber factory in which ETU had been used since 1952.

While no definite conclusions can be drawn from these limited studies, they do at least provide no positive evidence of any risk of teratogenic effects of thyroid cancer from the normal industrial use of ETU.

3180. Hexachlorophene and triethyl tin on the brain

Lock, E. A. (1976). Increase in cerebral fluids in rats after treatment with hexachlorophane or triethyltin. *Biochem. Pharmac.* **25,** 1455.

Both hexachlorophene (HCP) and triethyltin (TET) produce oedema of the white matter of the central nervous system. In both cases the accumulation of fluid is intramyelinic, and causes splitting of myelin at the intraperiod line (*Cited in F.C.T.* 1974, **12,** 790; *ibid* 1976, **14,** 642; *ibid* 1977, **15,** 258). In TET-treated rats this fluid has been shown to contain sodium chloride (Magee *et al. J. Path. Bact.* 1957, **73,** 107). Biochemical changes in the brain of HCP-treated rats included an increase in glucose levels and a slight decrease in glutamate levels, unaccompanied by changes in redox states or oxidative phosphorylation (*Cited in F.C.T.* 1976, **14,** 643), although the potency of HCP in uncoupling oxidative phosphorylation has been demonstrated elsewhere (*ibid* 1974, **12,** 566). TET is also a potent inhibitor of oxidative phosphorylation, and this has been suggested as the cause of the lesions it produces (*ibid* 1966, **4,** 617). Further biochemical observations on the brain oedema produced by the two compounds are now available.

When rats were given a single ip injection of HCP (78·5 μmol/kg) and killed at intervals up to 24 hr later, brain water was found to increase continuously from 3 hr after the injection. The water content of the spinal cord also increased after 24 hr, as did levels of sodium and chloride in both brain and spinal cord, but potassium levels were unaffected. Glucose concentrations in the brain were also increased at this stage, with a corresponding increase in blood glucose, but haematocrit values were unchanged. The levels of sodium and chloride found in the additional brain water suggested that it could have been derived either from the plasma or the cerebrospinal fluid, and glucose levels tended to support the latter origin. In addition there was a significant decrease in cerebral 2-oxoglutarate and malate, but glutamate and aspar-

tate levels were only slightly depressed and there was no change in the redox state. No evidence of uncoupling of oxidative phosphorylation, such as a lowering of cerebral ATP or phosphocreatine, was found.

In rats given a single injection of TET sulphate (40 μmol/kg), brain-water levels and glucose levels in brain and blood showed after 24 hr increases of the same order as in HCP-treated rats. Again the glucose concentration in the additional brain water was closer to that in the cerebrospinal fluid than in the plasma, although as with HCP it could have originated from either source. Brain glutamate was also significantly decreased, in confirmation of earlier findings (Cremer, *J. Neurochem.* 1964, **11**, 165).

3181. Biphenyl metabolism

Meyer, T., Aarbakke, J. & Scheline, R. R. (1976). The metabolism of biphenyl. I. Metabolic disposition of ^{14}C-biphenyl in the rat. *Acta pharmac. tox.* **39,** 412.

Meyer, T. & Scheline, R. R. (1976). The metabolism of biphenyl. II. Phenolic metabolites in the rat. *Acta pharmac. tox.* **39,** 419.

Meyer, T., Larsen, J. C., Hansen, E. V. & Scheline, R. R. (1976). The metabolism of biphenyl. III. Phenolic metabolites in the pig. *Acta pharmac. tox.* **39,** 433.

Biphenyl is among the compounds that have limited approval for the prevention of surface mould growth on certain fresh fruits. Although a comfortable safety margin separates its no-untoward-effect-level in experimental animals and its use level in citrus fruits for human consumption, it is not altogether clear how closely its metabolism in man resembles that in laboratory animals. In rats and rabbits, biphenyl has been reported to be completely absorbed and excreted in the urine in the form of glucuronides or ethereal sulphates of hydroxybiphenyl compounds (Williams, *Annls Biol. clin.* 1965, **23,** 7; *Cited in F.C.T.* 1973, **11,** 702). Other relatively early work located the enzyme systems responsible for biphenyl hydroxylation in the liver microsomes of several rodents and other species, and demonstrated that in man and the rat, 4-hydroxylation is the principal reaction, although in some species 2-hydroxylation is equally prominent (*ibid* 1964, **2,** 435; *ibid* 1966, **4,** 225). These observations have now been extended in three papers (cited above) covering biphenyl metabolism in rats and pigs.

When rats were given an oral dose of [^{14}C]biphenyl (100 mg/kg, 0·7–1·0 μCi), the total excretion of radioactivity after 96 hr, measured by liquid scintillation counting, was 92·2% of the dose. Urinary excretion accounted for 84·8% and faecal excretion for 7·3% of the dose, of which 81·6% was excreted within 24 hr. Trace amounts of $^{14}CO_2$ appeared in the expired air, and only 0·6% of the dose remained in the tissues after 96 hr. Fractionation of 24-hr urine samples revealed that almost 30% of the dose consisted of conjugated phenolic metabolites, with a further 25% consisting of acidic metabolites. In view of the virtual absence of expired $^{14}CO_2$, decarboxylation reactions were thought to be of minor importance in the metabolism of biphenyl in this species.

A second experiment traced in more detail the metabolic conversion of biphenyl to phenolic metabolites in the rat. A combination of mass spectrometry and gas chromatography facilitated the identification of metabolites and the quantification of their trimethylsilyl ethers. Rats were given biphenyl in a single oral dose of 25 mg (approximately 100 mg/kg). The total urinary recovery was 29.5% of the dose after 96 hr, although most of this (22·3% of the dose) was excreted in the first 24 hr. The two major urinary metabolites were 4-hydroxybiphenyl (7·7%) and 4,4′-dihydroxybiphenyl (11·4%), other compounds detected being either conjugates of mono-, di- and trihydroxybiphenyl derivatives or *m*- and *p*-methyl ethers of the catecholic compounds. Hydroxylation and conjugation were also essential steps in biliary metabolism, which accounted for 5·2% of the dose in the first 24 hr. Although 4·7% of the dose was detected in the faeces after 24 hr, faecal excretion of phenolic biphenyl derivatives was not important. Four metabolites of biphenyl were identified for the first time, namely 3,4-dihydroxy-, 3,4,4′-trihydroxy-, 3,4′-dihydroxy-4-methoxy- and 4,4′-dihydroxy-3-methoxybiphenyl.

In a third study, the phenolic metabolites of biphenyl in the urine of male and female pigs given a single oral dose of 100 mg/kg either in soya oil or in propylene glycol were examined by methods similar to those used in the previous study. The major metabolites were monohydroxylated biphenyls (particularly 4-hydroxybiphenyl); but in this species only small amounts of di- and trihydroxylated derivatives were detected, exclusively in the urine. The total urinary recovery after 96 hr was 44·8% in males and 27·6% in females, most being excreted in the first 24 hr after dosing. No faecal excretion of phenolic metabolites was detected. The overall qualitative metabolic picture of biphenyl in the pig was similar to that in the rat, although there were quantitative differences.

3182. Administration routes and methylchloroform toxicity

Shah, H. C. & Lal, H. (1976). Effects of 1,1,1-trichloroethane administered by different routes and in different solvents on barbiturate hypnosis and metabolism in mice. *J. Toxicol. envir. Hlth* **1,** 807.

Previous inhalation studies have supported the idea that 1,1,1-trichloroethane (methylchloroform; MC) is relatively innocuous under the conditions in which it is most likely to be used as an industrial solvent (*Cited in F.C.T.* 1975, **13,** 402). However, acute exposure to high levels of the material may affect cardiovascular function (*ibid* 1975, **13,** 675) and it is possible that repeated exposure to low concentrations has similar effects (*ibid* 1976, **14,** 75). It is evident that inhalation studies have provided much of the toxicological background on MC and other volatile industrial solvents. The paper cited above points out the need for testing by alternative routes of administration.

Swiss albino random-bred male mice were exposed to MC either by inhalation (1500–5700 ppm for periods ranging from 12 to 96 hr), by ip administration (injected dose 1 ml MC/kg body weight undiluted

or 0·05–2·5 ml MC/kg diluted with olive oil or dimethylsulphoxide (DMSO) to a final volume of 5 ml/kg body weight) or by local application in DMSO to the unshaved abdominal region (MC–DMSO, 1:1 v/v, in a volume of 10 ml/kg body weight). *In vitro* hexobarbitone-oxidase activity and hypnosis produced by sodium pentobarbitone (50 mg/kg, ip) were adopted as indices of MC effects.

Inhalation of MC at 3000 ppm reduced pentobarbitone-hypnosis time and increased the rate of barbiturate metabolism by hepatic microsomal enzymes. Increasing duration of exposure to MC tended to increase the reduction in pentobarbitone-hypnosis time. Similarly, three local topical applications of MC reduced pentobarbitone hypnosis in mice. In contrast, there was a potentiation of pentobarbitone hypnosis and inhibition of hexobarbitone metabolism following ip administration of MC (1 ml/kg). This potentiation was not apparent, even with a larger dose (2·5 ml/kg), when MC was dissolved in olive oil. However, pretreatment with olive oil or simultaneous treatment with the oil at a different site did not afford protection against the potentiation. It is possible, therefore, that the protection given by a mixture of MC and olive oil is due to physical factors affecting absorption or release of MC from the oil.

DMSO given ip before or together with the test compound enhanced the toxicity of MC. Increasing the percentage concentration of DMSO while keeping the dose of MC constant increased the death rate in mice. The hepatotoxic effects and local damage to organs in the peritoneal cavity by DMSO, alone or in combination with MC, may have contributed to the observed marked potentiation of pentobarbitone hypnosis. Compounds administered ip are known to be absorbed into the portal circulation and to reach the liver in high concentration. This present study implies that a direct access to the liver by way of ip injection is necessary for MC and DMSO to potentiate pentobarbitone hypnosis. Further investigations are required to measure liver concentrations of MC and its metabolites following administration by these different routes.

3183. Allergenicity and TDI

Butcher, B. T., Salvaggio, J. E., Weill, H. & Ziskind, M. M. (1976). Toluene diisocyanate (TDI) pulmonary disease: Immunologic and inhalation challenge studies. *J. Allergy clin. Immun.* **58,** 89.

It appears that toluene diisocyanate (TDI) may exhibit serious pulmonary toxicity at levels below the current US and UK TLV of 0·02 ppm (*Cited in F.C.T.* 1971, **9,** 279), but the pathogenesis of these effects is still ill-defined. However, fragmentary evidence of a hypersensitivity mechanism is available (*ibid* 1969, **7,** 272; *ibid* 1970, **8,** 235; *ibid* 1976, **14,** 218) and the present study of workers in a TDI-manufacturing plant attempts to elucidate further the immunological nature of TDI disease.

Immediately before TDI production began, all subjects who were to work on the plant underwent blood tests and were skin-tested against 15 common inhalant allergens. The workers, who were monitored again after 18 months, were classified into three groups. comprising those continuously exposed to TDI (84 subjects), those intermittently exposed (28) and unexposed control individuals (55). There were no marked differences between the three exposure groups in the proportion of the workers who were atopic (as measured before plant start-up). Although no subjects reacted specifically to TDI-antigen in intradermal tests before the plant was on stream, three subjects reacted positively, in the form of wheal-and-flare reactions, when tested after 18 months on the plant. Nevertheless no further TDI-specific responses could be demonstrated in a battery of immunological tests, including eosinophil counts, immunoglobulin levels, *in vivo* tests for anti-TDI antibodies and radioimmunoassays.

Of the 14 subjects who developed 'clinical sensitivity' to the work process, 11 were tested in provocative inhalation tests with 0·005–0·02 ppm TDI. Seven of these exhibited pulmonary responses to the inhaled TDI in the form of airways obstruction; in some cases these reactions were dose related and occurred even at the lowest TDI level tested. As in a previous study, the responses included immediate, delayed and dual types. However, no TDI-induced lymphocyte stimulation could be detected in clinically sensitive individuals and there was no correlation between clinical sensitivity and atopic status.

As the sole immunological change attributable to TDI was a low incidence of wheal-and-flare responses to a prick test with a TDI-antigen, the premise that the pathogenesis of TDI-induced pulmonary disease has an immunological basis must be considered still unproven.

[This is a useful addition to TDI epidemiological studies in that the workers were assessed prior to all industrial TDI exposure. This work is described as the initial phase of a comprehensive study, and it is to be hoped that later stages will include the monitoring of this well-defined human sample a few years hence in order to identify possible changes in responses as a result of prolonged exposure. Unfortunately, no workroom TDI levels were reported in this paper, so an opportunity to relate the findings to the present TLV of 0·02 ppm has been missed.]

NATURAL PRODUCTS

3184. The fate of intravenous coumarin

Ritschel, W. A., Hoffmann, K. A., Tan, H. S. & Sanders, P. R. (1976). Pharmacokinetics of coumarin upon i.v. administration in man. *Arzneimittel-Forsch.* **26,** 1382.

Coumarin (1,2-benzopyrone) occurs naturally in tonka beans, lavender oil, woodruff and sweet cloves and is known to cause liver damage to rats at high doses. In metabolism studies carried out in man, most of a single oral dose of 200 mg coumarin was eliminated in the urine during the first 24 hr, 68–92% as

7-hydroxycoumarin and a further 1–6% as *o*-hydroxyphenylacetic acid (*Cited in F.C.T.* 1969, **7**, 681). These results emphasized the weakness of animal toxicity data as the sole basis for assessing the potential toxicity of coumarin in man, since coumarin is not only absorbed rapidly from the human gut without subsequently entering the enterohepatic circulation, as occurs in the rat, but the products of its metabolism are very different in the two species. The paper cited above attempts to describe the pharmacokinetics of coumarin in man after iv administration.

Five men and one woman were given doses of 0·125, 0·2 or 0·25 mg coumarin/kg iv, and blood samples taken at frequent intervals over the following 6 hr were analysed for coumarin, 7-hydroxycoumarin and its glucuronide. Creatinine was also assayed in a blood sample taken prior to coumarin injection. The mean biological half-life of coumarin was found to be 1·81, 1·45 and 1·49 hr, respectively, at the three dose levels. The blood-level data for coumarin suggested that distribution might best be represented by an open two-compartment model, in which the central compartment comprised the plasma, highly perfused organs and the extracellular and intracellular fluid and the peripheral compartment comprised the deeper tissues. Coumarin appeared to be equilibrated evenly within the central compartment and widely distributed in the peripheral phase. However, the finding that the amount transferred to the tissues did not increase in direct proportion to dose was not in keeping with such a model, since it suggested that tissue saturation was occurring.

The total clearance (total removed by excretion and metabolism) was larger than the creatinine clearance, a finding which could be interpreted either by additional active tubular secretion or by extrarenal disposition. In this case it is likely that the high level of clearance could be attributed to extensive metabolism, data on which the authors intend to present in a further paper.

3185. Tracking down the locoweed toxin

James, L. F. & Van Kampen, K. R. (1976). Effects of locoweed toxin on rats. *Am. J. vet. Res.* **37**, 845.

The ingestion of locoweeds (species of Astragalus and Oxytropis found in the United States) by livestock produces marked neurotoxic and foetotoxic effects, which can be reproduced in laboratory animals (*Cited in F.C.T.* 1972, **10**, 604; *ibid* 1973, **11**, 344). Cytoplasmic vacuolation also occurs in a number of organs, of which the kidney is the first to be affected (Van Kampen *et al. Path. vet.* 1970, **7**, 503). This finding suggested that the toxic factor might be concentrated by the kidney and excreted in the urine, a hypothesis that the present study was designed to confirm.

Rats were given either an aqueous extract of locoweed or the urine from sheep intubated daily with 227 g ground locoweed in place of drinking-water for 30 days. Two further groups were maintained for the same period on feed containing 10 or 25% locoweed. The 25% level caused weight loss and an initial excitability followed by depression, and at autopsy there was evidence of severe cytoplasmic vacuolation of the proximal renal tubular epithelial cells and the hepatocytes. Similar but less marked histological changes occurred at the 10% level. In the two other groups, findings were almost identical to those at the 25% level, but in addition the group given urine from treated sheep showed vacuolar changes in the neurons of the central nervous system. In the sheep, signs of locoweed poisoning began to appear after about 2 wk, and autopsy after 30 days revealed erosion of the abomasal mucosa, thyroid enlargement and severe vacuolation of the neurons of the central and autonomic nervous systems, the epithelial cells of the proximal (and to a lesser extent also the distal) convoluted tubules and other visceral organs.

The study indicated that the locoweed toxin is water-extractable and is not detoxified by the body, but is excreted, and indeed appears to be concentrated in the urine.

[A further control group of rats receiving urine from sheep not given locoweed would have rounded off this study, since the possibility of effects of other components of sheep's urine, although unlikely, cannot be ruled out of the results for the group given urine from the treated sheep.]

COSMETICS, TOILETRIES AND HOUSEHOLD PRODUCTS

3186. Teratology and percutaneous toxicity of hair dyes

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

Earlier long-term tests on three oxidation-hair-dye formulations by topical application to mice gave no evidence of systemic toxicity or carcinogenicity (Burnett *et al. Fd Cosmet. Toxicol.* 1975, **13**, 353), and percutaneous absorption of directly acting (semipermanent) hair dyes is low (*Cited in F.C.T.* 1972, **10**, 884). Feeding studies in rats and rabbits have provided no evidence of teratological effects from direct hair dyes (*ibid* 1972, **10**, 884).

Twelve hair-dye composite formulations covering a broad spectrum of dyes were tested in two separate studies. Three of the dyes were of the semipermanent type and nine were of the oxidation type; the latter are mixed with an equal volume of 6% hydrogen peroxide just before use. These studies form part of a continuing American hair-dye industry safety-testing programme, undertaken as a result of some speculation that hair dyes may be mutagenic and carcinogenic to man.

The 12 hair-dye formulations were applied at a dose rate of 2 ml/kg to a shaved area of skin on groups of 20 female rats, on days 1, 4, 7, 10, 13, 16 and 19 of gestation. There were no signs of toxicity throughout the study. Changes in body weight and food consumption were similar for treated and con-

trol rats. At day 20 of gestation, the dyes had produced no significant changes in the mean numbers of corpora lutea, implantation sites or live foetuses or in the sex ratio of the foetuses, nor were there any significant changes in the number of resorptions. Minor skeletal anomalies in three of the 20 litters examined after treatment, of the dams with one of the dye formulations were not thought to be due to the treatment.

No evidence of compound-induced toxicity was found in groups of 12 adult rabbits after twice-weekly topical application of the 12 hair-dye formulations for 13 wk at dose rates of 1 ml/kg. The animals were weighed weekly during the study and haematological and clinical chemistry determinations and examinations of urine were performed on all animals at 0, 3, 7 and 13 wk. No toxic effects were observed with any of the dyes. After 13 wk there was no evidence of dye-induced changes in organ-to-body weight ratios, in the incidence of disease or in the appearance of gross or microscopic lesions. No discoloration of the urine by dye was seen at any time during the tests. Treated skin showed slight thickening in some groups, probably as a result of the frequency of dye application.

The authors considered the dose rates used to be well above those resulting from normal use of the products.

3187. More light on detergent foetotoxicity

Inouse, K. & Masuda, F. (1976). Effects of detergents on mouse fetuses. *J. Fd Hyg. Soc., Japan* **17**, 158.

Branched-chain alkylbenzene sulphonates (ABS) have recently been largely replaced in detergents by linear alkylbenzene sulphonates (LAS) because of the latter's biodegradability. The teratogenic potential of both types of compound has been widely investigated (*Cited in F.C.T.* 1976, **14**, 152). Most of the work so far reported suggests that adverse effects on the foetus occur only at levels that are toxic to dams, although some rather limited Japanese studies suggested that LAS-based liquid detergent for domestic use had teratogenic potential when given orally, sc or dermally to mice or rats. In the work cited above, the effects of two types of domestic liquid detergent, designated A and B, were determined by dermal application and sc administration to pregnant mice. Detergent A contained LAS (17%), ethanol (7%) and urea (15%), and detergent B contained a polyoxyethylene alkyl ether (8%), sodium alkyl ethoxy sulphate (7%) and ethanol (4%).

For dermal application, detergents A and B were diluted with distilled water to give 0·5, 5 and 15% solutions. Both detergent A and the distilled water control were applied to a shaved dorsal area of groups of 11–20 female mice at a rate of 0·5 ml/mouse on days 1–13 of gestation. Solutions of detergent B and its control were similarly applied on days 0–13 of gestation. For sc administration, both detergents were diluted with distilled water and given in a volume of 10 ml/kg. Detergent A was given once daily at doses of 30 and 150 mg/kg from day 7 to 13 or 0 to 13 of gestation to groups of 16–17 dams. Doses of 5, 50 or 500 mg detergent B/kg were similarly given from day 7 to 13 of gestation to groups of 15–16 dams. Animals given detergent A dermally were killed on day 17 of gestation while all others were killed on day 18, and the foetuses were removed surgically.

There were no statistically significant differences between treated groups and controls in weight gain, pregnancy rate or toxic effects on the dams. With a few exceptions there were no significant differences in numbers of implantations, live and dead foetuses, sex ratio or body weight of live foetuses between treated groups and controls. The exceptions were a significantly higher number of foetal deaths in the group given 15% detergent A dermally, higher placental weights in the groups given 0·5 or 15% detergent A dermally and a significantly greater number of implantations in the group given 150 mg detergent A/kg sc from day 0 to 13. There were no differences between treated groups and controls in the numbers of external and internal anomalies, apart from skeletal anomalies. There was a significantly low incidence of 14th rib in the group given 0·5% detergent A dermally and a significantly high incidence in the group given 15% detergent B dermally when compared with controls. There were also several skeletal anomalies in the animals given the detergents sc. There was a significantly high incidence of 14th rib in the group given 30 mg detergent A/kg from day 7 to 13 of gestation and a significantly increased incidence of foetuses with extra sternebrae in the group given 150 mg detergent A/kg from day 7 to 13 of gestation; no such results were found within groups given detergent A from day 0 to 13 of gestation. The group given 50 mg detergent B/kg showed a significantly greater incidence of 14th rib than did the control.

The skeletal malformations encountered showed no clear relationship to detergent dose and the results of this study thus provide no conclusive evidence of embryotoxic or teratogenic effects attributable to the detergents.

BIOCHEMICAL PHARMACOLOGY

3188. Cadmium and the liver enzyme systems

Yoshida, T., Ito, Y., Suzuki, Y. & Uchiyama, M. (1976). Inhibition of hepatic drug metabolizing enzyme by cadmium in mice. *Bull. env. contam. & Toxicol. (U.S.)* **15**, 402.

Cadmium, administered either sc or orally, has been shown to accumulate in the livers and kidneys of experimental animals. Previous studies have indicated that the cadmium accumulated in these organs is bound to a soluble cytoplasmic protein fraction, metallothionein (*Cited in F.C.T.* 1975, **13**, 471). In the

liver the synthesis of the protein appears to be greatly stimulated in response to cadmium uptake (*ibid* 1976, **14,** 508). The study cited above examines the effect of cadmium on hepatic drug-metabolizing enzymes and attempts to ascertain the possible role of metallothionein as a protective agent against the impaired activity of these enzymes by cadmium administration.

Amidopyrine *N*-demethylation was monitored in male mice following the administration of a dose of 3 mg cadmium chloride/kg. After 24 and 48 hr a significant inhibition of activity occurred, but activity returned nearly to control values after 72 hr. In further experiments the interval chosen for investigation was 48 hr, as the potentiation of the inhibition of amidopyrine *N*-demethylation was then maximal. Both amidopyrine *N*-demethylation and aniline hydroxylation were examined after the administration of 1, 3 and 5 mg cadmium chloride. The intensity of the inhibition of drug-metabolizing enzymes was dose-dependent, suggesting that the inhibition resulted from the direct action of cadmium on the drug-metabolizing enzymes.

Mice that have been exposed to low levels of cadmium are known to survive longer than untreated mice when exposed to a higher dose (Yoshikawa, *H. Ind. Hlth* 1973, **11,** 113). In an attempt to demonstrate this effect in the enzyme system, mice were pre-dosed with 0·5, 1·0 or 3·0 cadmium chloride/kg and 24 hr later were injected ip with a 5 mg/kg challenging dose. After a further 48 hr, the rates of amidopyrine *N*-demethylation and aniline hydroxylation and the levels of cytochrome *P*-450 in the liver were determined. Results suggested that inhibition of the drug-metabolizing enzymes by cadmium was due to a decrease in cytochrome *P*-450 content, although the mechanism involved needs clarification. The inhibition observed and the decrease in cytochrome *P*-450 content by the challenging dose were diminished when mice were pre-dosed and the degree of protection afforded was dose-related. Effects on the hepatic drug-metabolizing enzymes and cytochrome *P*-450 content were completely abolished when mice were pre-dosed with 3 mg cadmium chloride/kg.

The investigators conclude that metallothionein may well be involved in protection against cadmium poisoning but further studies are required to elucidate the contributory mechanisms.

PATHOLOGY

3189. Macromolecules and the gut

Leary, H. L., Jr. & Lecce, J. G. (1976). Uptake of macromolecules by enterocytes on transposed and isolated piglet small intestine. *J. Nutr.* **106,** 419.

Worthington, B. S. & Syrotuck, J. (1976). Intestinal permeability to large particles in normal and protein-deficient adult rats. *J. Nutr.* **106,** 20.

The suspicion that macromolecular absorption may be involved in the pathophysiology of various diseases has promoted interest in this field, and the established concern of the immunologist has recently been replaced by a broader experimental approach to intestinal absorption.

In the neonate, the intestinal uptake of macromolecules derived from the colostrum of the maternal animal plays an important role in the acquisition of passive immunity. In the first study cited above, the differential ability of parts of the gut of the neonatal piglet to absorb a marker protein and the effect of the digesta on the 'closure' of the gut towards this protein were examined.

A portion of the ileum of neonatal piglets was surgically transferred to the proximal third of the small intestine. The transposed segment absorbed fluorescent porcine γ-globulin by pinocytosis, at a time when the proximal intestine on either side was no longer active. The activity of the transposed ileal segment was similar to that of the area of the intestine from which it came. Thus, near the time of birth, the piglet's small intestine appears to be differentiated in its capacity to absorb macromolecules.

Regions of the small intestine were isolated from the direct digestive pathway to evaluate the effects of the digesta on the 'closure' of the gut. The ability of these isolated segments to absorb the marker protein was compared with that of corresponding areas within the digestive pathway. Isolated segments from all areas of the gut continued to take up marker protein actively for 23 days. In contrast, the absorption activity of the intestine within the digestive pathway was dependent on position, the proximal, middle and distal regions absorbing the marker for only 8, 6–16 and 18 days, respectively. The authors conclude that contact with digesta accelerates the rate of termination of the differentiated capacity of the intestinal epithelium of the neonatal piglet to absorb macromolecules.

The second study cited investigated the absorption of a range of macromolecules from the ileum and jejunum of mature rats. Large (mol wt >1,000,000), medium (mol wt 650,000) and small (mol wt 40,000) tracer macromolecules were absorbed by pinocytosis in rats maintained on a standard diet. The relative amounts of tracer absorbed by these control animals decreased as the molecular weight increased and, with the exception of the smallest polymer, which was demonstrated in the extracellular mucosal region, the tracer particles were believed to be present exclusively in the lysosomes.

Rats maintained for up to 5 months on a 0·5%-protein diet exhibited morphological evidence of intestinal deterioration, specifically villous atrophy and periodic separation of the junctional complexes between absorptive cells. Although quantitatively absorption was comparable between control and protein-deficient animals, it was thought likely that direct intercellular movement of macromolecules had occurred in the gut of severely protein-deficient rats (deprived of sufficient protein for 4 months or more)

as tracer particles were present between and beneath the intestinal epithelial cells.

[The second study indicates that there may be significant macromolecular absorption in some circumstances in the mature gut. There is a need to investigate further the extent of this absorption and the conditions in which it arises.]

CANCER RESEARCH

3190. Activation of aromatic acetamides

King, C. M., Olive, C. W. & Cardona, R. A. (1975). Activation of carcinogenic arylhydroxamic acids by human tissues. *J. natn. Cancer Inst.* **55**, 285.

Tumour induction by certain *N*-arylacetamides has been shown to involve a two-step activation process, initial *N*-hydroxylation being followed by $N \rightarrow O$ aryl transfer. The *N*-acyloxyarylamines thus formed are capable of spontaneous covalent combination with both proteins and nucleic acids (Bartsch *et al. Biochim. biophys. Acta* 1973, **304**, 42). The paper cited above investigates the ability of human tissues to perform the second (aryl transfer) step of this activation. Arylhydroxamic acid acyltransferases have been identified in the tissues of several animals.

Labelled *N*-hydroxy-*N*-2-fluorenylacetamide (HFA) or *N*-hydroxy-*N*-4-biphenylacetamide (HBPA), and tRNA were incubated with cytosols (105,000 **g**) of human tissue. When transferase activity was present in the cytosol, the arylamine groups of either HFA or HBPA became covalently bound to the nucleic acid. All specimens of small intestine obtained during surgery were capable of inducing the formation of these adducts and some activity was also apparent in the colon and in a caecal carcinoma. However, only one out of four samples of small intestine obtained at autopsy showed significant activity, and adduct formation was not observed in assays of any other post-mortem tissues apart from liver specimens. It appears, therefore, that the human acyltransferases, like those previously obtained from the tissues of the rat (Bartsch *et al. loc. cit.*; King, *Cancer Res.* 1974, **34**, 1503), have a limited stability.

Cytosols obtained from biopsy specimens of small intestine were examined further to characterize the activity of the human enzyme. Adduct formation was found to be a function of incubation time and was independent of the concentration of the *N*-hydroxy substrate. Under optimum conditions HFA had a much greater affinity for the nucleic acid than did HBPA. Incorporation of the labelled acetyl group of HFA into the RNA was less than 10% of that of the labelled fluorene nucleus. These findings were consistent with previous reports on the $N \rightarrow O$ acyltransferase activity of tissues from various animal species and, together with the relative inactivity of most autopsy specimens, support the view that the adduct formation between nucleic acids and *N*-hydroxyarylacetamides in human tissues is a result of acyltransferase activity in the human tissues, rather than of enzyme activity arising from bacterial contamination, for example.

This study indicates one pathway by which possible arylamine carcinogens could alter macromolecules in human tissue. The carcinogenic process, however, is a complex one and it is difficult to establish the significance of acyltransferase activity or other activation mechanisms in the induction of tumours.

3191. Sinister synergism for the smoker

Wehner, A. P., Busch, R. H. & Olson, R. J. (1976). Effects of diethylnitrosamine and cigarette smoke on hamsters. *J. natn. Cancer Inst.* **56**, 749.

Argus, M. F. & Arcos, J. C. (1976). Hydrocarbon-nitrosamine synergism as a possible amplifying factor in lung tumorigenesis by tobacco smoke. *J. theor. Biol.* **56**, 491.

Nitrosamines are versatile and sometimes potent carcinogenic agents, and may be present in smoked and fried foods (*Cited in F.C.T.* 1976, **14**, 205). Their possible interaction with other compounds to which an animal may simultaneously be exposed has received some attention (*ibid* 1970, **8**, 79), and the question of interaction between nitrosamines and exposure to cigarette smoke has recently been investigated.

The first paper cited above describes the results of 12 consecutive weekly sc injections of 0·25 mg diethylnitrosamine (DENA) given to male hamsters, half of the group also being exposed to cigarette smoke for 10 min three times/day, 5 days/wk for life. Control groups of hamsters received either smoke or sham smoke exposures only. Animals given DENA developed more epithelial lesions of the larynx than did the controls, and cigarette smoke enhanced this effect. Emphysema was more common in the group exposed to both DENA and cigarette smoke than in those receiving DENA alone. Lung lesions were milder in animals receiving DENA only than in either of the smoke-exposed groups. Necrosis of the liver was commoner in smoke-exposed animals than in those exposed to DENA alone. No high incidence of tumours of the nasal cavity, trachea and lower respiratory tract was observed, and no significant incidence of malignant tumours was recorded. Lifespan was not significantly affected by DENA but showed a significant increase, accompanied by lower body weights, in smoke-exposed hamsters.

The second paper considers the report that pulmonary tumours have been produced in rats and mice by simultaneous administration of 3-methylcholanthrene and dimethylnitrosamine (DMNA) in doses that separately are not carcinogenic for the lung. Two possible mechanisms of this synergism are discussed. One is based on the assumption that tumorigenesis is due to methylcholanthrene and that DMNA increases the topically available active epoxide by

reducing the level of available pulmonary epoxide hydrases; the other involves the possible inhibition by one carcinogen of the enzyme responsible for the hepatic metabolism of the other, thus making more carcinogen available for tumour induction in extrahepatic tissues. The authors argue that since tobacco smoke contains both nitrosamines (including DMNA) and hydrocarbons (benzo[*a*]pyrene and benzo [*β*]fluoranthene) similar in carcinogenic properties to 3-methylcholanthrene, synergism between carcinogenic hydrocarbons and nitrosamines may be involved in the genesis of lung tumours in smokers. The suggestion is also made that readier induction of lung cancer in smokers might accompany the choice of a diet rich in nitrosamines or nitrosamine precursors.

3192. Sarcoma and the shape of nickel

Jasmin, G. & Riopelle, J. L. (1976). Renal carcinomas and erythrocytosis in rats following intrarenal injection of nickel subsulphide. *Lab. Invest.* **35**, 71.

Sunderman, F. W., Jr. & Maenza, R. M. (1976). Comparisons of carcinogenicities of nickel compounds in rats. *Res. Commun. chem. Path. Pharmac.* **14**, 319.

It has previously been demonstrated that nickel and some nickel compounds, including nickel subsulphide (Ni_3S_2), produce tumours at the site of intramuscular injection (*Cited in F.C.T.* 1964, **2**, 33; Gilman, *Cancer Res.* 1962, **22**, 158). The carcinogenic potential of nickel compounds seems generally to be inversely proportional to their solubility in water (*Cited in F.C.T.* 1969, **7**, 334). The two recent studies cited above shed further light on the potential of various nickel compounds to induce tumours.

In the first study cited, rats were injected intrarenally with Ni_3S_2 and other metallic compounds. The first experiment involved four groups of young female rats; one group was injected with 10 mg Ni_3S_2 in 1 ml saline in the jugular vein, two groups were injected with 10 mg Ni_3S_2 intrarenally in either 0·1 ml glycerine or 0·1 ml saline (half of the dose into each pole of the right kidney), and a control group received intrarenal glycerine only. None of the animals injected iv had increased numbers of red blood cells or renal neoplasms, but intrarenal administration of Ni_3S_2 in either glycerine or saline caused an increase in red blood cells and an average renal tumour incidence of 40% after 12 months.

Despite the complex morphology of the renal parenchyma, the neoplastic renal cells appeared to be epithelial in origin but showing a pronounced tendency to evolve towards an anaplastic spindle-cell variant. There was no indication that the erythrogenic and carcinogenic responses to Ni_3S_2 were interrelated, as the high haemoglobin and erythrocyte values tended to fall again with the development of the renal carcinoma.

In the second experiment, rats were given renal injections of Ni_3S_2, nickel monosulphide (NiS), metallic nickel, cobalt sulphide, metallic cobalt, metallic chromium, metallic cadmium, metallic lead or metallic gold (5 mg suspended in 0·05 ml glycerine being injected into each pole of the right kidney, as before). Controls were injected with glycerine only. No renal carcinogenic or erythrogenic response was provoked after 12 months except in the case of Ni_3S_2. There were no histological effects with the other compounds, except intense fibrosis around the metal salt deposits and occasional calcification in the lower nephron.

In the second study cited, rats were given single intramuscular injections of various insoluble nickel-containing powders (particles less than 2 μm in diameter) suspended in a penicillin solution. The insoluble nickel compounds NiS, Ni_3S_2, a partially converted nickel-iron sulphide (Ni_4FeS_4) matte (resembling an intermediate encountered in the refining of pentlandite ore) and metallic nickel produced markedly different incidences of local sarcomas after 2 yr. Thus sarcomas developed in 17 of 19 rats given a dose of 5 or 20 mg Ni_3S_2, while amorphous NiS induced no tumours, findings in agreement with the results of the study described above. With the partially converted Ni_4FeS_4 matte in a dose of 9·2 or 36·8 mg, the incidence of sarcomas lay between the incidence with Ni_3S_2 and that with metallic Ni. The tumours were classified as fibrosarcomas (50%), rhabdomyosarcomas (32%), undifferentiated sarcomas (11%) and pleomorphic sarcomas (7%). It is suggested that differences in the chemical bonding in amorphous NiS and in the rhombohedral crystals of Ni_3S_2 used in this study may be a factor in the different sarcoma-inducing potential of these two materials, since both compounds have a very low solubility in water.

3193. Carcinogens in the colon

Renwick, A. G. & Drasar, B. S. (1976). Environmental carcinogens and large bowel cancer. *Nature, Lond.* **263**, 234.

There is strong evidence that environmental and probably dietary factors are involved in large-bowel cancer (*Cited in F.C.T.* 1972, **10**, 440). Diet influences both bile-acid levels and the nature of the gut flora, both of which have been implicated in colon carcinogenesis. It has been demonstrated (*loc. cit.*) that certain known carcinogens are absorbed in the upper reaches of the gut, that they are *N*-hydroxylated and conjugated with glucuronic acid in the liver, and that the conjugate is then excreted in the bile from which the gut flora liberates the free *N*-hydroxy compound, the postulated active carcinogen. The paper cited above describes preliminary investigations on an analogous mechanism by which the carcinogen benzo[*a*]pyrene may reach the large bowel without causing cancer in other parts of the gut.

Two bile-duct-cannulated rats were each given 1 mg [7,10,14-^{14}C]benzo[*a*]pyrene. The bile was collected for 4 hr and contained 6·7 and 12·5% of the ^{14}C. Analysis by thin-layer chromatography showed a single peak of radioactivity corresponding to conjugated metabolites. Aliquots of the pooled bile containing the ^{14}C-labelled metabolites of benzo[*a*]pyrene were incubated with samples of human and rat faeces, or with pure cultures of bacteria representing the major types found in the intestine, for 72–96 hr under anaerobic conditions. The reaction mixtures

were then analysed by thin-layer chromatography, which revealed that almost all the bacteria hydrolysed the conjugates to release the oxidative metabolites of benzo[*a*]pyrene. These reactions are a reversal of the detoxication processes occurring within the liver. The organism releasing most parent hydrocarbon in the test, *Clostridium paraputrificum*, is found in larger numbers in the faeces of high-risk ('western') subjects than in those of low-risk groups.

Diet probably affects the metabolism of polycyclic aromatic hydrocarbons through its influence on the bile acids and on mucosal enzymes such as aryl hydrocarbon hydroxylases. Bile acids may influence in two ways the metabolism of polycyclic aromatic hydrocarbons excreted in the bile. They are important in the formation of mixed micelles in fat digestion, and polar conjugates of benzo[*a*]pyrene may be incorporated into the micelles, aiding their passage through the gut. Further, polycyclic aromatic hydrocarbons are lipid soluble, which could make them inactive in the aqueous gut environment. Bile acids are powerful solubilizing agents and would maintain the carcinogens in solution, thus aiding their interaction with cell surfaces.

The authors conclude with the suggestion that large-bowel cancer results from retoxification, by the gut flora, of liver metabolites of polycyclic aromatic hydrocarbons, a process influenced by diet. The disease is thus more common in western countries because western diets favour this retoxification and because industrial activity increases the amount of polycyclic aromatic carcinogens in the environment.

Fd Cosmet. Toxicol. Vol. 15, p. 373. Pergamon Press 1977. Printed in Great Britain

MEETING ANNOUNCEMENTS

ENERGY USE

The first International Conference on Energy Use Management is to be held on 24–28 October 1977 at the Marriott Hotel and Tucson Community Center, Tucson, Arizona, USA. Organized by The Interdisciplinary Group for Ecology, Development, and Energy (EDEN), Los Angeles, and the University of Arizona, the conference will focus on the need for efficient use of energy and the ways in which this may be achieved. The programme will have a technical emphasis, but will also be concerned with relevant social and economic considerations. A Community Workshop, to be held in conjunction with the technical programme, will include demonstrations, discussions, films and displays relating to the more efficient use of energy in the home and community.

Further information may be obtained from the General Chairman: Dr. Craig B. Smith, Energy Use Management Conference, P.O. Box 64369, Los Angeles, California 90064, USA.

IMPORTANCE OF DIETARY FIBRE

A Nutrition Symposium entitled "New Developments in the Importance of Dietary Fibre and Health" has been arranged by the Kellogg Company of Great Britain, Ltd, and will be held at the Royal Society of Medicine, 1 Wimpole Street, London W1, on 5 and 6 December 1977. Admission will be by invitation only and programmes and invitation cards will be available on request in September.

Further information may be obtained from Ms Suzanna Hammond, Lexington International, 38 Berkeley Square, London W1X 6BS, England.

POLISH SYMPOSIUM ON OCCUPATIONAL SAFETY

"SECURA-78", an international exhibition and scientific symposium on problems of occupational safety and health will be held in Poznań, Poland, on 16–21 April 1978. The symposium, which is being organized in collaboration with the International Labour Office and the International Social Security Association, will last from 17 to 20 April and will be divided into two main sections. Section I will deal with the control of risks encountered in chemical processes and Section II with the control of risks arising from in-plant transport and working at heights. Among the topics to be considered in Section I are the evaluation of chemical hazards in the working environment, methods for their elimination or limitation and protective measures for personnel. The conference languages will be English, German, Polish and Russian, with provision of simultaneous translations, and material will be published in the English, Polish and Russian versions.

Correspondence concerning the symposium should be sent to: Sympozjum "SECURA-78", Centralny Instytut Ochrony Pracy, ul. Tamka 1, 00-349 Warszawa, Poland.

Fd Cosmet. Toxicol. Vol. 15, p. 375. Pergamon Press 1977. Printed in Great Britain

NOTICE

COSMETIC INGREDIENT REVIEW

The Cosmetic Ingredient Review, begun in late 1976, has developed from a private initiative aimed at determining the safety of ingredients used in cosmetics. The vehicle for this review will be a panel consisting of experts whose disciplines and scientific achievements are particularly relevant to the concerns of the review. On the basis of all the information available from both published and unpublished scientific sources, from laboratory-animal investigations, from human experience and from knowledge about the patterns of use of the chemical, the Panel will render a judgement as to whether or not the ingredient is safe for use in cosmetics. A lack of information will not serve as an adequate basis for a determination of safety. For those ingredients on which there is insufficient information to allow an opinion to be reached, the Panel will recommend additional investigations which must be put in hand in order to fill the gaps in information.

The process of the review of cosmetic ingredients is expected to continue over several years, since the total number of ingredients to be reviewed is large. The spectrum of ingredients is expected to change with time, and it may be anticipated that new scientific questions will be raised from time to time.

With the exception of information involving frankly proprietary material and trade secrets, the review will be an open process. Meetings will generally be open to the public and each meeting will include a period set aside for an open public hearing during which interested parties will be invited to present data and views to the Expert Panel. Further, public comments will be requested on the data and materials used for the Panel's deliberations and on the reports issued by the Panel before they are made final. Finally, the Expert Panel will be assisted by persons serving in a liaison role from consumer interests, the FDA and the cosmetic industry.

The course of the review is governed by procedures which describe in detail the manner in which the review is to be conducted. Copies of the procedures, as well as the Tentative First Priority List of Cosmetic Ingredients for Review by the Expert Panel, are available from Linda L. Broadwater, Administrator, Cosmetic Ingredient Review, 1133—15th Street, N.W., Suite 1275, Washington, D.C., 20005, USA.

Fd Cosmet. Toxicol. Vol. 15, p. 377. Pergamon Press 1977. Printed in Great Britain

FORTHCOMING PAPERS

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

One-year toxicity study of Orange G in the ferret. By P. G. Brantom, I. F. Gaunt and J. Hardy.

Short-term toxicity of furfuryl mercaptan in rats. By J. C. Phillips, I. F. Gaunt, J. Hardy, I. S. Kiss, S. D. Gangolli and K. R. Butterworth.

Short-term toxicity study of di-(2-ethylhexyl) phthalate in rats. By T. J. B. Gray, K. R. Butterworth, I. F. Gaunt, P. Grasso and S. D. Gangolli.

Morphological changes in monkeys consuming a diet containing low levels of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. By J. R. Allen, D. A. Barsotti, J. P. Van Miller, L. J. Abrahamson and J. J. Lalich.

Mycotoxic diseases produced in mice by species of the *Aspergillus ochraceus* group. By J. L. Zimmermann, W. W. Carlton, J. Tuite and D. I. Fennell.

Inhibition of yeast alcohol dehydrogenase by dehydroretronecine. By P. S. Sun, M.-T. S. Hsia, F. S. Chu and J. R. Allen.

N-Nitrosodiethanolamine in cosmetics, lotions and shampoos. By T. Y. Fan, U. Goff, L. Song, D. H. Fine, G. P. Arsenault and K. Biemann.

Effect of L-tryptophan on diethylnitrosamine and 3'-methyl-4-*N*-dimethyl-aminoazobenzene hepatocarcinogenesis. By R. P. Evarts and C. A. Brown.

Volatile nitrosamines from ion-exchange resins. By T. A. Gough, K. S. Webb and M. F. McPhail.

The presence of dimethyl- and diethyl-nitrosamines in deionized water. By W. Fiddler, J. W. Pensabene, R. C. Doerr and C. J. Dooley. (Short paper)

Short-term toxicity study of *Paxillus involutus* in the rat. By L. Nieminen, K. Bjondahl, H. Ojanen and E. Ohenoja. (Short paper)

Teratogenicity of 2,5-diaminotoluene, a hair-dye constituent, in mice. By M. Inouye and U. Murakami. (Short paper)

Absorption of ingested talc by hamsters. By A. P. Wehner, T. M. Tanner and R. L. Buschbom. (Short paper)

Criteria employed by the expert panel of FEMA for the GRAS evaluation of flavouring substances. By B. L. Oser and R. L. Hall. (Review 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.

Illustrations and Diagrams. These should be kept to a *minimum* and they should be numbered and marked on the back with the author's name. Legends accompanying illustrations should be typewritten on a *separate* sheet. Diagrams and graphs must be drawn in Indian ink on good quality paper or tracing linen. The following standard symbols should be used on line drawings since they are easily available to the printers:

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Photographs and photomicrographs should be submitted unmounted and on glossy paper. When colour plates are to be printed, payment for blockmaking is the responsibility of the author.

Tables and Figures. These should be constructed so as to be intelligible without reference to the text, each table and column being provided with a heading. The same information should not be reproduced in both tables and figures.

Chemical Nomenclature. The fundamental principles of organic and inorganic chemical nomenclature are laid down in the I.U.P.A.C. 1957 Rules (Butterworths Scientific Publications, London, 1958, 1959). These are given in *Handbook for Chemical Society Authors* (1961), pp. 16–163.

Other Nomenclature, Symbols and Abbreviations. In general, authors should follow the recommendations published in the *Handbook for Chemical Society Authors* (1961), p. 164 and in the *I.U.P.A.C. Information Bulletin*, No. 13, p. 64, Appendix B (1961). In the title and summary, abbreviations should be avoided; in the Introduction, Results and Discussion they should be used sparingly.

Page Proofs. These will be sent to the first-named author for correction.

Reprints. Reprints can be ordered on the form accompanying proofs.

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