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Aims and Scope

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

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

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Research Section

DOSE-RESPONSE STUDY OF THE CARCINOGENICITY OF DIETARY SODIUM NITRITE AND MORPHOLINE IN RATS AND HAMSTERS

R. C. SHANK and P. M. NEWBERNE

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(*Received 8 June 1975*)

Abstract—Long-term feeding studies were conducted in Sprague–Dawley rats and Syrian golden hamsters using various dietary concentrations of nitrite and morpholine (up to 1000 ppm of each) or *N*-nitrosomorpholine (5 or 50 ppm). Most combinations of the two chemicals induced a high incidence of hepatocellular carcinoma in rats and a lower incidence in hamsters. The highest level of nitrite and morpholine (1000 ppm of each) had a stronger potential for carcinogenesis in both rats and hamsters than did a dietary level of 50 ppm of preformed *N*-nitrosomorpholine. Nitrite and morpholine also induced angiosarcomas in both species, most frequently in the liver, with the lung as the next most common site. The nitrite concentration in the diet seemed to have a greater effect on the incidence of hepatocellular carcinoma and angiosarcoma in the rat than did the concentration of morpholine. Dietary concentrations of 5 ppm each of nitrite and morpholine induced hepatocellular carcinoma and angiosarcoma in some rats. High concentrations of sodium nitrite alone were associated with a relatively high incidence of lymphoreticular tumours.

INTRODUCTION

What began as the study of a few isolated industrial poisonings (Barnes & Magee, 1954; Freund, 1937; Hamilton & Hardy, 1949; Watrous, 1947; Wrigley, 1948) soon became a tool for investigating chemical carcinogenesis (Magee & Barnes, 1956) and led, subsequently, to the current wave of interest in *N*-nitroso compounds. An outbreak of dimethylnitrosamine poisoning among sheep fed nitrite-treated herring meal was recognized as resulting from *in vitro* nitrosation of dimethylamine (Ender, Havre, Helgebostad, Koppang, Madsen & Ceh, 1964; Sakshaug, Sognen, Hansen & Koppang, 1965), a conclusion which suggested that nitrosamines and their precursors occurred in the environment and posed a toxicological threat to human health.

Investigators later demonstrated that nitrosamines were indeed present in the environment and that nitrites and amines might form nitrosamines under certain conditions. This discovery prompted numerous articles on the potential hazards of these agents. For example, one report stated that, under acidic conditions, nitrite could form nitrous acid and nitrous anhydride, resulting in a low-yield nitrosation of weakly basic secondary amines (Sander, Schweinsberg & Menz, 1968). The increased specificity and sensitivity of methods involving gas chromatography–mass spectrometry (as compared with chemical assays) has permitted the detection and identification of *N*-nitroso compounds in such varied materials as gastric contents, tobacco smoke and mushrooms (*Food and Cosmetics Toxicology*, 1968). They have also been found, more significantly, in staple foods such as grains, pasteurized milk and cheese (Hedler & Marquardt, 1968), and in nitrite-treated cheese, fish, smoked fish and meat (Ender & Ceh, 1968; *Food and Cosmetics Toxicology*, 1968).

In addition, many authors have made the disturbing suggestion that nitrosamines may be formed from nitrite and secondary amines under the acidic conditions in the human stomach (Druckrey, Steinhoff, Beuthner, Schneider & Klärner, 1963; Sander, 1967). This finding is particularly distressing because the carcinogen may not be in the food itself, but may be formed only after ingestion of the food, and may therefore remain undetected in food-screening programmes. Sander *et al.* (1968) demonstrated the *in vivo* formation of nitrosamines in the stomachs of rats simultaneously fed nitrite and secondary amines; the interest in this phenomenon increased considerably when Sander & Seif (1969) showed that human patients given nitrite and diphenylamine produced nitrosodiphenylamine (which is not a demonstrated carcinogen) in the stomach. These considerations lent an urgency to this area of environmental carcinogenesis paralleled only by the recently recognized mycotoxin hazard (Ciegler, Kadis & Ajl, 1971).

It is a matter of debate whether the amounts of nitrosamines formed in the human stomach from nitrite and amines are biologically significant. However, research to date strongly indicates a need for more information about *in vivo* nitrosation and its relation to human carcinogenesis. Only a few studies have dealt with the long-term administration of

secondary amines and nitrite to intact animals. Druckrey *et al.* (1963) reported negative results when sodium nitrite and diethylamine were fed to rats; Sander *et al.* (1968) observed that neither diethylamine nor *N*-methylaniline displayed carcinogenic effects when fed with nitrite to rats. On the other hand, Sander & Bürkle (1969) reported the occurrence of hepatic and oesophageal tumours in rats fed sodium nitrite and either *N*-methylbenzylamine or morpholine. More recently, Greenblatt, Mirvish & So (1971) presented data suggesting that an increased incidence of lung adenomas in mice resulted from *in vivo* nitrosation of secondary amines by dietary nitrite. Lijinsky, Taylor, Snyder & Nettesheim (1973) described the induction of liver and oesophageal tumours in rats given nitrite and either aminopyrine or heptamethyleneimine.

Morpholine, a cyclic amine that was used until recently as an anticorrosive agent in boiler water, represents an unintentional additive to foods such as canned ham. Nitrite and morpholine, when combined at concentrations found in food products, could result in the formation of nitrosated morpholine at levels high enough to be significantly carcinogenic. Greenblatt *et al.* (1971) fed morpholine (in the diet) along with high levels of sodium nitrite (in the drinking-water) to Swiss mice characterized by a high incidence of spontaneous lung adenomas; these animals showed an increased incidence of lung adenoma when compared to control mice. *N*-Nitrosomorpholine (*N*NM) also affects mice as a hepatocarcinogen, but the absence of liver tumours in the animals treated in this experiment was not explained by the authors. Sander (1971) conducted similar experiments, in which rats fed 5 g morpholine/kg diet and 5 g sodium nitrite/litre drinking-water developed livers tumours 150–234 days after a 56-day exposure.

The study described in this paper demonstrated a dose-response relationship for the induction of liver and lung tumours by dietary sodium nitrite and morpholine, these tumours being identical to those induced by *N*NM, a preformed nitrosamine. The rat was shown to be a good animal model for studying this phenomenon because of its sensitivity. On the other hand, the hamster was found to be resistant, developing few tumours when exposed to identical treatment. Preliminary results of some of this work in rats have been published (Newberne & Shank, 1973).

EXPERIMENTAL

Animals. Pregnant Sprague–Dawley CD rats (from Charles River Breeding Laboratories, Wilmington, Mass.) and pregnant random-bred Syrian golden hamsters (Trenton Experimental Laboratory Animal Colony (Telaco), Bar Harbor, Maine) were fed from the time of conception on an agar-gel diet (Table 1), containing various concentrations of nitrite, morpholine or *N*NM (Table 2). When the F_0 females littered, rats and hamsters of both sexes were randomly selected from the F_1 generation for long-term carcinogenicity studies and, in addition, for the rat studies an F_2 generation was derived from the F_1 mothers. The studies were terminated with the killing of surviving (F_3) rats at wk 125 and of surviving 110-wk-old hamsters.

Table 1. *Formulation of agar-gel diets for rats and hamsters*

Ingredient*	Concn (g/kg dry weight) in Rat diet	Hamster diet
Casein	220	150
Corn starch	—	417
Dextrin†	197	—
Sucrose	147	210
Dextrose	197	—
Salt mix‡	40	50
Alpha-cell†	—	50
Oil§	150	50
Choline chloride	4	3
Cyanocobalamin	—	0·005
Vitamin mix‖	10	20
Agar	35	30
Soya-protein isolate	—	50

*Each diet also contained distilled water, 1000 ml/kg dry ingredients in the rat diet and 1100 ml/kg in the hamster diet.

†Test compounds were added in place of this component.

‡Wesson-Modified Osborne–Mendel Salt Mix from General Biochemicals, Chagrin Falls, Ohio (Wesson, 1932) for the rat diet and Rogers–Harper Salt Mix from General Biochemicals (Rogers & Harper, 1965) for the hamster diet.

§Corn oil (Mazola) for the rat diet and cottonseed oil (Wesson) for the hamster diet.

‖As described by Wogan & Newberne (1967) for the rat diet and by Newberne & Young (1966) for the hamster diet.

Diets. The diets were prepared fresh every 2–7 days and stored in covered polyethylene tubs at 4 C. Animals were fed once a day after removal of any stale diet remaining in the cages. The diets were prepared by adding the protein, carbohydrate, oil and salts premix to molten 3% agar in water and beating the mixture in a commercial blender until it had cooled to just above the gelling point; the vitamins, sodium nitrite, morpholine and *N*NM were then added as

Table 2. *Dietary levels of additives given to rats and hamsters*

Test group and diet no.	Dietary level (ppm)* of $NaNO_2$	Morpholine	*N*NM
1	0	0	0
2	1000	0	0
3	0	1000	0
4	1000	1000	0
5	1000	50	0
6	1000	5	0
7	50	1000	0
8	5	1000	0
9	50	50	0
10	5	5	0
11	0	0	5
12	0	0	50

*Molar concentrations (g-mol weight/kg (dry weight) of diet) corresponding to given (ppm) concentrations are $1{\cdot}4 \times 10^{-2}$, $0{\cdot}7 \times 10^{-3}$ and $0{\cdot}7 \times 10^{-4}$ for 1000, 50 and 5 ppm $NaNO_2$, respectively, $1{\cdot}1 \times 10^{-2}$, $0{\cdot}6 \times 10^{-3}$ and $0{\cdot}6 \times 10^{-4}$ for 1000, 50 and 5 ppm morpholine, respectively, and $0{\cdot}5 \times 10^{-3}$ and $0{\cdot}5 \times 10^{-4}$ for 50 and 5 ppm *N*NM, respectively.

required, and the diet was poured into labelled plastics containers to harden. Sodium nitrite, *N*NM and morpholine were each dissolved in approximately 30 ml water before addition to the diet.

Several times after preparation, the diets were assayed chemically for nitrite and *N*NM to determine the stability of the nitrite and to ascertain whether the carcinogen was formed after the diet had been mixed and stored. The concentration of nitrite in the diet samples was measured by the Official Method of Analysis (First Action) of the Association of Official Analytical Chemists (Fan & Tannenbaum, 1971). *N*NM was detected in defatted dichloromethane extracts of the diet, according to the combined gas chromatography–mass spectrometry method of Essigmann & Issenberg (1972).

Crystalline sodium nitrite (ACS reagent grade) and morpholine (ACS reagent grade; Fisher Scientific Co., Fairlawn, N.J.) were added according to the schedule in Table 2. *N*NM was purchased from Dr. Theodor Schuchardt (Schuchardt GmbH, Munich, Federal Republic of Germany). The dietary levels of nitrite and morpholine have been expressed as molar concentrations, or, more specifically, as the number of gram molecular weights in 1 kg of diet (dry weight). The schedule incorporated a titration of morpholine against a high concentration of sodium nitrite, a titration of sodium nitrite against a high concentration of morpholine and a titration of morpholine and sodium nitrite together. Also included were a negative control group and two positive control groups.

RESULTS

Stability of sodium nitrite in the experimental diets

Because nitrite ion is unstable when added to foods, it was necessary to determine how much nitrite remained in the diet that was being fed to the animals. All diets were assayed for nitrite immediately after preparation and up to 1 wk after refrigerated storage in sealed containers, 1-g samples extracted with phosphate buffer (pH 7·0) being used for this assay. Table 3 lists the results of these nitrite determinations at various concentrations and times.

Formation of NNM in the rat diet

Various mixes of the rat diet were analysed for *N*NM. After storage at 4°C for 4 days, the diets that contained 1000 ppm sodium nitrite and 1000 ppm morpholine were extracted with dichloromethane, cleaned up to remove oils and analysed as described above. The diets prepared with 1000 ppm of both nitrite and morpholine contained 1–2 ppm *N*NM, but other diets yielded no detectable *N*NM.

Long-term studies in rats

Survival times. For most groups, the feeding studies were terminated when the number of survivors fell to 20% of the original population. The rate of survival was fairly high, except in the groups fed the highest concentration of both sodium nitrite and morpholine (group 4) or *N*NM (group 12). In six instances, 23–44% of the F_2 generation were still living at wk 125 (28 months) and were killed to terminate the study. Groups 4 and 12 demonstrated liver carcinoma rates of almost 100%; rats within these groups died relatively early in the experiment, after being permitted to live their lifespans in order to see whether slower-growing tumours would develop. Because survival in the F_1 and F_2 generations was essentially the same, the results for both are combined in Table 4, which lists the mean survival times and the times of each group's first and last deaths. In order to compare survival rates between various groups, Table 4 presents the ages of the youngest and oldest rats to die and the median age at death, i.e. the age by which 50% of the population had died. The median age at death for the negative control group and groups 2 and 3 were approximately the same. The titration of mor-

Table 3. *Stability of sodium nitrite in the rat diet*

Diet no.‡	$NaNO_2$ concentration (ppm)* in diets on day†							
	0	1	2	3	4	5	6	0–6 (mean)
Determination 1§								
2	878	164	164	131	—	125	118	263
4	426	164	184	170	—	170	164	214
5	465	205	205	170	—	215	223	247
6	460	164	230	275	—	210	197	256
Determination 2§								
2	410	290	275	255	205	185	100	246
5	290	275	272	260	200	140	92	218
6	380	310	290	288	220	179	110	254
7	15	12	12	12	8	0	0	8
8	5	0	5	3	0	0	0	2
9	19	12	15	16	10	0	0	10
10	2	—	2	2	2	1	0	2

*Determined according to Fan & Tannenbaum (1971).
†Time after preparation.
‡The theoretical concentrations of sodium nitrite and morpholine are given in Table 2.
§Assays were performed on two separate batches of diet, stored in covered plastics tubs at 4–6°C.

Table 4. *Mean survival data for rats fed diet containing various concentrations of sodium nitrite, morpholine and NNM*

Group no.†	Age at death (wk)*		
	First death	Last death‡	Median§
1	38	(129)	109
2	52	(129)	108
3	51	(130)	117
4	19	50	38
5	22	(112)	90
6	24	(129)	105
7	40	142	116
8	36	137	115
9	55	128	114
10	46	130	113
11	44	(126)	96
12	21	80	56

*Data for F_1 and F_2 generations combined.
†The theoretical concentrations of sodium nitrite, morpholine and *N*NM in the diets are given in Table 2.
‡Brackets indicate that survivors were killed at the indicated age.
§Age (in wk) by which 50% of the population had died.

pholine against a high nitrite level demonstrated a clear dose response on the median age at death, but the reverse was not true, various doses of nitrite against a constant high level of morpholine having no effect on the median age at death (Table 4). Low concentrations of both reactants also showed no effect. The reduction in median age at death among the high-nitrite groups (4–6) indicates the increased carcinogenicity of these diets.

Incidence of hepatocellular carcinomas and angiosarcomas in rats. Hepatocellular carcinoma was the most frequent tumour associated with dietary nitrite and morpholine or with preformed dietary *N*NM. Angiosarcomas were also numerous in the rats, but did not show as clear a dose response as the liver-cell tumours. Angiosarcomas of the liver and lung were the most common, but angiosarcomas also appeared in the mesentery, intestine, diaphragm, heart and subcutis.

The tumours induced by nitrite and morpholine were morphologically identical to those induced by *N*NM. They were massive, multicentric nodular tumours with a remarkably vascular component, and usually with extensive haemorrhage or large haematomas (Fig. 1). Grey-white nodules and blood-filled cavities had massively destroyed the liver; the tumours usually extended rapidly throughout the abdominal cavity. With lower levels of reactants or with *N*NM, smaller liver tumours were typical. In areas of the liver free from haemorrhage and necrosis, soft, tumorous, rapidly-growing nodules predominated and were anaplastic or, to a lesser extent, trabecular (Fig. 2). Angiosarcomas were solid and firm (Fig. 3); the cells varied morphologically but tended to be hyperchromatic and spindle-shaped with abundant mitotic figures (Fig. 4).

Rates of incidence of hepatocellular carcinoma and angiosarcoma in the negative control and in groups 2 and 3 are given in Table 5. The controls given neither additive (group 1) developed no tumours of the liver or lung, a finding further supported by thousands of additional rats of this strain used in other experiments in our laboratory.

In the sodium nitrite control group (group 2), one rat from the F_2 generation (44 rats) developed a hepatocellular carcinoma, and one subcutaneous angiosarcoma was observed in a rat from the F_1 generation (52 rats). Despite the low probability of spontaneous development of these two tumours, their occurrence is not regarded as significant. The livers of these animals at death were congested and necrotic, with diffuse and focal nodular hyperplasia of the parenchyma and large hyperplastic nodules.

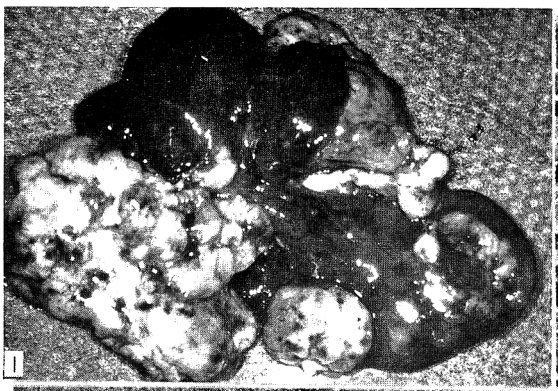
The morpholine control group (group 3) included three animals with hepatocellular carcinoma and one with lung angiosarcoma out of an F_1 population of 50. No liver tumours were observed in the F_2 generation (54 rats) but a single lung angiosarcoma was again observed. The diet of these animals contained no detectable nitrite and they were given only distilled water; it would seem that either morpholine itself is weakly carcinogenic or that nitrite reached the stomach from an unknown source. One rat in group 8, fed a diet to which was added 1000 ppm morpholine and 5 ppm sodium nitrite, developed a liver carcinoma.

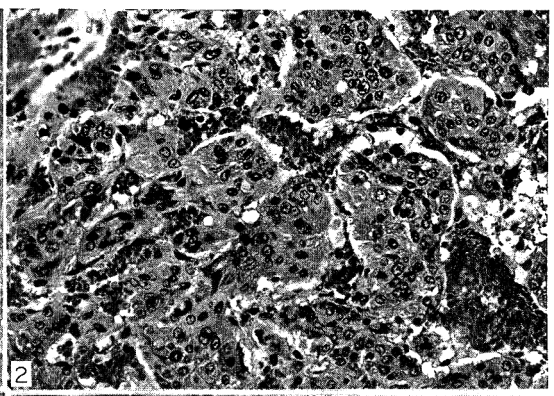
Table 5 also shows that when the dietary levels of both reactants were high (group 4), the incidence of liver tumours was essentially 100%, with many angiosarcomas of liver and lung and a high frequency of metastases of the liver-cell tumour to the lung. Liver tumours killed animals as young as 19 wk, and 50% of both generations in this group died with liver cancer by the age of 38 wk. As the morpholine level was reduced (with maintenance of the high nitrite concentration), the liver-cell carcinoma rate decreased with a linear dose-response relationship. This relationship corresponded to that observed when response was defined as the median age at death (Table 4). The high positive control (group 12; 50 ppm *N*NM) developed nearly 100% liver tumours, a rate greatly exceeding the percentage of tumours in group 5, indicating that probably less than 100% of the morpholine in diet 5 was nitrosated. On the other hand, group 4 had a median age at death of only 38 wk.

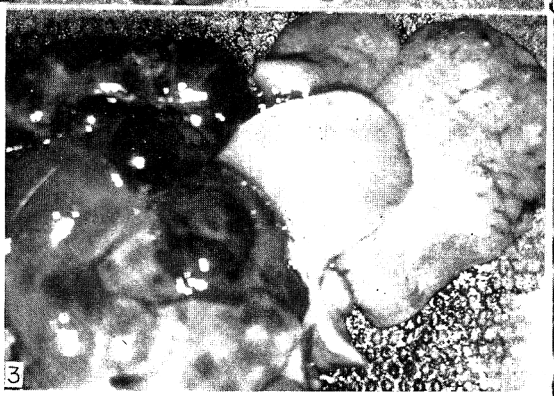
Table 5. *Incidence of hepatocellular carcinoma and angiosarcoma among rats fed experimental diets*

Group*	No. of rats†	Incidence (%) of: Liver-cell carcinoma	Liver angio-sarcoma	Lung angio-sarcoma	Other angio-sarcoma	Metastases from liver to lung	Age at death with liver carcinoma (wk): First death	Median‡
1	156	0	0	0	0	—	—	—
2	96	1	0	0	1	0	123	—
3	104	3	0	2	1	0	68	—
4	159	97	14	23	1	49	19	38
5	117	59	5	6	0	17	47	111
6	154	28	12	8	1	7	24	—
7	109	3	2	1	0	0	89	—
8	172	1	2	1	1	0	64	—
9	152	2	1	1	1	0	65	—
10	125	1	2	2	1	0	88	—
11	128	58	15	9	1	22	53	106
12	94	93	21	20	1	58	30	56

*See Table 2 for dietary concentrations of sodium nitrite, morpholine and *N*NM.
†F_1 and F_2 generations combined.
‡Age (in wk) by which 50% of the population had died with hepatocellular carcinoma.







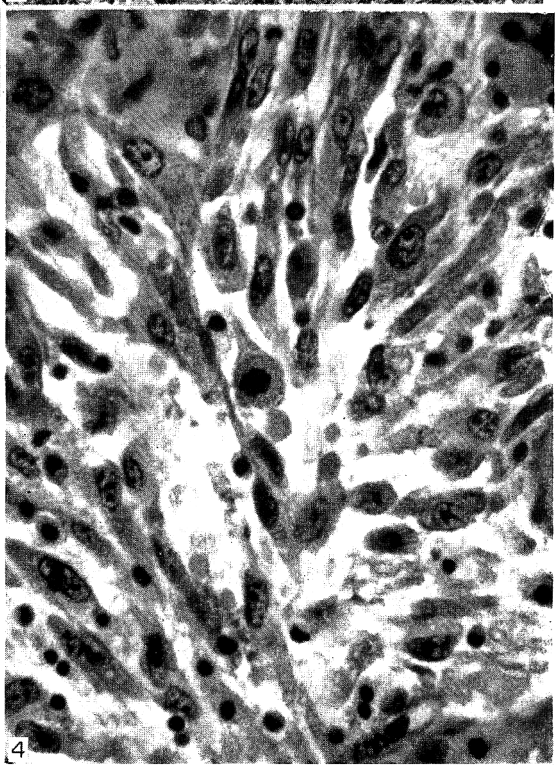


Fig. 1. Liver tumour typical of hepatocellular carcinomas induced by dietary nitrite and morpholine, showing a moderate degree of vascularity in the soft tumorous mass.

Fig. 2. Trabecular pattern of the nodule of the hepatocellular carcinoma shown in Fig. 1. Blood channels are present in spaces between the trabeculae. Haematoxylin and eosin × 210.

Fig. 3. Gross appearance of angiosarcoma (dark area) with pale lobe attached. These occurred in a significant number of animals fed nitrite and morpholine or preformed *N*NM. The hepatocellular component varied from one animal to another.

Fig. 4. Solid angiosarcoma from a rat fed nitrite and morpholine. The tumour was solid and glistening on gross appearance; vascular structures were small but abundant on microscopic examination, and mitotic figures were frequent. Haematoxylin and eosin × 590.

compared with 56 wk for group 12, demonstrating that the feeding of a diet prepared with 1000 ppm sodium nitrite and 1000 ppm morpholine was more potent than the feeding of one prepared with 50 ppm *N*NM.

Decreasing the nitrite concentration and maintaining a high morpholine level sharply reduced the frequency of hepatocellular carcinoma and angiosarcoma. This result was expected, because the nitrosation of secondary amines such as morpholine has been shown to vary with the square of the nitrite concentration (Mirvish, 1970).

Incidence of tumours other than hepatocellular carcinomas and angiosarcomas in rats. A great variety of tumours appeared in the twelve groups, and their incidence is summarized for each group in Table 6. The tumours have been classified broadly according to categories set forth in the *Manual of Tumor Nomenclature and Coding* (Subcommittee of the Statistics Committee, 1953). Because the F_1 and F_2 generations differed only very slightly, the data for these generations have been combined. Table 6 does not list data for liver-cell carcinomas or angiosarcomas because these findings are reported in Table 5. Tumours of the glandular epithelium included ductal and papillary tumours, neoplastic cysts, functionally active tumours and others. Non-glandular epithelial tumours consisted of squamous epithelium. Leukaemias were lymphocytic or granulocytic, and, in most cases, lymphomas accompanied the infiltrating leukaemic cells; in Table 6, they are all combined for convenience and listed as tumours of the lymphoreticular system. Nervous-system tumours included tumours of the neuroepithelium, paraganglion and glia. The smooth-muscle tumours refer to leiomyomas, myofibrosarcomas and leiomyosarcomas. Connective-tissue tumours were those of fibrous tissues, mucinous connective tissue and lipoid tissue. The embryonal mixed-tissue tumours included those of epithelial, mesodermal or mesenchymal origin.

Animals fed the high-nitrite diet (group 2) showed a high incidence of tumours in the lymphoreticular system: 27% for the entire group (Table 6) compared with 6% in the no-additive controls. These lymphatic tumours were often accompanied by infiltrating leukaemic cells and were responsible for widespread damage throughout several organs; they were observed only in animals at least 1 yr old. These tumours had a low incidence in the high-nitrite groups (4–6) that developed hepatomas and angiosarcomas. The higher lymphoma rate in group 2 cannot be explained solely by greater longevity, because the group 6 animals lived about as long as those in group 2. Group 7 (Table 6) also had a high rate of lymphatic tumours, but only 3% of the animals developed hepatocellular carcinomas. These animals were continuously exposed to diets to which 50 ppm sodium nitrite and 1000 ppm morpholine had been added. Although the nitrite concentration was markedly reduced during the week after the diet was mixed (Table 3), the rats developed extensive liver damage and, it appears, were also at an increased risk of developing malignant lymphomas, even though the incidence of liver cancer among them was low (Table 5).

Not only was the incidence of lymphoreticular tumours high in the high-nitrite group (2), but the total number of animals that developed tumours other than hepatomas and angiosarcomas was also high (61%, compared with 18% in the no-additive control group).

The high-morpholine control (group 3) developed two malignant brain gliomas, a rare finding in this study. The numbers were too small to be statistically significant, but when the liver-cell carcinomas and angiosarcomas are also considered, it appears that morpholine itself may be a hazardous compound.

Long-term studies in hamsters

The feeding studies in hamsters were terminated when the number of survivors in each group de-

Table 6. *Summary of incidence of tumours other than hepatocellular carcinomas or angiosarcomas in rats*

Site of tumour	Incidence (%) in group*											
	1	2	3	4	5	6	7	8	9	10	11	12
Glandular epithelium	10	17	11	2	2	12	15	25	16	15	7	2
Non-glandular epithelium	1	2	0	0	0	1	1	1	1	2	1	0
Lymphoreticular system	6	27	9	2	10	5	20	11	5	12	12	1
Nervous system	1	2	2	1	2	1	0	1	0	0	1	0
Muscle	0	0	0	0	0	0	0	0	1	1	0	0
Connective tissue	0	2	3	1	3	6	3	3	4	0	0	0
Embryonal and mixed tissues	0	11	13	0	1	6	8	10	11	7	4	0
Total.....	18	6	38	6	18	31	37	51	38	37	25	3

*Data for F_1 and F_2 generations are combined; dietary concentrations of sodium nitrite, morpholine and *N*NM for the different groups are given in Table 2.

Table 7. *Mean survival data for hamsters fed diet containing various concentrations of sodium nitrite, morpholine and* N*NM*

Group no.*	Age at death (wk)		
	First death	Last death†	Median‡
1	40	(110)	72§
2	40	(110)	104
3	37	(109)	68
4	24	100	70
5	25	(110)	85
6	25	(110)	73
7	34	(110)	97
8	52	(110)	104
9	55	(110)	99
10	45	(110)	97
11	39	(110)	73
12	39	(94)	65

*Dietary concentrations of sodium nitrite, morpholine and *N*NM for the different groups are given in Table 2.
†Brackets indicate that survivors were killed at the indicated age.
‡Age (in wk) by which 50% of the population had died.
§Out of 31 control hamsters, eight died from intercurrent enteritis and six died from severe renal deterioration.

Table 8. *Incidence of hepatocellular carcinoma, angiosarcoma and other tumours among hamsters fed experimental diets*

Group*	No. of animals	Liver-cell carcinomas		No. of angiosarcomas	No. and type of other tumours
		No.	%		
1	23	1	4	4	0
2	30	0	0	1 (sc)	0
3	22	0	0	0	0
4	16	5	31	0	1 lung cyst adenoma
5	32	0	0	0	1 malignant lymphoma 1 keratinizing acanthoma of stomach 1 adrenal adenoma
6	40	0	0	0	1 liver cyst adenoma
7	22	0	0	1 (spleen)	1 tumour of adrenal glomerulosa
8	19	0	0	0	0
9	30	0	0	1 (spleen)	1 malignant lymphoma
10	40	0	0	0	1 malignant lymphoma with leukaemia 1 ovarian adenocarcinoma 1 cyst adenoma of bile duct
11	35	0	0	0	0
12	18	1	6	1 (liver)	0

*See Table 2 for dietary concentrations of sodium nitrite, morpholine and *N*NM for each group.

creased to 20% of the starting population (10% in the case of group 12, the 50-ppm *N*NM positive control). In most cases this level occurred when hamsters reached the age of approximately 110 wk. The survival time for this strain of hamster is 24–30 months, a reasonable longevity for the species. Table 7 summarizes the survival data for hamsters in this study. The relatively low survival rate in the no-additive controls (group 1; median age at death, 72 wk) can be attributed to a high rate of intercurrent enteritis and severe renal deterioration of undetermined origin.

Table 8 lists the tumours obtained in the hamster study. Groups 1 and 12 both had a single hepatocellular carcinoma, a tumour which rarely occurs spontaneously in this species, and group 4 had five liver-cell tumours (an incidence of 31%). Apart from one hepatic angiosarcoma in group 12, three angiosarcomas were seen—a subcutaneous angiosarcoma in group 2, and a single spleen angiosarcoma in each of groups 7 and 9. Although the high dietary concentration of sodium nitrite and morpholine (group 4) induced liver cancer in some animals, the hamster seemed to be considerably more resistant to *N*NM carcinogenesis than did the Sprague–Dawley rat. Moreover, the high-nitrite, high-morpholine diet appeared to be more potent in inducing liver-cell carcinomas in hamsters than the 50 ppm *N*NM (a finding which agrees with the rat study, although the rates here are much lower).

Table 9. *Nodular hyperplasia in livers of hamsters fed experimental diets*

Group*	Incidence (%) of	
	Nodular hyperplasia of parenchyma	Bile-duct hyperplasia
4	23	0
5	3	18
6	0	20
2	0	0
4	23	0
7	0	29
8	0	16
3	0	9
4	23	0
9	6	19
10	3	13
1	0	9
12	16	5
11	3	11
1	0	9

*See Table 2 for dietary concentrations of sodium nitrite, morpholine and *N*NM for each group.

Tables 8 and 9 summarize the histopathological observations on the hamster tissues, but few patterns emerge. There appeared to be no clear dose-response relationship between the contents of nitrite and/or morpholine in the diets and the incidence of necrosis or liver parenchymal and bile-duct hyperplasia. Nodular hyperplasia of the liver parenchyma, however, followed an apparent dose-response relationship (Table 9, groups 4, 9 and 10). In addition to the lesions described above, nephrosis, myocardial necrosis and mineralization were observed, but these did not demonstrate a relationship with the concentrations of sodium nitrite and morpholine in the diet.

DISCUSSION

Sodium nitrite breaks down rapidly when added to food products, although the pathways for the nitrite loss have not yet been fully defined (Fox & Nicholas, 1974; Sebranek, Cassens, Hoekstra, Winder, Podebradsky & Kielsmeier, 1973; Walters & Casselden, 1973). All diets showed a progressive loss of nitrite with storage; although variability was large, nitrite seemed less stable in the rat diet. The rats were ingesting only a quarter to half the nitrite added to the diets; thus, for diet 4, 1000 ppm sodium nitrite and 1000 ppm morpholine were added, but only 240–460 ppm nitrite could be detected after preparation. It is not known whether this loss of nitrite represented a real loss in the diet or a failure of the Griess reaction to detect all the nitrite. Pertinent to

the latter point is a report by Perigo, Whiting & Bashford (1967) suggesting that nitrite, upon heating in a bacteriological medium, reacts with a component of the medium to produce an unknown substance not detectable as nitrite but highly inhibitory to the vegetative growth of Clostridia. This observation may help to explain why rats fed nitrite–morpholine diets, in which most of the nitrite was not detectable and little or no *N*NM could be found, still had a high incidence of liver cancer. Perhaps the nitrite was sequestered, in terms of the Griess reaction, but still remained biologically available for the intragastric nitrosation reaction.

Early studies on nitrite-induced nitrosation were criticized for using reactants at levels far in excess of those encountered in food processing. The results here show a clear dose-response relationship between nitrite and morpholine concentrations and the incidence of hepatocellular carcinomas. These tumours and angiosarcomas were seen in animals exposed to levels of dietary nitrite and morpholine that are not in excess of those that can occur in the food supply.

Similar dose-response relationships probably occur with amines other than morpholine. Morpholine was used in this study because it represented a food contaminant and because its *N*-nitroso derivative is relatively easy to detect quantitatively. Similar nitrosations are being demonstrated not only in food products, but also with various drugs (Lijinsky, Conrad & Van de Bogart, 1972) and pesticides (Eisenbrand, Ungerer & Preussmann, 1974). The potential for nitrite to react with environmental amines to form carcinogens appears to be great.

The chronic toxicity of morpholine requires more study. Although the numbers were small, hepatocellular carcinomas and angiosarcomas were seen in the groups of rats on the low-nitrite, high-morpholine diets. Because such tumours were also observed in the morpholine control (group 3) but not in the baseline control (group 1), one cannot determine with certainty whether the tumours resulted from the high morpholine level or from a high morpholine level combined with the presence of nitrite (coming from an unknown source in the case of group 3). The results in Table 5 tend to support the view that nitrite must have been present, since although the morpholine concentration in the diets varied 200-fold, the cancer rate remained low in most groups and was related more closely to the nitrite concentration. Presumably, then, the tumours in group 3 indicate the presence of an unknown source of nitrite, which reacted with the morpholine to form *N*NM, or the food may have contained some other reactant, which interacted with morpholine to create a carcinogenic effect.

The relatively low incidence of 'spontaneous' tumours in this strain of Sprague–Dawley rat is a finding consistent with numerous other experiments using thousands of these same animals over 15 yr. Although the incidence was low, the variety of tumour types suggests that some of them may represent sites where nitrosation of an endogenous tissue component occurred or, in the case of group 2, where the nitrate triggered neoplasia in a specific tissue.

Survival data for the rats are equally interesting. Except for the two groups fed the high levels of nitrite plus morpholine and the two groups fed the preformed *N*NM, all the groups registered more than 50% survival after 2 yr on the same experiment (Table 4). Good survival rates again recommend this strain of rat for carcinogenicity studies.

The choice of hamster strain was less fortunate, since this animal exhibited a marked resistance toward tumour induction by nitrite and morpholine, as well as by preformed *N*NM (Table 8). Although the survival rate (Table 7) of this strain was less than desirable, it was not sufficiently low to account for the low rate of tumour induction. Furthermore, the incidence of spontaneous tumours (Table 8) was low, with six of the twelve groups exhibiting none. Bile-duct hyperplasia (Table 9) was a common observation in the hamster liver but did not show a dose-response relationship to either of the three chemicals. This species difference suggests the need for comparative metabolism studies on *N*NM, in order to help elucidate the bioactivation pathway for this compound.

The original concepts of Sander (1967) and Lijinsky & Epstein (1970) are provocative, and it is likely that *in vivo* nitrosation occurs in the stomach of rats and hamsters. Moreover, it is fairly certain that nitrosation of amines occurs in other biological compartments and sites where the pH is appreciably higher than in the stomach. It should be pointed out, however, that nitrates, nitrites and amines have been widespread in our environment for many years without any apparent increase in chemically related cancer of the types produced in experimental animals. Our findings, along with those of others, raise important questions, but we must use caution in attempting to extrapolate animal data to the human situation.

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EFFECTS OF FOOD SEASONINGS ON THE CELL CYCLE AND CHROMOSOME MORPHOLOGY OF MAMMALIAN CELLS *IN VITRO* WITH SPECIAL REFERENCE TO TURMERIC*

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Abstract—Direct application of some common food seasonings, and of solubilized filtered extracts of these seasonings, has toxic effects on cells in culture. Asynchronous populations of several mammalian cell strains were grown in media containing one of the three food seasonings, cayenne pepper, curry and paprika. The cells exhibited decondensed and banded metaphase chromosomes. The spice turmeric arrested mitosis, altered chromosome morphology and interfered with nucleic acid synthesis. The metaphase chromosomes showed a progression of changes including uncoiling, chromatid separation, fragmentation and disintegration. This progression was dose- and time-dependent. Monitoring of the cell cycle using autoradiographic techniques suggested that turmeric interfered with cell-cycle progression. The rate of incorporation of [^{3}H]thymidine and [^{3}H]uridine was reduced to less than 25% of control values within 30 min when turmeric (10 μg/ml) was added to asynchronous Chinese-hamster cells.

INTRODUCTION

The toxic potential of food-flavouring components has interested many investigators. For example, severe hepatotoxicity and gastro-intestinal irritation has followed ingestion of safrole and coumarin by rodents (Hagan, Jenner, Jones, Fitzhugh, Long, Brouwer & Webb, 1965; Homburger, Kelley & Friedler, 1961; Jenner, Hagan, Taylor, Cook & Fitzhugh, 1964), while capsaicin, a component of peppers, has been found to induce immediate subcellular disorganization upon contact with the mucosal cells of the rat (Nopanitaya & Nye, 1974; Sirsat & Khanolkar, 1960). Although the composition of many spices and food seasonings is well defined and their components are highly stable in marketed products as well as being freely soluble in hot water or common organic solvents (*Merck Index*, 1968), the direct effects of these substances on mammalian cells are largely unknown.

The value of cell cultures for the toxicity testing of chemicals and for the evaluation of potential hazard to higher animals has been well demonstrated (Ferguson & Prottey, 1974; Metcalfe, 1971). The present paper describes the effects of several commonly-used food seasonings on chromosome morphology and the cell cycle of mammalian cells in culture. Our investigations are an outgrowth of previous work undertaken in this laboratory to demonstrate *in vitro* alterations of chromosome morphology and behaviour brought about by chemical agents known to intercalate DNA (Hsu, Pathak & Shafer, 1973; McGill, Pathak & Hsu, 1974; Pathak, McGill & Hsu, 1975). Data available at the start of these studies suggested that other compounds, with totally unknown effects on cellular macromolecules, could induce chromosomal alterations similar to those of the intercalating agents. Preliminary observations of chromosome crossbanding induced by the addition of powdered spices to cell cultures indicated that some spices might have cytotoxic effects on mammalian cells *in vitro*. Accordingly we tested the ability of several of these agents to alter chromosome morphology and influence cell-cycle progression. Turmeric was selected for a detailed analysis of cytotoxicity because its chemical composition has been relatively well defined (Honwad & Rao, 1964; Rupe, Clar, Pfau & Plattner, 1934) and because it is in common use in many parts of the world, in condiments, meats and pickles and as one of the main components of curry (Hall & Oser, 1965).

EXPERIMENTAL

Cells and culture techniques. Cells of the Chinese hamster, *Cricetulus griseus*, cell line Don, of the cactus mouse, *Peromyscus eremicus*, and of the Indian muntjac, *Muntiacus muntjac*, were used, as well as short-term human lymphocyte cultures. The cactus-mouse cells and Don cells were cultured in modified McCoy's 5a medium and the muntjac cells in Ham's F10 medium. Both media were supplemented with 20% foetal bovine serum. Lymphocytes were cultured in GIBCO chromosome medium 1A. All experiments were performed on cells in the exponential growth period.

Seasonings and seasoning treatments. The food seasonings studied included curry powder, onion juice, paprika, cayenne pepper and turmeric. In one set of experiments the seasonings were added directly to the cultures. In the other experiments, saturated solutions of the seasonings were prepared using

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various solvents (distilled water, Hanks' balanced salt solution or 95% ethanol) and were filtered or centrifuged to remove particles. With turmeric, an additional extraction procedure was used to obtain an alcohol-soluble fraction from the spice powder. This simple procedure involved refluxing powdered turmeric in 70% ethanol at 90 ± 2°C for 1 hr and then adding cold distilled water, whereupon two fractions formed. These were separated and the precipitate was oven-dried for 24 hr at 37°C. This fraction (the turmeric extract) was completely soluble in absolute ethanol. This extraction procedure eliminated initial problems due to fine particles of turmeric which adhered so tenaciously to the cells that it was impossible to make good cytological preparations. The turmeric extract was added to the cultures in specific amounts while control cultures received a similar amount of the solvent.

Nucleoside incorporation. Asynchronous Don cells were treated with the turmeric extract at a level of 1–100 μg/ml in conditioned media. The radioactive nucleoside was added before, at the same time as, or at various times after the addition of turmeric. The concentration of [^{3}H]thymidine ([^{3}H]TdR) was 1 or 2 μCi/ml (sp. activity 6·7 μCi/mmol). Cultures were treated with [^{3}H]uridine ([^{3}H]UR) at 10 μCi/ml (sp. activity 28 μCi/mmol). In experiments using pulses of [^{3}H]TdR prior to treatment with the turmeric extract, thymidine at 10 μg/ml was added after the cultures had been washed twice with Hanks' balanced salt solution to remove the labelled compound.

Cytological preparations and autoradiography. Conventional cytological air-dry or squash preparations were made. In some cases (mitotic index determinations) the cells were fixed without pretreatment with Colcemid or hypotonic solution. When used, the concentration of Colcemid was 0·06 μg/ml. Autoradiographs were prepared using Kodak AR-10 stripping film and an exposure period of 3 or 7 days.

RESULTS

Alteration of chromosome morphology

Morphological alterations induced in metaphase chromosomes by the various seasonings tested are shown in Figs 1–12 and summarized in Table 1. Figure 1 shows a normal metaphase cell of the Chinese hamster from a control sample, while Figs 2–4 illustrate the changes in morphology following treatment for 4 hr with water-solubilized powdered curry, paprika, and cayenne pepper, respectively. These agents apparently inhibited normal chromosome condensation and the chromosomes appeared banded or uncoiled. In some cells the gyres of a supercoiled structure could be seen (Fig. 2). In many metaphase plates, the chromosomes exhibited a lattice of closely spaced crossbands, as seen in the chromosomes of Figs 3 and 4. When clear-cut bands were discernible, the pattern corresponded to the conventional G-band karyotype of this species. In addition to banding, chromosomal clumping (Fig. 3) or stickiness (Fig. 12) was frequently observed (McGill *et al.* 1974; Pathak *et al.* 1975). It should be noted that alterations in chromosome morphology were the same whether powdered seasonings or water-soluble filtered extracts were added to the cultures.

Table 1. *Changes in chromosome morphology following the direct addition of food seasonings to cell cultures*

Seasoning*	Stated ingredients	Effect on chromosome morphology†
Onion juice	Onion juice, Sodium benzoate, 0·1% Potassium sorbate, 0·1%	None
Paprika	Unstated	Abnormal condensation, indistinct crossbanding
Cayenne	Ground red pepper	Abnormal condensation, indistinct crossbanding
Curry	Unstated	Abnormal condensation, indistinct crossbanding
Turmeric	Unstated	Chromatid separation, breakage, disintegration

*Turmeric manufactured by McCormick & Spice Islands, other seasonings by McCormick.

†Culture media contained 1% onion juice or 1% water-soluble filtrates of the powdered spices; treatment time was 4 hr.

Several experiments were designed to define seasoning-induced cytotoxicity more completely, using turmeric as the test agent. The effects of various extracts and concentrations of turmeric on chromosome morphology are seen in Figs 5–9, 11 and 12.

Figure 10 shows chromosomes of a muntjac cell from a control sample. Unlike other seasonings tested, turmeric did not induce banding patterns comparable to conventional G-band preparations. However, abnormalities were observed, including chromosome condensation, the presence of C anaphases and eventual disintegration of the chromosomes. Figure 11 shows a muntjac cell with a chromatid break in the X chromosome. In short-term cultures (1–4 hr), many cells had either highly condensed chromosomes (Fig. 5) or greatly despiralized or stretched chromosomes (Fig. 12). After only a 1-hr exposure to Colcemid following turmeric treatment, C anaphase was observed (Fig. 6). Usually C anaphases were observed in these cells after Colcemid treatment for 4 hr. With longer turmeric exposures (8–24 hr), fragmenting and disintegrating chromosomes (Figs 7 & 8) as well as cells with micronuclei (Fig. 9) occurred frequently. After about 16 hr, most metaphase plates were in an advanced state of disintegration and separated chromatids and chromosome fragments appeared to be represented by condensed areas surrounded by a loose mass of fibrils.

Effects on cell cycle

Tables 2 and 3 present the results of microscopic analyses of changes in mitotic indices and other mitotic abnormalities induced in Chinese-hamster cells, cactus-mouse cells and human lymphocytes treated

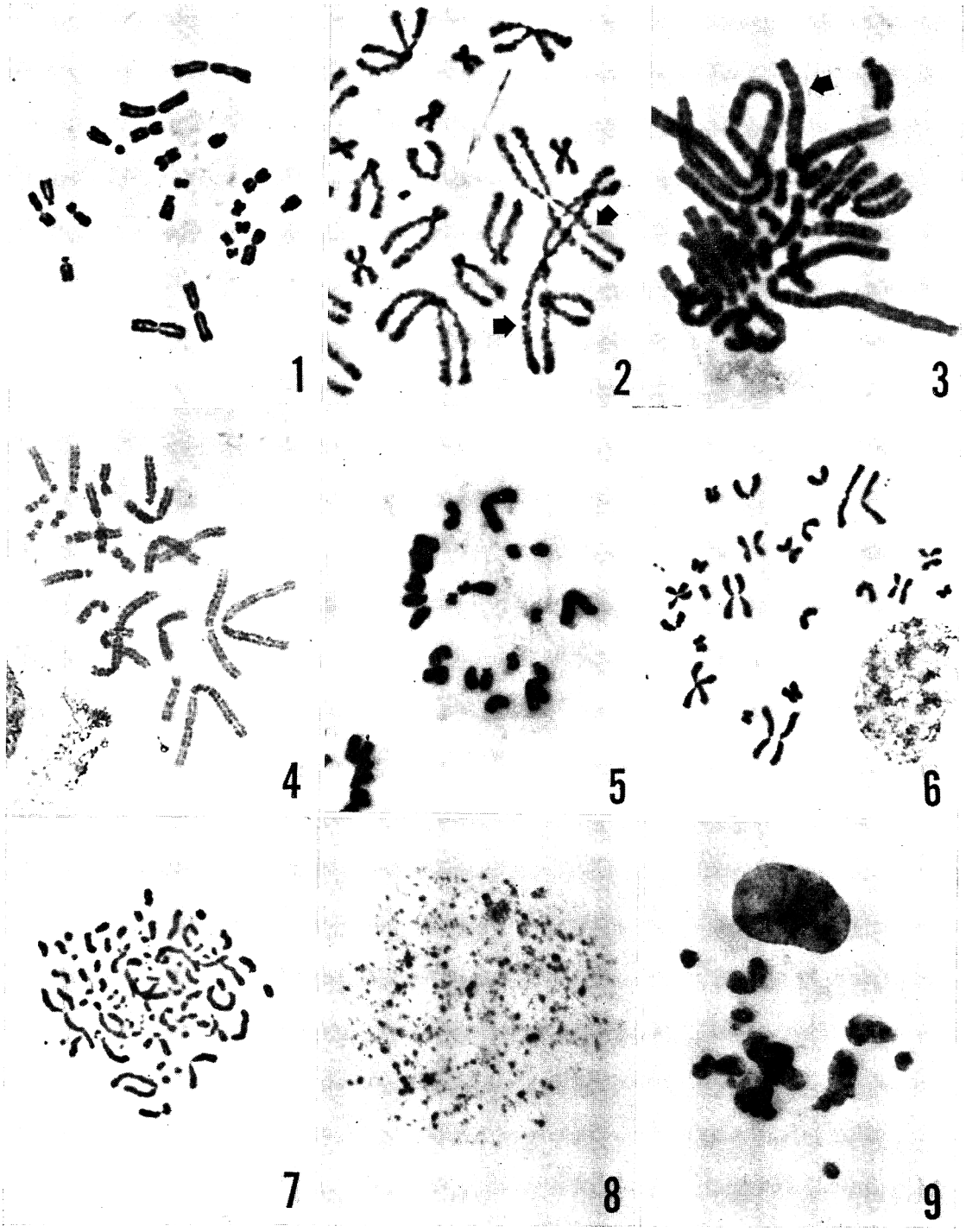

Fig. 1. Metaphase chromosomes of normal Don cells. Colcemid treatment. Giemsa × 800.

Fig. 2. Partial metaphase plate of Don cell treated with curry powder (1 mg/ml) for 2 hr, showing the coiling effect (arrowed) in these cells. Colcemid treatment. Giemsa × 2400.

Fig. 3. Metaphase cell of Don culture treated with paprika (1 mg/ml for 4 hr), showing banding pattern similar to G-banding (arrowed). Colcemid treatment. Giemsa × 2400.

Fig. 4. Partial metaphase plate of Don culture treated with cayenne pepper (5 mg/ml for 4 hr), showing distinct bands on the chromosomes. Colcemid treatment. Giemsa × 800.

Fig. 5. Metaphase cells from Don culture showing intense condensation of chromosomes after treatment with turmeric (50 μg/ml) for 4 hr. No Colcemid treatment. Giemsa × 800.

Fig. 6. Cell from Don culture showing some chromosomes exhibiting C anaphase and others with intact chromatids, after treatment with turmeric (50 μg/ml) for 4 hr. Colcemid treatment (1 hr). Giemsa × 800.

Fig. 7. Early stage of chromosome fragmentation in Don cells treated with turmeric (50 μg/ml) for 8 hr. No Colcemid treatment. Giemsa × 800.

Fig. 8. Shattered chromosomes of Don cell after turmeric treatment (50 μg/ml) for 24 hr. No Colcemid treatment. Giemsa × 800.

Fig. 9. Cells of Don culture after turmeric treatment (50 μg/ml) for 8 hr) and an intact nucleus at top. No Colcemid treatment. Giemsa × 800.

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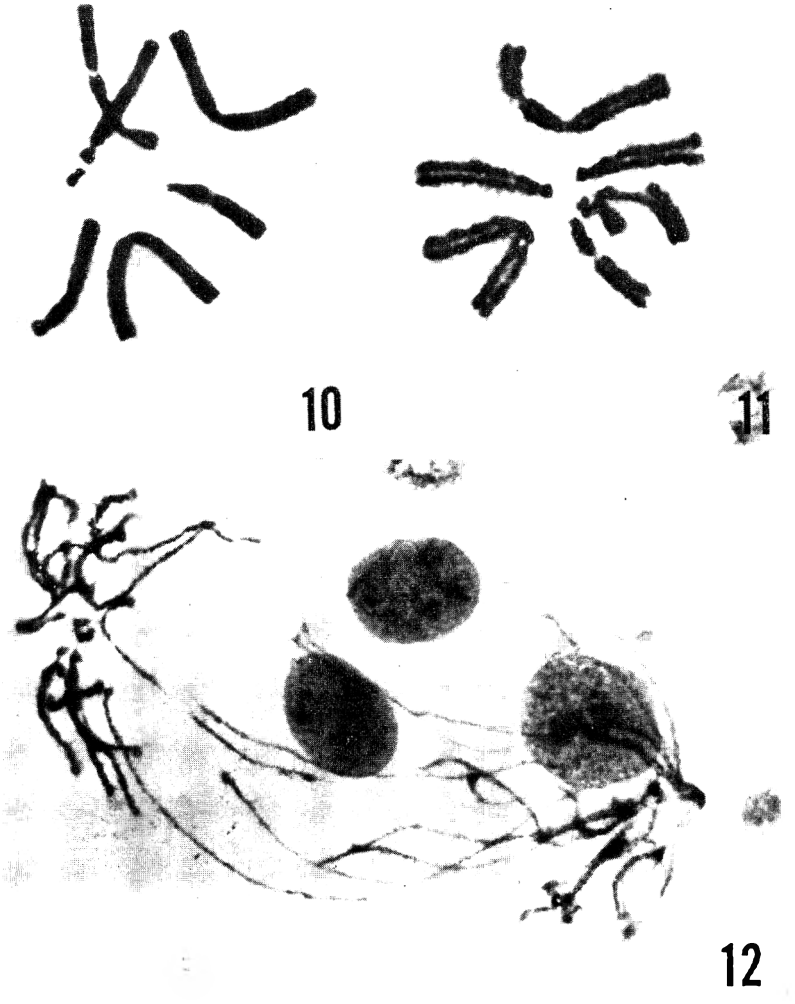

Fig. 10. Metaphase cells showing normal number of chromosomes of muntjac (male, 2n = 7). Colcemid treatment (1 hr). Giemsa × 1000.

Fig. 11. Metaphase cell from muntjac culture showing chromatid break in X chromosome after treatment with turmeric (10 μg/ml) for 4 hr. Colcemid treatment (1 hr). Giemsa × 1000.

Fig. 12. Anaphase cell of muntjac culture showing extremely stretched chromosomes after turmeric treatment (10 μg/ml) for 4 hr. No Colcemid treatment. Giemsa × 1000.

Table 2. *Distribution of normal and abnormal metaphase figures following continuous treatment of cell cultures from the Chinese hamster or cactus mouse with 50 μg turmeric/ml*

Duration of treatment (hr)	No.* of Normal metaphases	C-anaphases	Fragmented metaphases	Cells with micronuclei
	Chinese hamster			
0	99	0	0	1
4	50	49	0	1
8	13	67	18	2
16	2	3	90	5
24	32	1	27	40
	Cactus mouse			
0	100	0	0	0
4	17	77	5	1
8	4	13	83	0
12	0	0	99	1
16	0	0	97	3

*For each sample, 100 cells (including only metaphase cells and those with micronuclei) were scored.

Table 3. *Effects of continuous treatment with turmeric on the mitotic characteristics of human blood cultures*

Dose of turmeric (μg/ml)	Mitotic index	Percentage of mitotic cells damaged
	24 hr*	
0	2·30	0
6	0·80	16·4
12	0·23	66·0
25	0·075	100
38	0·025	100
	48 hr*	
0	3·35	0
1	3·55	2·0
6	2·07	24·0
12	0·15	96·5

*Cultures were harvested at 24 or 48 hr, 4000 mononucleate cells being counted for the mitotic indices while 50 metaphase plates were scored for chromosome damage.

with various concentrations of turmeric and harvested at intervals after introduction of the spice. The immediate response of Don and cactus-mouse cells was an increase in the mitotic index. For example, Don cells treated with 50 μg turmeric/ml and studied up to 6 hr after treatment (Fig. 13) showed an increase in mitotic figures somewhat comparable to metaphase arrest by Colcemid. However, the total number of cells arrested was lower than that seen with Colcemid treatment alone (Fig. 14). As the dose and the length of treatment increased, the mitotic index decreased and damage to metaphase chromosomes increased.

The progression of cells from metaphase to anaphase appeared to be slowed by turmeric treatment (Fig. 15). The number of cells in anaphase 8 hr after treatment was distinctly lower than in the control sample, which showed the normal constant rate. Since turmeric exhibited cytotoxic properties, a study was made of the incorporation of [^{3}H]UR and [^{3}H]TdR

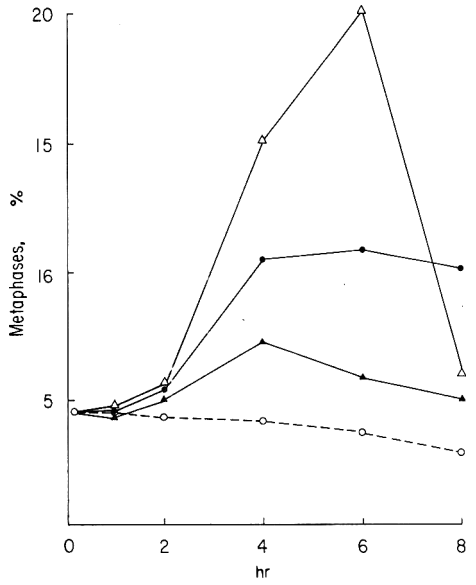

Fig. 13. Effect of 0 (○), 10 (▲), 25 (●) and 50 (△) μg turmeric/ml on the mitotic index of Chinese hamster (Don) cells. No Colcemid or hypotonic treatment preceded cell harvest; 4000 cells were counted at each time.

in turmeric-treated cultures to determine the effect of turmeric on DNA and RNA synthesis. The number of labelled cells (Fig. 16) as well as the grain counts (Fig. 17) indicated a decrease in the uptake of [^{3}H]TdR and [^{3}H]UR (Fig. 18). Inhibition of incorporation was less marked with the lower concentrations, especially 1 μg/ml, than with the higher (25 μg/ml).

A decrease in the uptake of the tritiated precursors of either RNA or DNA predisposes to a decrease in the number of labelled mitoses. Figure 19 shows that the number of labelled metaphase cells following turmeric treatment was less than the control value at 2 hr. However, at 4 hr, the number of labelled mitoses with a turmeric sample of 10 μg/ml approached the control value. The number of labelled mitoses with 25 μg turmeric/ml did not approach the control value

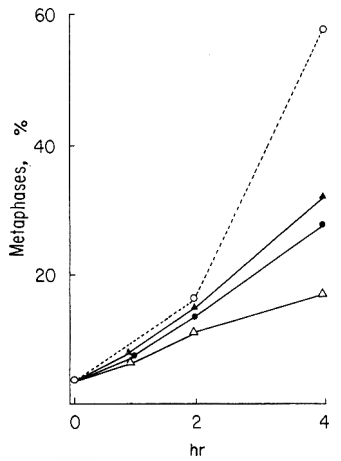

Fig. 14. Comparison of metaphase arrest in Don cells by 0·06 μg Colcemid/ml (○) and by 10 (▲), 25 (●) and 50 (△) μg turmeric/ml plus Colcemid. Each point represents a count of 4000 cells.

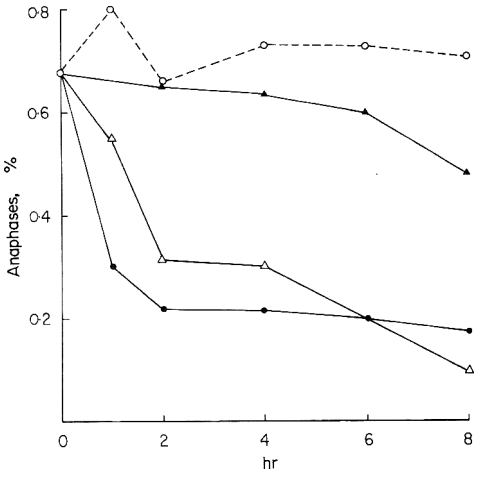

Fig. 15. Effect of 0 (○), 10 (▲), 25 (●) and 50 (△) μg turmeric/ml on the progression of metaphase Don cells into anaphase stage. No Colcemid or hypotonic treatment preceded cell harvest; 4000 cells were counted at each time.

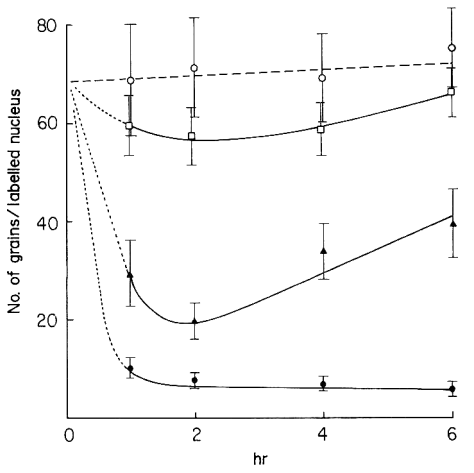

Fig. 17. Effect of 0 (○), 1 (□), 10 (▲) and 25 (●) μg turmeric/ml on the incorporation of [^{3}H]TdR in Don cells. Pulse label was added at 2 μCi/ml for 10 min prior to harvest; no Colcemid or hypotonic treatments preceded harvest. Each point represents the mean number of reduced silver grains found in 100 labelled nuclei.

until 8 hr after treatment. Since the uptake of [^{3}H]UR was reduced after 30 min (Fig. 18) and that of [^{3}H]TdR at 1 hr (Fig. 17), the number of labelled mitotic cells represents that population of cells that took the label during the 10-min pulse. Since the number of labelled mitoses did not reach the number in the control sample, progression of these cells through the remainder of S and G_2 must have been slowed.

In Chinese-hamster cultures, normal metaphase cells reappeared 24 hr after the removal of the turmeric, while the number of damaged metaphases decreased, indicating at least a partial recovery. Table 2 shows that in Chinese-hamster cultures the number of normal metaphase cells was also increasing after continuous turmeric treatment for 24 hr. In the cactus mouse, metaphase cells were not seen after 24 hr of treatment. Recovery after removal of the turmeric was not as good as in the Chinese hamster cells. In human lymphocyte cultures the numbers of mitotic cells and the mitotic indices fell sharply with increasing concentrations and exposure time (Table 3). No indication of recovery was noted in these cultures.

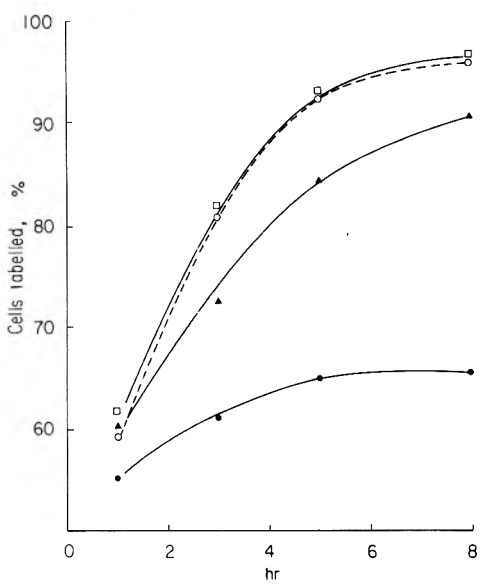

Fig. 16. Effect of continuous treatment with 1 μCi [^{3}H]TdR/ml and 0 (○), 1 (□), 10 (▲) or 25 (●) μg turmeric/ml on the progression of Don cells through S phase. No Colcemid or hypotonic treatments preceded cell harvest; autoradiographs were exposed for 1 wk.

DISCUSSION

The data presented in this paper indicate that some common food seasonings are cytotoxic when they are in direct contact with mammalian cells *in vitro*. These cytotoxic effects include interference with chromosome condensation, indications of chromosome banding, chromosome breakage, fragmentation and disintegration, mitotic arrest, formation of micronuclei and reduction in nucleic acid synthesis.

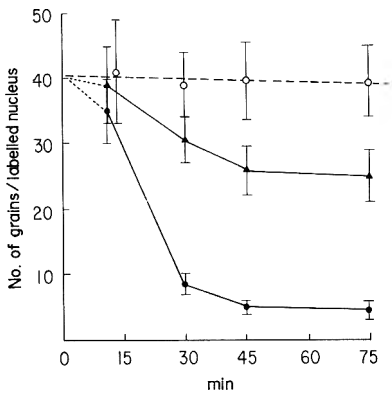

Fig. 18. Effect of 0 (○), 10 (▲) and 25 (●) μg turmeric/ml on the incorporation of [^{3}H]UR in Don cells. Pulse label was added at 10 μCi/ml for 10 min prior to harvest; no Colcemid or hypotonic treatments preceded harvest. Each point represents the mean number of grains from a count of 100 labelled nuclei.

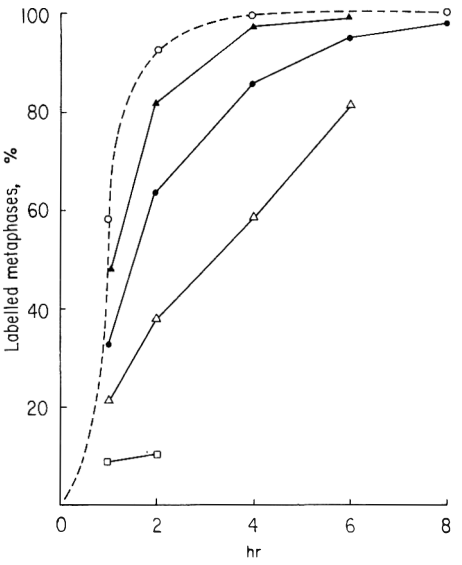

Fig. 19. Percentage of labelled metaphase cells in a Don-cell culture pulse-labelled with [^{3}H]TdR at 2 μCi/ml for 10 min, rinsed twice with culture medium and then treated continuously with 0 (○), 10 (▲), 25 (●), 50 (△) or 100 (□) μg turmeric/ml until harvest. Colcemid (1 hr) and hypotonic treatments preceded harvest and 1000 cells were counted at each time.

The uncoiled and banded nature of the chromosomes affected by the spices tested can be interpreted as a failure of the final stages of chromosomal condensation during metaphase. The incomplete contraction as well as overcontraction of metaphase chromosomes observed here may reflect the absence or abnormality of protein constituents responsible for normal metaphase configuration of the chromosomes. Several DNA-intercalating agents are known to induce banding patterns *in vitro* and it has been suggested that compounds that bind to DNA at specific sites during G_2 or prophase may interfere with the binding of chromosomal proteins which are necessary for condensation of the chromosomes in their preparation for mitosis (Hsu *et al.* 1973).

The immediate mitotic arrest induced by turmeric suggests that this spice may have effects on centrioles and spindle protein, as is the case with many well-known mitotic poisons (Kohlman, 1966). The lower rate of accumulation of metaphase cells compared with that following Colcemid treatment alone is in agreement with the autoradiographic data suggesting that turmeric also interferes with the progression of cells through the cell cycle. The observation that, in inducing mitotic arrest, combinations of Colcemid and turmeric are less efficient than Colcemid alone may indicate antagonistic action between the two mitotic arrestants. In addition to mitotic arrest, turmeric produced chromosomal abnormalities including non-condensation and, after longer exposure, fragmentation and disintegration. Chromosome damage induced by turmeric is similar to the damage induced by a number of C-mitotic agents and prophase poisons (Brinkley, Barham, Barranco & Fuller, 1974; Shaw, 1970).

In the evaluation of turmeric-induced cytotoxicity, the number of grains per labelled nucleus appears to provide a more sensitive and accurate estimate of toxicity than do the mitotic parameters of chromosome morphology, mitotic index and cell-cycle progression. For example, autoradiographs indicated that incorporation of labelled nucleosides into Chinese-hamster cells was greatly inhibited by concentrations that did not detectably alter chromosome morphology. However, reduced rates of nucleoside incorporation indicated by lowered grain counts are subject to differences in interpretation. It is not known whether reduced incorporation is a reflection of interference with nucleic acid synthesis or with cellular permeability and/or transport mechanisms (Cutroneo & Bresnick, 1971; Grunicke, Hirsch, Wolf, Bauer & Kiefer, 1975; Skehel, Hay, Burke & Cartwright, 1967). The observation that cells grown in the presence of turmeric continue to progress slowly through S phase, while the rate of incorporation of [^{3}H]TdR is greatly reduced, may suggest that turmeric prevents cellular utilization of exogenous thymidine or uridine.

It is not yet known whether similar cytotoxic effects may occur *in vivo* when food seasonings are ingested. Our *in vitro* system provides no information on possible chemical changes in these agents or on the responses of different tissues or individuals. It is also difficult to compare the levels of seasonings used in cell-culture systems with levels encountered *in vivo*, while persons who ingest these spices frequently may build up a resistance to their cytotoxic effects. Nevertheless, toxic effects of food additives, however mild, should be monitored, and the simple system used in our experiments would be useful for future screening programmes. Furthermore such data may be useful also in basic cell research. For example, the fact that turmeric can arrest mitosis suggests that some of the components of this spice may interact with specific cellular components such as microtubule proteins. Biochemical analysis and electron-microscopic observations on these interactions should be initiated.

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CHOLESTEROL LEVELS, ATHEROSCLEROSIS AND LIVER MORPHOLOGY IN RABBITS FED CYCLOPROPENOID FATTY ACIDS

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Abstract—Male New Zealand rabbits were fed diets containing either 0·27% cyclopropenoid fatty acids (CPFA) or 0·5% cholesterol or both in combination. Compared with control animals, those receiving CPFA tended to have higher plasma- and liver-cholesterol levels and a higher incidence of aortic atherosclerosis. A similar pattern was seen when animals fed cholesterol and CPFA were compared with animals fed cholesterol alone. The observed increases in liver cholesterol, plasma cholesterol and plasma triglycerides indicated a wide range of sensitivity to CPFA. Some animals had plasma-cholesterol levels eight to ten times as high as normal after feeding had continued for 3 wk, while others showed only slight changes after 5 wk. Liver cells from all CPFA-fed animals had altered morphology and often showed extensive damage.

INTRODUCTION

The cyclopropenoid fatty acids (CPFA), sterculic and malvalic, occur naturally in oils from plants of the order Malvales (Phelps, Shenstone, Kemmerer & Evans, 1965). Cottonseed oil, an important food item, contains a small amount of both the 18-carbon malvalic acid and the 19-carbon sterculic acid. There have been many reports of adverse physiological effects following the feeding of pure CPFA, or of oils containing CPFA, to animals. Abnormalities in reproductive processes and alterations in lipid metabolism were among the effects noted in work reviewed by Phelps *et al.* (1965).

Reiser & Raju (1964) reported that the fatty acyl desaturase system was inhibited in rats dosed with sterculic acid. Johnson, Pearson, Shenstone & Fogerty (1967) found that sterculic acid reduced the ability of hen-liver homogenates to convert stearic to oleic acid. They speculated that this inhibition was responsible for the increase in stearic acid and the relative decrease in oleic acid in lipids from animals fed cyclopropenes. Nixon, Eisele, Wales & Sinnhuber (1974) found altered lipid metabolism and possibly also membrane changes in rats fed CPFA. Erythrocyte haemolysis in 0·3 M-glycerol was increased, glutathione-induced mitochondrial swelling was completely inhibited and microsomal codeine-demethylase activity was depressed in the CPFA-fed rats.

Sinnhuber, Lee, Wales & Ayres (1968) and Lee, Wales & Sinnhuber (1971) reported that CPFA greatly enhanced the incidence and rate of growth of aflatoxin-induced hepatoma in rainbow trout. Other changes noted in CPFA-fed trout were lipid accumulation and morphological changes in liver cells (Roehm, Lee, Wales, Polityka & Sinnhuber, 1970), decreased liver-protein levels and a reduction in the activity of soluble dehydrogenases (Taylor, Montgomery & Lee, 1973).

Tennent, Zanetti, Siegel, Kuron & Ott (1959) found that hens fed cottonseed oil had higher serum-cholesterol levels and a greater degree of atherosclerosis than would have been predicted from the content of saturated fatty acids in the oil. Goodnight & Kemmerer (1967) demonstrated that CPFA fed as *Sterculia foetida* oil altered cholesterol metabolism in cockerels and increased plasma cholesterol, aortic atherosclerosis, liver weight, bile volume and bile-acid excretion. The reaction of mammalian cholesterol synthesis to CPFA is not well established.

This report describes the effects of CPFA (fed as *S. foetida* oil) on cholesterol levels, atherosclerosis and liver morphology in rabbits during a 5-wk exposure.

EXPERIMENTAL

Animals and diets. Male New Zealand rabbits, approximately 14 wk old and with a mean weight of 1800 g, were kept in individual wire cages. A pelleted semi-purified diet, which included 3% dehydrated alfalfa meal, 2% ground barley and 5% molasses, produced normal weight gains in the animals. Addition of 0·5% *S. foetida* oil did not reduce consumption of the diet. The composition of the basal diet (A) is given in Table 1. The *S. foetida* oil used in diets B and D, at a level of 0·27%, contained 48% sterculic and 7% malvalic acids and replaced 0·5% corn oil. Diet C contained 0·5% cholesterol, replacing an equal amount of cellulose in the basic formulation. The fourth diet, D, contained both 0·5% *S. foetida* oil and 0·5% cholesterol. All four diets were isocaloric at 3·41 kcal/g and contained approximately 21% saturated fatty acids.

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Table 1. *Composition of basal diet*

Component	Content (g/kg diet)
Casein	200
Sucrose	164·5
Corn starch	295
Cellulose	123
Alfalfa meal	30
Molasses	50
Barley	20
Mineral mix*	40
Vitamin mix†	22
α-Tocopherol concentrate	1·5‡
Choline chloride	2
D,L-Methionine	2
Corn oil	25
Hydrogenated vegetable oil	25

*Jones-Foster Mineral Mix, Nutritional Biochemical Corp., Cleveland, Ohio.
†Vitamin fortification mixture, Nutritional Biochemical Corp.
‡Supplying 375 IU as D,L-α-tocopherol acetate/kg feed.

Preliminary feeding studies indicated that experimental animals would consume 80–85 g diet containing 0·5% *S. foetida* oil when fed *ad lib*. Animals receiving diets C and D were fed identical amounts based on the average amount the animals given diet D ate in a 24-hr period. Consumption was 60 g/day for the first 3 wk and then 50 g/day to the end of the trial.

Conduct of experiment. Food consumption and weight gains were recorded throughout the feeding study. Blood samples (approximately 2 ml) were collected initially, after 3 wk and at wk 5, when the feeding trial was terminated. Blood taken from the marginal ear vein was transferred to centrifuge tubes containing 0·1 ml heparin solution (1000 units/ml) and immediately centrifuged for 20 min at top speed using an I.E.C. clinical centrifuge. After the plasma had been transferred to vials, it was purged with nitrogen and frozen for subsequent analysis. In all cases, animals were starved but given free access to water for 12 hr before blood samples were taken.

After 5 wk on the test diets, animals were fasted overnight and killed by cervical fracture. The jugular vein was immediately severed, blood samples were taken and internal organs were removed for gross examination. Pieces of liver, heart and aortic tissue were collected for histological examination. The thoracic and abdominal aorta was removed down to the femoral branch, divided longitudinally and fixed in 10% formalin to facilitate lipid staining.

Total cholesterol (Rudel & Morris, 1973) and non-esterified cholesterol (Crawford, 1958) were determined in plasma. For the measurement of total triglycerides in plasma a Calbiochem Triglyceride Stat-Pack was used (Calbiochem, La Jolla, Cal.). Liver cholesterol was extracted by the method of Folch, Lees & Sloane-Stanley (1957) and the quantity was determined by the method of Rudel & Morris (1973). Liver protein content was determined by the Kjehldahl method.*

Liver tissue was preserved in Bouin's solution for histological examination and sections were stained with haematoxylin and eosin.

The fatty-acid composition of diet and tissue lipids was determined by gas chromatography (GLC) using a column (length 9 ft) packed with 10% DEGS on Anakrom (110/120 mesh). CPFA content was measured using the Halphen reaction as described by Hammonds, Cornelius & Tan (1971).

Sections of aorta were stained according to Holman, McGill, Strong & Greer (1958) and atherosclerotic lesions were graded on a 0–4 scale as described by Adams, Gaman & Feigenbaum (1972), grade 1 indicating mainly fatty streaks, grade 2, elevated intimal thickening, grade 3, extended thickened areas with apparent fibrous plaques and grade 4, ulcerated complicated plaques with haemorrhages and calcium deposits.

*Proximate analyses of livers were conducted by the Department of Agricultural Chemistry, Oregon State University.

RESULTS

The mean plasma-cholesterol level of group B animals (Table 2) was more than double the mean level in group A (control) animals at wk 3, and was three times as high as the levels in control animals at wk 5. Because of the large variation within groups, differences between groups A and B and those between groups C and D were not statistically significant. Mean plasma-cholesterol levels of rabbits in groups B, C and D increased throughout the trial. The increase in plasma cholesterol in control rabbits was apparently due to the change from commercial pellets to the basal test diet.

At wk 3 and 5, the percentage of non-esterified cholesterol in the plasma of group B animals was higher than that in the control animals. Group D animals had higher free cholesterol levels than group C at wk 5. We have no explanation for the high initial level of free cholesterol in group D. When groups A and C (control and control plus cholesterol) were compared, cholesterol-fed animals had higher total plasma- and liver-cholesterol levels, but the percentage of free cholesterol in the plasma of both groups was nearly identical. In a study of rabbit lipoproteins, Camejo, Bosch, Arreaza & Mendez (1973) reported 39% non-esterified cholesterol in the plasma of control rabbits. Increased levels of non-esterified cholesterol in plasma have been found in cases on familial lethicin–cholesterol acyl transferase (LCAT) deficiency and in cholesterol-fed guinea-pigs (Myant, 1971).

Post-absorptive plasma-triglyceride levels (Table 2) varied widely between individual animals in each treatment and showed no direct relationship to plasma-cholesterol levels. The CPFA-containing diets produced plasma-triglyceride levels higher than those associated with similar diets without cyclopropenoids. The Calbiochem method could not be used to determine the wk-5 levels of plasma triglycerides in animals fed 0·5% cholesterol because of the extremely high cholesterol content.

There were no statistically significant differences in liver cholesterol content (Table 2) but an obvious trend was established. Control livers contained an average of

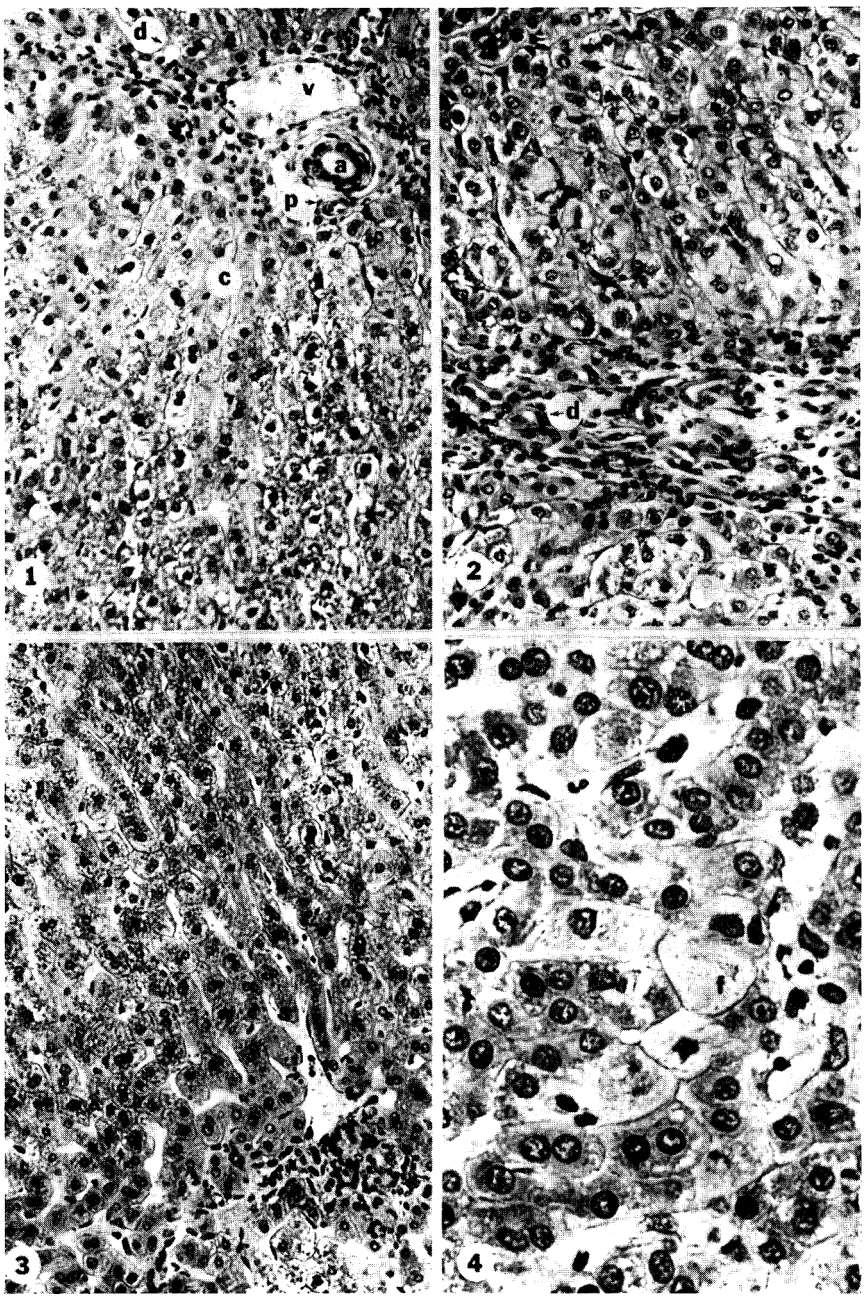

Fig. 1. Section from liver of control rabbit (group A), showing an artery (a), a vein (v), at least two bile ductules (d), a portal branch of connective tissue (p) and normal cords of parenchymal cells (c). Haematoxylin and eosin × 250.

Fig. 2. Section from liver of CPFA-fed rabbit (group B), depicting an area containing a tangentially cut branch of a portal tract showing marked ductular proliferation (d). The cords of parenchymal cells have lost their orderly arrangement and the area shown is a mass of enlarged bizarre cells with abnormal nuclei, some of which are vacuolated. Haematoxylin and eosin × 250.

Fig. 3. Section of liver of cholesterol-fed rabbit (group C). No abnormalities are evident. Haematoxylin and eosin × 250.

Fig. 4. Section of liver from rabbit fed cholesterol and CPFA (group D), showing several dying parenchymal cells with fibres (f) and cells that are irregular in shape and size. Haematoxylin and eosin × 560.

Table 2. *Effect of CPFA on plasma- and liver-cholesterol levels and on plasma-triglyceride levels in the rabbit*

Determination	Duration of feeding (wk)	Mean values* for rabbits in group†			
		A	B	C	D
Plasma cholesterol					
Total (mg/100 ml)	0	85 ± 26·8	87 ± 29·5	84 ± 16·8	83 ± 28·2
	3	188 ± 85·5	405 ± 294	1170 ± 467	1231 ± 503
	5	148 ± 59·0	461 ± 453	1442 ± 600	1719 ± 615
Free (%)	0	32	30	30	43
	3	42	59	45	45
	5	37	46	38	48
Plasma triglycerides					
(mg/100 ml)	0	49 ± 35·0	33 ± 11·8	31 ± 15·3	30 ± 13·7
	3	25 ± 7·1	58 ± 61·2	20 ± 14·5	97 ± 39·2
	5	43 ± 37·1	64 ± 30·3	ND	ND
Liver cholesterol					
In mg/g tissue‡	5	4·5 ± 1·0	7·7 ± 6·0	18·3 ± 4·6	20·9 ± 8·5
In mg/g protein	5	22·2 ± 5·0	44·2 ± 34·6	98·6 ± 24·7	114·8 ± 46·8

ND = Not determined

*Except for percentage values, results are expressed as the means ± SD, groups A, B and C each consisting of eight animals and group D of ten.

†Group A, basal diet; B, basal diet plus *S. foetida* oil; C, basal diet plus cholesterol; D, basal diet plus *S. foetida* oil and cholesterol.

‡Wet weight.

22·2 mg cholesterol/g protein (4·5 mg/g wet weight), a value in agreement with published values for the cholesterol content of normal rabbit liver (Gupta, Tandon & Ramalingaswami, 1971). This value was doubled when CPFA was fed, although there was a large individual variation. A similar pattern was seen when liver-cholesterol levels of animals fed cholesterol were compared with those of animals receiving cholesterol and CPFA.

Table 3 summarizes the results obtained when aortic atherosclerosis was graded and compared in the four groups. Rabbits receiving the control diet for 5 wk showed no sign of plaque formation, fatty streaks or any other degeneration of the intimal surface. Five of the eight animals in group B developed fatty streaks (accumulated foam cells under the intact endothelial surface) and small plaques in the abdominal aorta and aortic arch. The extent of the atherosclerotic disease was associated with increases in plasma-cholesterol levels in the CPFA-fed rabbits. Animals in groups C and D had much more severe atherosclerosis, as reflected in mean aortic grades of 1·31 and 2·42, respectively. Animals receiving cholesterol plus CPFA tended to have more extensive atherosclerotic disease, although individual variations were large. Except for one individual, all animals in group D were found to have an aortic grade of at least 1·5, indicating the presence of some plaques elevated above the intimal surface.

Table 3. *Effect of CPFA on the degree of atherosclerosis in the rabbit*

Group*	Incidence of aortic lesions†	Mean aorta scores‡
A	0/8	0
B	5/8	0·50 ± 0·46
C	8/8	1·31 ± 1·0
D	9/10	2·42 ± 1·3

*Group A, basal diet; B, basal diet plus *S. foetida* oil; C basal diet plus cholesterol; D, basal diet plus *S. foetida* oil and cholesterol.

†No. of animals affected/total no. in group.

‡Graded on a 0–4 scale as described by Adams *et al.* (1972), following staining by the method of Holman *et al.* (1958), and expressed as the mean ± SD for the group.

Histological examination showed that liver cells from animals fed diets A and C were normal. Parenchymal cells of group C animals contained inclusion bodies, probably accumulated lipid. Liver tissue from group-B rabbits showed alteration of parenchymal cells in most livers. In some cells, the subcellular organelles were organized into laminar arrays that appeared similar to abnormalities found in trout fed CPFA (Lee *et al.* 1971; Sinnhuber *et al.* 1968). Liver cells from animals of group D were abnormal and showed many signs of toxicity, including disintegrated cell walls, pyknotic nuclei, binucleate cells and abnormal, folded bile-duct walls. Typical liver cells from animals on each of the four diets are shown in Figs 1–4.

GLC analysis of a limited number of samples of liver lipids showed an increase in the ratio of saturated to unsaturated fatty acids (primarily 18:0/18:1) in CPFA-fed rabbits. Analysis of lipid from the adipose tissue of animals fed CPFA showed that the cyclopropenoids were deposited in adipose tissue. After 5 wk, CPFA levels were approximately 2% of the total lipid.

Feed-efficiency and weight gains varied widely between individuals, but no differences were detected between CPFA-fed and control animals. Two animals in group D lost weight (59 and 141 g) during the 5-wk period. Average gains per group were 634 ± 299, 652 ± 263, 237 ± 96 and 173 ± 280 for groups A, B, C and D, respectively. Feeding CPFA together with cholesterol produced effects that were

much more severe than those produced by either component alone.

DISCUSSION

The effects of CPFA in rabbits were similar to those in cockerels (Goodnight & Kemmerer, 1967; Tennet *et al.* 1959). In the rabbit, however, both liver and plasma-cholesterol levels were increased, while in cockerels liver-cholesterol levels were reduced. A species difference among mammals is also suggested by the fact that rats do not exhibit elevated cholesterol levels when fed *S. foetida* oil (Coleman & Friedman, 1971). The deleterious effects of CPFA in rabbits were increased when cholesterol was fed concurrently. In general, elevated liver-cholesterol levels were associated with slow growth and the more severe histopathological changes. Animals in groups B and D, which had large increases in plasma-cholesterol levels compared with their controls, were also found to have the most extensive atherosclerosis. However, induction of atherosclerosis in CPFA-fed rabbits cannot be accounted for solely by increased cholesterol levels. Two animals in group A had plasma-cholesterol levels above the levels noted in group B animals, but showed no signs of atherosclerosis.

The unusual, striated appearance of certain liver cells observed in this study is similar to that found in rainbow trout fed CPFA (Sinnhuber *et al.* 1968). Scarpelli, Lee, Sinnhuber & Chiga (1974) reported that this peculiar striated appearance is caused by rearrangement of the rough endoplasmic reticulum. Associated with this change was the appearance of granular material and needle-like crystals, which were tentatively identified as cholesterol. It is interesting that this cytoplasmic change has not been seen in the rat, a species that does not develop hypercholesterolaemia when fed CPFA.

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THE RENAL HANDLING OF 2,4,5-TRICHLOROPHENOXY-ACETIC ACID (2,4,5-T) IN THE DOG

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Abstract—The herbicide 2,4,5-T is actively transported by renal cortical slices of dogs and rats, suggesting that the compound should be rapidly eliminated from the body via the kidneys. The prolonged plasma half-life of 2,4,5-T in the dog (77 hr) indicates that factors other than secretion into the urine are important determinants of elimination in the dog. This study was designed to determine the renal handling of 2,4,5-T in anaesthetized dogs, and an attempt was made to increase excretion of the herbicide with sodium acetate. Injection of 2,4,5-T decreased clearance of *p*-aminohippurate in a dose-dependent manner, suggesting that the compound was actively secreted. The clearance of the herbicide, however, was exceedingly low, being less than 1% of inulin clearance. The clearance of 2,4,5-T was increased by sodium acetate and by acetazolamide. Additional studies with mannitol, sodium bicarbonate and ammonium chloride demonstrated that clearance of 2,4,5-T was related to urinary pH, but only when the pH exceeded 6, and was not affected by changes in urine volume. Addition of plasma inhibited the transport of 2,4,5-T by renal cortex slices *in vitro*, suggesting that the low clearance *in vivo* was due to very tight binding of the herbicide to plasma protein.

INTRODUCTION

2,4,5-Trichlorophenoxyacetic acid (2,4,5-T) is a plant-growth regulator and herbicide of relatively low toxicity, a property apparently due in part to its rapid excretion via the kidneys. 2,4,5-T is more toxic to dogs than to rats. Piper, Rose, Leng & Gehring (1973) demonstrated that the rate of renal excretion of 2,4,5-T was greater in rats than in dogs and suggested that this difference could account for the differences in toxicity. Hook, Bailie, Johnson & Gehring (1974) used an *in vitro* technique to demonstrate that 2,4,5-T is actively transported by renal cortical tissue of the rat and dog. They suggested that the primary route of renal elimination of 2,4,5-T is active secretion of the compound. The ability of adult rat tissue to transport 2,4,5-T rapidly was given as the explanation for the shorter biological half-life of 2,4,5-T in the rat. Addition of sodium acetate to the incubation medium markedly augmented the transport of 2,4,5-T by renal cortical slices from the dog (Hook *et al.* 1974). Acetate also stimulated the incorporation of other organic anions *in vitro* and increased clearance and T_m in intact animals (Cross & Taggart, 1950; Mudge & Taggart, 1950). This suggested that sodium acetate might serve to enhance the elimination of 2,4,5-T from the plasma of dogs.

The purpose of this investigation was twofold—first, to determine the mechanisms involved in the renal handling of 2,4,5-T in anaesthetized dogs and, secondly, to determine whether the clearance of 2,4,5-T could be enhanced by the infusion of sodium acetate.

*Supported by a World Health Organization Fellowship.

EXPERIMENTAL

Animals and treatment. Mongrel dogs of either sex were anaesthetized with sodium pentobarbitone (30 mg/kg) given iv. A cuffed endotracheal tube was inserted and the animals were ventilated artifically on a Harvard Respirator. A femoral artery and vein were cannulated for purposes of recording blood pressure and infusion of fluids, respectively. Both ureters were cannulated through a low abdominal midline incision and the urine from both kidneys was collected in a single graduated cylinder. All animals received a basal infusion of 0·9% sodium chloride containing 0·4% inulin at 5 ml/min. In some experiments, *p*-aminohippurate (PAH) was added to the infusion (0·04%). Five groups of experiments were carried out.

Series 1. The effect of a single iv injection of 2,4,5-T on the clearance of PAH and inulin was determined. After approximately 1 hr of iv infusion, 10-min urine samples were collected, and blood samples were drawn at the midpoint of the collection period. After two or three collection periods, a single bolus of 2,4,5-T (5 or 10 mg/kg) was administered iv. Urine and blood samples were then collected for 2–3 hr.

Series 2. The clearance of 2,4,5-T was determined in a similar manner. After surgery, an infusion of 0·4% inulin in saline was begun at 0·25 ml/kg/min. When urine volume had stabilized, ^{14}C-labelled 2,4,5-T was injected iv in a dose of 2·5 mg/kg (10 μCi/dog). After

a 30-min equilibration period, urine and plasma samples were collected at 20-min intervals. After two or three collection periods, the animals were infused with sodium acetate (50, 100 or 210 μmols/kg/min) for 2–3 hr. In four animals, acetazolamide was administered 60 min after the 2,4,5-T injection as a single 10-mg/kg dose followed by an infusion of 10 mg/kg/hr for 2 hr.

Series 3. Following the administration of 2,4,5-T, urine volume was increased gradually by infusing 0·9% sodium chloride at 0·5 ml/kg/min for approximately 1 hr. The infusion was then changed to 5% mannitol at the same rate for 30 min followed by sodium acetate at 100 μmols/kg/min for 1 hr. The acetate was then discontinued and mannitol was again infused.

Series 4. To determine the effect of urinary pH on the clearance of 2,4,5-T, three dogs were given ammonium chloride (11·64 g/day) orally for 1, 2 or 3 days. The animals were then anaesthetized and infused as before. After appropriate control clearance periods, the infusion was changed to one containing sodium bicarbonate (5%) infused at 0·5 ml/kg/min. Urine samples were collected under oil and pH was measured with a hydrogen-sensitive pH electrode (Instrumentation Laboratories).

Series 5. The effect of plasma on the *in vitro* transport of 2,4,5-T was determined in renal cortical slices from dogs. Kidneys were removed from anaesthetized dogs and placed in ice-cold saline (0·9% NaCl), and plasma was collected from the same animals. Renal cortical slices were prepared free-hand and kept briefly in cold saline until incubated. Slices (100–200 mg) were evenly divided between beakers containing $7{\cdot}4 \times 10^{-5}$ M-PAH and other beakers containing 2×10^{-5} M-2,4,5-T in 2·5 ml phosphate buffer (Cross & Taggart, 1950). Within each of these groups, plasma (0·5 ml) was added to half the beakers to produce a 1:6 dilution, while saline (0·5 ml) was added to control beakers. The slices were incubated for 90 min at 25°C under a gas phase of 100% oxygen. Following incubation the slices were removed from the beakers, blotted, weighed and analysed for PAH as described previously (Hook *et al.* 1974).

Analytical methods. PAH was estimated by the method of Smith, Finkelstein, Aliminosa, Crawford & Graber (1945) and inulin by the method of Walser, Davidson and Orloff (1955). 2,4,5-T concentration was estimated by liquid scintillation spectrometry using a Beckman LS-100 liquid scintillation counter. Clearance was the product of minute volume multiplied by urine concentration divided by the total concentration in arterial plasma. Clearance data for PAH and 2,4,5-T are reported as the ratio of the clearance relative to that of inulin (i.e. C_{PAH}/C_{IN} and $C_{2,4,5\text{-}T}/C_{IN}$) to normalize data and to account for small fluctuations in blood pressure.

RESULTS

Administration of 5 or 10 mg 2,4,5-T/kg significantly depressed PAH secretion without influencing glomerular filtration rate. The ratio C_{PAH}/C_{IN} was depressed in the first clearance period following the administration of the herbicide (Fig. 1). The effect appeared to be dose-dependent in that the depression of PAH clearance by 10 mg/kg was much greater than that produced by 5 mg/kg. In both cases the effect was long lasting, being obvious 2 hr after this single dose (Fig. 1).

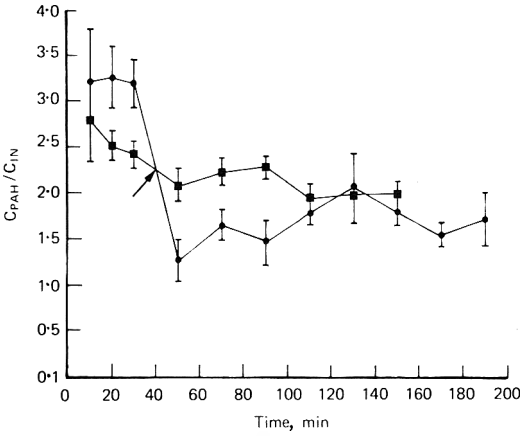

Fig. 1. Effect of a single iv injection of 2,4,5-T, in a dose of 5 (■——■) or 10 (●——●) mg/kg, on PAH clearance. Each point represents the mean ± SEM for three dogs, and the point of 2,4,5-T administration is arrowed.

Infusion of acetate was followed by a significant increase in the urine volume and pH and the clearance of 2,4,5-T. During the control phase, the clearance of 2,4,5-T was exceedingly small. Infusion of sodium acetate increased $C_{2,4,5\text{-}T}/C_{IN}$ in a dose-dependent manner (Fig. 2). However, even at the peak of the response the clearance ratio did not reach 1.

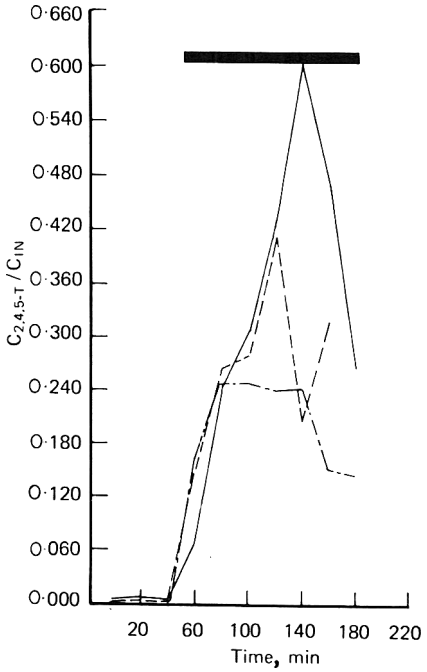

Fig. 2. Effect of sodium acetate infusion on the clearance of 2,4,5-T. Each line represents the average of two experiments, involving infusion of 50 (— — —), 100 (— · —) or 210 (——) μmols sodium acetate/kg/min during the period indicated by the horizontal bar.

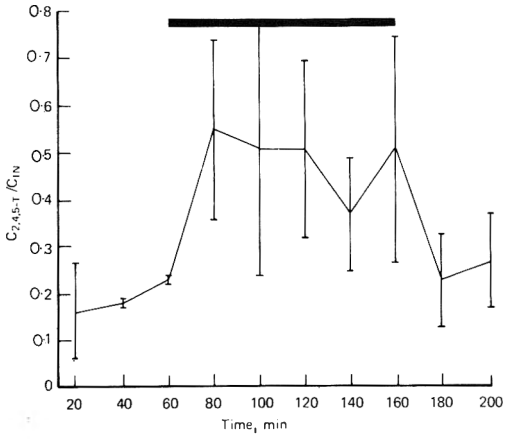

Fig. 3. Effect of acetazolamide (in a single iv dose of 10 mg/kg and iv infusion of 10 mg/kg/hr) on the clearance of 2,4,5-T. Each point represents the mean ± SEM for three dogs. The horizontal bar indicates the duration of acetazolamide infusion.

Infusion of acetazolamide also increased the $C_{2,4,5\text{-}T}/C_{IN}$ ratio, but the effect was extremely variable (Fig. 3). In an attempt to determine the specific effects of urine volume and urine alkalinity, a series of experiments was conducted in which urine volume was increased with saline and mannitol followed by sodium acetate. A representative experiment is illustrated in Fig. 4. During the control period, clearance of 2,4,5-T was almost negligible. As urine volume increased there was only a slight increase in the clearance of 2,4,5-T. During sodium acetate infusion, urine volume remained relatively constant and the clearance of 2,4,5-T increased, reaching a peak during the second administration of mannitol. However, even in the situation where urine volume was high and the urine alkaline, the clearance of 2,4,5-T was not as high as the clearance of inulin.

To determine the effect of urinary volume and pH in the same animals, three dogs were pretreated with ammonium chloride for 1–3 days to acidify the urine. The animals were anaesthetized, control observations

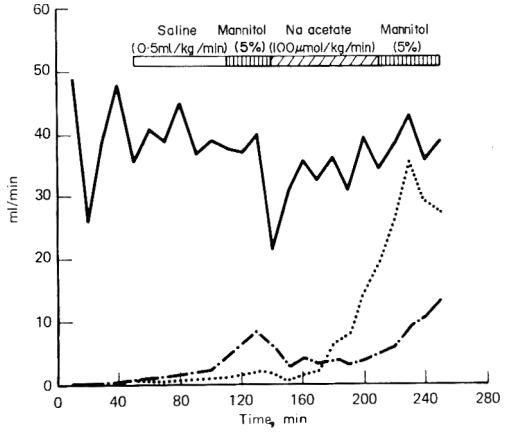

Fig. 4. Effect of urine volume (- - - -) and sodium acetate infusion on 2,4,5-T clearance (·····) in a typical dog. The horizontal bar indicates the duration and content of iv infusions, and inulin clearance is also shown (——).

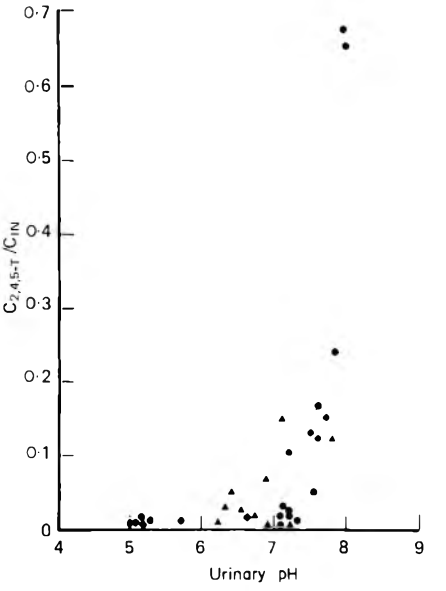

Fig. 5. Relationship between 2,4,5-T clearance and urinary pH. Dogs were pretreated with ammonium chloride and infused with sodium bicarbonate to change the urinary pH. The different symbols represent separate animals.

were made and then sodium bicarbonate was infused to increase the pH and volume of the urine. The clearance of 2,4,5-T was sensitive to urinary pH, for at pH values exceeding 6·0 there appeared to be a direct relationship between the clearance and the pH (Fig. 5). However, in no case did the clearance of 2,4,5-T exceed that of inulin. In the same animals, there was no relationship between the clearance of 2,4,5-T and the urine volume (Fig. 6).

To determine the effect of the plasma-binding of 2,4,5-T on transport, renal cortical slices were prepared from dogs. Half of the slices prepared from

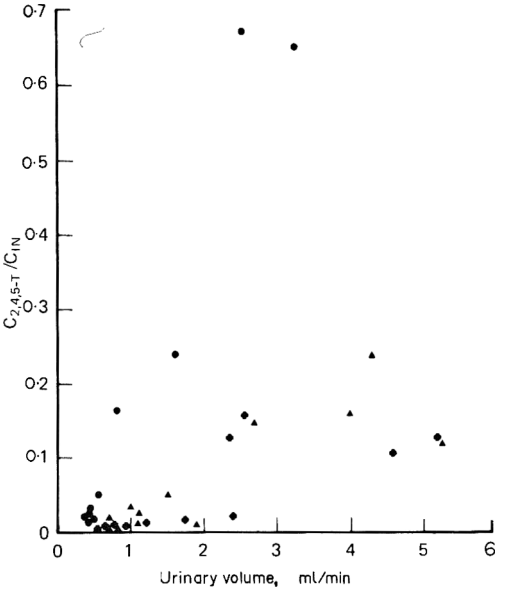

Fig. 6. Relationship between 2,4,5-T clearance and urinary volume in the dogs involved in Fig. 5.

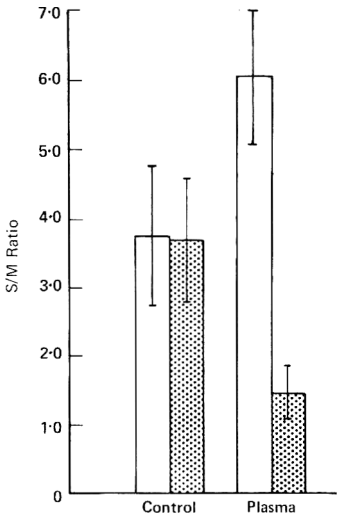

Fig. 7. Effect of plasma (1:6 dilution) on the accumulation (S/M ratio) of PAH (□) and 2,4,5-T (▨) by renal cortical slices from dogs. Each bar represents the mean ± SEM for three experiments. The effect of plasma on both PAH and 2,4,5-T was statistically significant ($P < 0{\cdot}05$).

a dog were incubated with PAH and half with 2,4,5-T. Incubation of these slices with plasma increased the apparent transport of PAH while significantly decreasing the transport of 2,4,5-T (Fig. 7).

DISCUSSION

Piper *et al.* (1973) demonstrated that the half-life of 2,4,5-T in the dog was approximately 77 hr, compared with 4·7 hr in the rat. They implied that this was due to a difference in the secretory capacity of the two species. This difference was confirmed by Hook *et al.* (1974), who observed that accumulation of 2,4,5-T *in vitro* was significantly greater in rat tissue than in dog tissue. Nevertheless, dog tissue did actively transport 2,4,5-T and this was enhanced by acetate. The long half-life in the dog suggested that processes other than active secretion were involved in the renal handling of the compound. Since the compound is very soluble in lipid, it was not unreasonable to assume that the compound would passively backdiffuse into the plasma as urine was concentrated. Thus, it might be expected that the clearance of 2,4,5-T would be regulated not only by active transport but by factors that influence passive diffusion, such as urinary volume and urinary pH.

The observation that 2,4,5-T selectively depressed PAH secretion *in vivo* demonstrated that the compound was capable of being actively transported *in vivo*. This, however, provided no quantitative information concerning the secretory mechanism. Thus, the second series of experiments was conducted to determine the clearance of 2,4,5-T. Interestingly, the clearance of 2,4,5-T was exceedingly low, being less than 1% of the clearance of inulin. This suggested either that the material was not being secreted or that a great deal of passive reabsorption of the herbicide had occurred. Administration of acetate increased 2,4,5-T clearance, yet even with doses of acetate approaching toxic levels the clearance of 2,4,5-T was less than that of inulin. This might suggest that acetate was stimulating transport. However, these doses of acetate increased urinary volume and made the urine alkaline. Either of these effects could also influence the clearance of the compound if it were passively reabsorbed. To test this hypothesis, the carbonic anhydrase inhibitor, acetazolamide, was used. This compound increased urine volume and made the urine alkaline without having the effect on intermediate metabolism that has been ascribed to acetate (Cross & Taggart, 1950). Acetazolamide did increase clearance somewhat, although the effect was erratic (see large standard errors in Fig. 3) and like acetate, acetazolamide did not raise the clearance of 2,4,5-T as high as inulin clearance. Therefore, the question of whether urine volume and/or alkalinity was influencing clearance was not resolved.

In the next group of experiments it became clear that the effect of acetate was not due to changes in urine volume. A marked increase in urine volume produced by infusion of saline and mannitol had only a minor effect on 2,4,5-T clearance (Fig. 4). Addition of acetate, however, produced a steep increase in 2,4,5-T clearance, suggesting that either the alkalinizing effect of acetate and/or its metabolic effect was responsible for this increase. However, as before, the clearance did not exceed that of inulin. Figures 5 and 6 demonstrated that clearance of 2,4,5-T was dependent on urinary pH but not on urinary volume. In two experiments, probenecid (30 mg/kg) was administered iv after a high urinary pH had been established and the 2,4,5-T clearance ratio was near 0·4. This inhibitor of anion secretion significantly reduced the clearance of 2,4,5-T, suggesting that even though the clearance was low, most of the 2,4,5-T in the urine arrived by means of active secretion.

Thus, the question remained as to why the clearance of 2,4,5-T was so low if, indeed, it was being secreted. Classical renal physiology suggests that a compound may be secreted even if it is bound to plasma protein. Between 80–90% of PAH, for instance, is bound to plasma proteins and yet it can be completely extracted from the plasma passing the cortex (Mudge & Taggart, 1950). This suggests that the affinity of the kidney for PAH is greater than that of the binding sites on the plasma protein. This, however, may not be the case for certain other compounds, including 2,4,5-T. Berndt, Wade & Mudge (1971) suggested that the long half-life of iophenoxic acid could be related to the highly tenacious binding of the material to plasma protein. Addition of plasma reduced active accumulation of iophenoxic acid by dog and rabbit renal cortical slices, suggesting that plasma binding sites had a greater affinity for the anion than did renal-transport protein (Berndt *et al.* 1971). In the dose used (2·5 mg/kg), most 2,4,5-T in plasma is bound to protein. Ultrafiltrates of plasma prepared from four dogs in this study contained only 2–4% of the total plasma-2,4,5-T concentration. Similarly, A. Sauerhoff & P. J. Gehring (unpublished results 1974) observed 96–98% protein-binding of 2,4,5-T over a wide range of plasma concentrations.

These data, however, provide no information regarding the characteristics of the binding. Addition

of plasma to renal cortical slices enhances accumulation of PAH possibly because of the presence in plasma of intermediates of renal metabolism, such as lactate, pyruvate or acetate (Kliger, Goldin & Preuss, 1975). Addition of plasma significantly enhanced accumulation of PAH and simultaneously depressed 2,4,5-T (Fig. 7). This strongly suggests that the addition of plasma provided binding sites for 2,4,5-T, effectively removing the material from the medium. Thus, 2,4,5-T was more tightly bound to plasma protein than to the kidney, a finding which provides the explanation for the low clearance of 2,4,5-T in the dog.

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MIREX: A TERATOGENICITY, DOMINANT LETHAL AND TISSUE DISTRIBUTION STUDY IN RATS

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Abstract—The teratogenic potential of Mirex was studied in rats given daily oral doses of 0, 1.5, 3·0, 6·0 and 12·5 mg/kg on days 6–15 of gestation. The 12·5-mg/kg dosage caused maternal toxicity, pregnancy failure, decreased foetal survival, reduced foetal weight and an increased incidence of visceral anomalies. Maternal effects and an increased foetal incidence of (visceral) anomalies were observed also with the 6-mg/kg doses. Lower doses produced minimal or no adverse effects. Mirex accumulated in the maternal tissues and readily crossed the placenta. Although Mirex was present in the testes of treated adult rats, a dominant lethal assay revealed no significant difference in reproduction parameters between controls and the groups treated with 1·5, 3·0 or 6·0 mg/kg.

INTRODUCTION

Mirex, an extremely stable insecticide, resists biodegradation in the soil (Van Valin, Andrews & Eller, 1968) and contaminates aquatic and terrestrial systems (de la Cruz & Naqvi, 1973; Wolfe & Norment, 1974). It has been detected in milk (Lofgren, Bartlett, Stringer & Banks, 1964), in beef (Baetcke, Cain & Poe, 1972; Ford, Hawthorne & Markin, 1973) and in fish and birds (Borthwick, Duke, Wilson, Lowe, Patrick & Oberheu, 1973). These residues have generally been restricted to the habitat of the southern United States, where Mirex is used in government-sponsored programmes. Recently, Mirex has been found in fish caught from Lake Ontario (Kaiser, 1974), suggesting that it might be more widely distributed in the environment than was hitherto suspected.

Biochemical changes induced in the liver by Mirex (Baker, Coons, Mailman & Hodgson, 1972; Byard, Koepke, Abraham, Golberg & Coulston, 1974) or a polychlorinated biphenyl–Mirex mixture (Abraham, Koepke, Golberg & Coulston, 1974) have been described. The fate of [^{14}C]Mirex in rats (Mehendale, Fishbein, Fields & Matthews, 1972) and in rhesus monkeys (Kennedy, Pittman & Stein, 1975) and the carcinogenicity of Mirex (Innes, Ulland, Valerio, Petrucelli, Fishbein, Hart, Pallotta, Bates, Falk, Gart, Klein, Mitchell & Peters, 1969) have been reported.

Progeny from female rats fed a diet containing 25 ppm Mirex (98% purity) had a reduced survival rate and a high incidence of cataracts, while progeny from females maintained on a level of 5 ppm appeared normal (Gaines & Kimbrough, 1970). Increased parental mortality and reduced litter size were noted in two strains of mice fed a dietary concentration of 5 ppm (Ware & Good, 1967).

In the absence of previous reports on the teratogenicity, dominant lethality and placental passage of Mirex, studies on these aspects were undertaken and the results obtained are reported here.

EXPERIMENTAL

Teratogenicity study. Female Wistar rats, weighing 175–200 g, were paired overnight with proven sires and the morning on which a sperm-positive vaginal smear was observed was considered to be day 1 of pregnancy. Twenty mated females were randomly assigned to each of the five experimental groups. Mirex of technical standard (98% purity; Hooker Chemicals & Plastics Corp. Niagara Falls, New York) was suspended in corn oil and administered orally by stomach intubation in single daily doses on days 6–15 of gestation. The doses, expressed in mg/kg with the percentage concentrations in parentheses, were 1·5 (0·03), 3 (0·06), 6 (0·13) and 12·5 (0·25). The volume of the oil per dose for all experimental groups was 10 ml/kg body weight and controls were given corn oil alone in the same amount.

The females were weighed on gestation days 1 and 6–15, as well as before and after Caesarean section. The body-weight determinations permitted correction of the dose for changing weight and the assessment of maternal toxicity. Females were killed on day 22 of gestation and their viscera, including the uteri, were examined for pathological changes. Samples of liver, heart, spleen, kidney, brain and fat from four or five females in each dosage group were collected and stored in the freezer for residue analysis. The foetuses were removed, weighed and examined for viability and external malformations. Two-thirds of the live foetuses from each litter were examined for skeletal anomalies. The remainder were studied for visceral changes or analysed for Mirex residues, using either the whole foetus or foetal brain and liver.

Dominant lethal studies. Male rats of proven fertility were randomly distributed into four groups, each consisting of 20 males. They were dosed by stomach tube with 0, 1·5, 3·0 or 6·0 mg Mirex/kg daily for ten consecutive days. The Mirex suspension was prepared as described for the teratogenicity study. After the end of the treatment, 14 sequential mating trials were con-

Table 1. *Maternal effects in rats dosed orally with 1·5–12·5mg Mirex/kg during days 6–15 of gestation*

Dose (mg/kg/day)	Numbers of mated females			
	Started on test	Dying during gestation	Not pregnant at term	Pregnant at term
0	20	0	1	19
1·5	20	0	1	19
3·0	20	1	4	15
6·0	20	4	3	13
12·5	18	7	5	6

ducted. In each mating trial, a treated male was caged with two untreated virgin females for 5 days. The females were killed 13–15 days after separation from the males, and viable embryos, deciduomas and corpora lutea were counted.

Tissue distribution. A tissue distribution study of Mirex was conducted separately in males assigned to three test groups, each consisting of five animals and given 3, 6 or 12·5 mg Mirex/kg in single daily doses for 10 days. All the rats were then killed, and samples of liver, brain, fat and testis were collected, frozen and stored for chemical analysis.

Residue analyses. Tissue concentrations of Mirex were determined by a gas–liquid chromatographic method (Villeneuve, Phillips, Panopio, Mendoza, Hatina & Grant, 1974) and are reported without correction for percentage recovery, which was generally more than 90%. The presence of Mirex was confirmed by combined gas chromatography–mass spectrometry (Finnigan 1015 D).

Statistical methods. The mean and standard error of the mean were calculated for all body-weight measurements obtained in the teratogenicity and dominant lethal studies as well as for all foetal parameters. Test-group *v.* control-group comparisons were made by computing critical values of *t*. Differences were considered significant when P was $<0{\cdot}05$. Foetal anomalies were analysed by the standard chi-squared

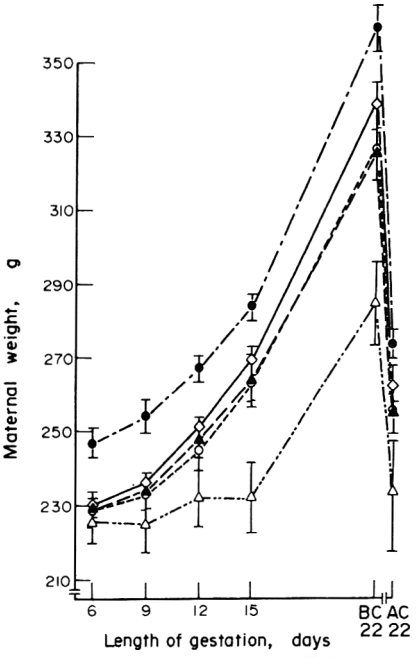

Fig. 1. Maternal body weights (means ± SEM) recorded on days 6–22 of gestation (on the last day, before (BC) and after (AC) Caesarean section) for groups of rats treated during organogenesis with daily oral doses of 0 (control; ◇——◇), 1·5 (●– – –●), 3·0 (○– – –○), 6·0 (▲– – –▲) or 12·5 (△– – – –△) mg Mirex/kg.

Table 2. *Foetal development in pregnant rats treated with 1·5–12·5 mg Mirex/kg on days 6–15 of gestation*

	Values for groups given doses (mg/kg) of				
	0	1·5	3·0	6·0	12·5
No. of rats pregnant at term	19	19	15	13	6
Live foetuses/pregnancy (mean ± SEM)	10·9 ± 3·4	12·1 ± 3·8	11·7 ± 1·8	10·1 ± 3·6*	7·5 ± 3·2*
Percentage resorptions†	3·7	4·4	7·4	8·4	40·0
Foetal weight (g: mean ± SEM)	5·1 ± 0·4	5·0 ± 0·4	4·9 ± 0·2	5·1 ± 0·3	4·0 ± 0·6*
Foetal anomalies‡					
Skeletal	15/109	10/108	10/92	11/71	4/22
Uni- and bilateral 14th rib	8	7	0	1	0
Wavy ribs, uni- and bilateral	6	1	9	9	3
Retarded ossification of: rib	1	0	1	0	0
sternum	2	2	0	1	0
skull	3	0	1	0	0
Bent ulna	0	0	0	0	2
Visceral	0/65	0/63	0/50	5/37*	10/14*
Subcutaneous oedema				2	9
Scoliosis				1	2
Cleft palate				0	6
Runts				1	0
Short tail				1	2
Heart defects: fleshy heart				0	2
enlarged atrium				0	1

†$\left(\frac{\text{No. of deciduomas and resorptions}}{\text{Total implantations}}\right) \times 100$.

‡No. of foetuses affected/total examined.

Values marked with an asterisk differ significantly (*$P < 0{\cdot}05$) from the controls.

Table 3. *Pregnancies and mean numbers of viable embryos and deciduomas/pregnancy in untreated females mated to Mirex-treated rats*

Mating trial no.	Time of mating (days after dosing)	Dose (mg/kg)...	Viable embryos/pregnancy				Deciduomas/pregnancy				No. of pregnancies (% of matings)			
			0	1·5	3·0	6·0	0	1·5	3·0	6·0	0	1·5	3·0	6·0
1	0–5		12·8	12·0	12·4	11·8	0·7	1·1	0·6	1·2	82·5	75·0	77·5	65·0*
2	5–10		12·6	12·2	12·9	12·4	0·8	0·6	0·7	0·6	87·5	82·5	87·5	75·0
3	10–15		11·9	12·5	13·0	14·0	0·7	0·5	0·7	0·5	95·0	82·5	82·5	77·5
4	15–20		12·2	12·4	11·6	12·1	0·5	0·5	0·7	0·5	87·5	80·0	75·0	86·6
5	20–25		11·1	12·8	11·9	12·1	0·7	0·5	0·7	0·8	78·0	75·0	87·5	87·2
6	25–30		11·5	12·2	11·4	11·6	0·9	0·7	0·6	0·8	78·0	85·0	90·0	89·7
7	30–35		11·6	10·5	10·9	11·6	0·9	1·0	0·8	0·9	82·5	87·5	90·0	81·5
8	35–40		11·7	12·3	12·2	10·9	0·6	1·0	0·9	0·9	85·0	82·5	77·5	86·8
9	40–45		12·1	12·4	11·4	10·8	0·6	0·6	0·8	0·8	80·0	77·5	77·5	84·2
10	45–50		12·4	12·7	12·5	11·2	0·9	0·8	0·8	0·5	92·5	77·5	87·5	79·0
11	50–55		12·3	11·0	11·4	11·4	0·3	0·7	0·8	0·9	85·0	77·5	80·0	81·1
12	55–60		12·4	11·4	11·2	11·6	0·6	0·5	0·7	0·7	90·0	77·5	80·0	94·7
13	60–65		11·2	11·3	11·4	12·3	0·7	0·7	0·8	0·6	80·0	90·0	77·5	76·3
14	65–70		11·7	11·6	11·8	11·6	0·8	0·5	0·4	0·5	87·5	90·0	82·5	89·4
		Mean...	12·0	12·0	11·9	11·8	0·71	0·66	0·72	0·72	85·1	81·4	82·3	82·4
		± SEM...	±0·5	±0·7	±0·6	±0·6	±0·17	±0·19	±0·14	±0·21	±5·2	±5·2	±5·2	±7·6

The value marked with an asterisk differs significantly ($P < 0.05$) from the control.

tests on foetal and litter ratios (number anomalous/total examined).

RESULTS

Maternal toxicity

The 3·0, 6·0 and 12·5 mg/kg doses were toxic to mated female rats, being associated with a number of deaths and a reduction in the incidence of pregnancy (Table 1). The severity of both of these effects was dose-related. Diarrhoea was a prominent sign in females dying of Mirex poisoning. At the 12·5-mg/kg level, a reduction in body-weight gain was observed during the treatment and post-treatment periods (Fig. 1).

Foetal effects

The number of live foetuses per litter was significantly reduced in the groups given doses of 6·0 or 12·5 mg/kg (Table 2). Only the highest dose produced an increased incidence of deciduomas and foetal deaths and a decrease in mean foetal weight. Visceral anomalies were seen in the groups given the 6- or 12·5-mg/kg doses, the frequency being significantly increased ($P < 0.05$) whether the foetus or the litter was considered as the experimental unit for statistical calculations. The anomalies observed at these two dose levels are listed in Table 2. The incidence of skeletal anomalies was comparable to the control incidence in all the test groups.

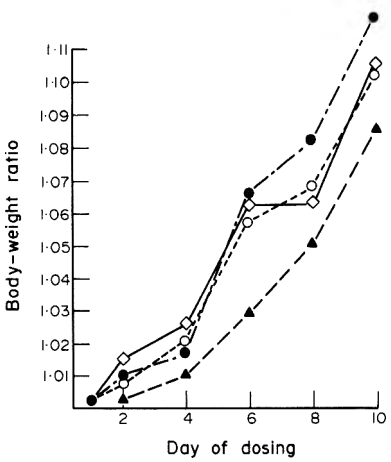

Fig. 2. Ratios of mean body weight (weight measured on day of dosing/that before dosing) for groups of male rats treated for 10 days with daily oral doses of 0 (control; ◇——◇), 1·5 (●– – –●), 3·0 (○– – –○) or 6·0 (▲– –▲) mg Mirex/kg.

Dominant lethal effects

The highest dose level used in this study, 6·0 mg/kg, produced a consistent reduction in body-weight gain during the entire dosing period (Fig. 2). One male in this group died. The data for the dominant lethal assay are summarized in Table 3. The numbers of viable embryos, deciduomas and pregnancies in the test groups were all within the control range except for a decrease in the incidence of pregnancies in the 6·0-mg/kg group in the first trial.

Mirex distribution in tissues

The amount of Mirex accumulating in the tissues of male rats given different daily doses for 10 days was dose-related (Table 4). Highest concentrations were found in the adipose tissue, followed in decreasing order by those in the liver and in the testis and brain, the levels in the latter two tissues being very similar.

Mirex was found to persist in a dose-dependent manner in all the tissues taken from pregnant females in the teratogenicity test (Table 5). The highest concentrations occurred in the fat and were followed in decreasing order by those in the liver, heart, kidney, brain and spleen. Residues were readily detected in the whole foetus and in foetal brain or liver following treatment at all the dose levels studied. The small number of live foetuses in the 12·5-mg/kg group

Table 4. *Residues in adult male rats dosed with 0–12·5 mg Mirex/kg daily for 10 days*

Dose (mg/kg)	Tissue levels (ppm) of Mirex			
	Testis	Brain	Liver	Adipose tissue
0	<0·05	<0·05	<0·05	<0·05
3·0	4·19 ± 0·3	4·1 ± 0·3	42·0 ± 2·8	48·1 ± 3·8
6·0	7·53 ± 0·6	9·1 ± 0·6	76·8 ± 8·1	137·4 ± 15·0
12·5	16·7 ± 3·7	15·3 ± 1·7	110·6 ± 16·0	281 ± 51

Values are means ± SEM for determinations on groups of five rats.

Table 5. *Maternal and foetal levels of Mirex in rats killed on day 22 of pregnancy following dosing with 1·5–12·5 mg Mirex/kg/day on days 6–15 of pregnancy*

Tissue	Mirex levels (ppm)* in rats given doses (mg/kg) of				
	0	1·5	3·0	6·0	12·5
Maternal					
Liver	<0·05	1·67 ± 0·22	2·89 ± 0·54	5·38 ± 0·57	28·1 ± 9·0
Brain	<0·05	1·00 ± 0·04	2·21 ± 0·28	3·15 ± 0·25	8·87 ± 2·0
Fat	<0·05	37·6 ± 6·7	65·1 ± 8·4	102·4 ± 16	169·0 ± 64
Heart	<0·05	1·86 ± 0·24	3·16 ± 0·37	6·9 ± 0·58	13·11 ± 1·7
Kidney	<0·05	1·32 ± 0·24	2·39 ± 0·32	4·00 ± 0·34	10·2 ± 1·8
Spleen	<0·05	1·88 ± 0·38	2·61 ± 0·17	2·90 ± 0·27	7·87 ± 1·1
Foetal					
No. analysed (foetuses/litters)....	10/5	28/5	25/5	18/5	10/4
Whole foetus	<0·05	0·28 ± 0·02	0·48 ± 0·02	1·00 ± 0·05	3·69 ± 0·86
Liver	<0·05	0·89 ± 0·12	1·36 ± 0·39†	3·33 ± 0·35	21·7‡
Brain	<0·05	<0·05	0·75 ± 0·30†	1·18 ± 0·18	31·5‡

*Values are means ± SEM for samples from four gravida and their litters in the 12·5-mg/kg group and from five in the groups given the other doses, except where indicated otherwise.
†Mean value for foetuses from two litters.
‡Mean value for foetuses from only one litter.

necessitated the pooling of tissues from a single litter to provide one value for the Mirex concentration in foetal brain and liver.

DISCUSSION

An accumulation of Mirex was found in all maternal and foetal tissues at term. The presence of Mirex in the foetuses indicated that the insecticide crossed the rat's placenta. Foetal residue levels in the liver and brain were lower than the corresponding maternal levels at all doses except 12·5 mg/kg. The amount in the foetal brain at this dose was 31·5 ppm, but the significance of this figure cannot be established since it was derived from a single analysis.

Foetal effects were associated with maternal toxicity. Reduced foetal weights and/or visceral anomalies were observed at the 6·0- and 12·5-mg/kg dose levels, which also produced varying degrees of maternal toxicity. At 1·5 mg/kg, no adverse effect either on the maternal organism or on foetal development was evident.

Higher tissue levels of Mirex were detected in the male than in the female rat, a difference most probably due to the length of time between termination of treatment and collection of tissues (1 day in males and 7 days in females). Although residues of Mirex were found in the testes, this did not affect reproduction parameters in subsequent mating trials. The observed decrease in the incidence of pregnancy in the 6·0-mg/kg group in the first trial may have been due to a sub-clinical toxic effect, as suggested by the reduction in body-weight gain.

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TISSUE DISTRIBUTION AND EXCRETION OF TRITIATED TETRACHLORODIBENZO-*p*-DIOXIN IN NON-HUMAN PRIMATES AND RATS

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Abstract—Tritiated 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was given to adult and infant rhesus monkeys and to rats to determine the distribution of this compound and to compare the tissue localization of the compound in the two species. Excretion, both daily and total, was similar in all three groups of animals, as was total tritium recovery. However, the concentration and total level of tritium in most tissues differed significantly between species. In the rat, retention in the liver accounted for over 40% of the administered dose, while this level was less than 10% in the livers of the monkeys. In contrast, a large percentage of the dose was located in the monkey in tissues with a high lipid content, particularly the skin, muscle and fat. In the rat, these tissues had much lower levels of TCDD.

INTRODUCTION

The compound 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the most toxic of the chlorinated dibenzo-*p*-dioxins, a group of widely dispersed environmental contaminants. Although a considerable volume of literature is devoted to the toxicity and biological effects of TCDD, virtually nothing is known about its mode of action.

The biological effects of TCDD observed in rats have included loss of weight, icterus, thymic, splenic and lymph-node regression, and haemorrhage and ulceration of the gastro-intestinal tract (Gupta, Vos, Moore, Zinkl & Bullock, 1973). Necrotic centrilobular lesions in the liver have also been reported (Buu-Hoï, Pham-Huu Chanh, Sesqué, Azum-Gelade & Saint-Ruf, 1972; Jones & Butler, 1974). Norback & Allen (1969) have reported an increase in smooth endoplasmic reticulum (SER) and the formation of concentric membrane arrays in the livers of rats treated with toxic fat containing chlorinated dibenzo-*p*-dioxins.

Little is known of the effects of TCDD in man. There are reports (Jones & Krizek, 1962; Kimmig & Schultz, 1957; Schwartz, Tulipan & Birmingham, 1957) that acne has developed in persons exposed to TCDD. Bleiberg, Wallen, Brodkin & Appelbaum (1964) reported porphyria cutanea tarda in workers exposed to 2,4,5-trichlorophenol, and Poland, Smith, Metter & Possick (1971) attributed this porphyria to TCDD present in the herbicide. Among the effects known to be caused by chlorinated dibenzo-*p*-dioxins in non-human primates are acne, alopecia, gastric hyperplasia and ulceration, atrophy of the bone marrow (Allen & Carstens, 1967) and hepatocellular changes, including an increase in SER (Norback & Allen, 1973).

While the distribution of TCDD has been studied extensively in rats (Allen, Van Miller & Norback, 1975; Piper, Rose & Gehring, 1973) and to a lesser extent in mice (Vinopal & Casida, 1973), there is a dearth of information on its distribution in non-human primates. The present work serves to fill this gap and in particular to stress the differences between the distribution of TCDD in non-human primates and in rats.

EXPERIMENTAL

Tritiated TCDD was administered to three groups of experimental animals, the first comprising three mature adult female rhesus monkeys weighing 4·5–5·5 kg, the second four male infant rhesus monkeys between 2 and 4 months of age and weighing 0·95–1·1 kg, and the third five male Sprague–Dawley rats weighing 192–203 g. All three groups received an ip dose of 400 μg TCDD/kg body weight in corn oil (in a volume of 1 ml for group 1 animals and 0·5 ml for groups 2 and 3). The radioactivity administered to each animal was 0·85 mCi in group 1, 0·65 mCi in group 2 and 24 μCi in group 3. Adult monkeys and rats were given normal food (Purina Monkey and Rat Chow, Ralston-Purina Co., Inc., St. Louis, Mo.) and water *ad lib*. Infant monkeys received daily 400–500 ml of commercial infant formula (Similac, Ross Laboratories, Columbus, Ohio). The animals were housed individually in metabolism cages. Daily faecal and urine samples were collected for 7 days, at the end of which the animals were killed.

All tissues were then collected for radioactivity analyses and microscopic evaluation. Radioactivity was analysed by oxidation of the tissue, excreta and blood in a Packard Model 306 sample oxidizer with Monophase 40 (Packard) as the scintillation cocktail. The samples were counted in a Nuclear Chicago Isocap 300 scintillation counter. Samples for light-microscopic evaluation were fixed in 10% neutral buffered formalin for 24 hr and subsequently embedded in paraffin. Sections (5 μm thick) were stained with haematoxylin and eosin for histological examination. Small sections of liver were prepared for electron

microscopy by fixing in osmium tetroxide buffered with veronal acetate for 1·5 hr (Caulfield, 1957) and the tissues were subsequently embedded in an epoxy-resin mixture (Mollenhauer, 1964). Sections of the tissue were cut on an ultratome, placed on uncoated copper grids, stained with uranyl acetate and examined with an RCA-EMU3G electron microscope.

RESULTS

Clinically, all of the experimental animals appeared normal, but they all lost weight over the 7-day test period. Weight losses expressed as a percentage of initial body weight were 10·8 ± 3·8, 20·7 ± 1·7 and 10·6 ± 5·3% for the adult monkeys, infant monkeys and rats, respectively. At autopsy the tissues of the animals appeared normal, except for a moderate thymic atrophy in the rats. Light microscopy indicated hypertrophied hepatocytes in the centrilobular area and smaller distinctly eosinophilic cells in the surrounding periportal areas in the livers of two of the adult monkeys, and a moderate amount of fatty infiltration in all of the rat livers. Electron microscopy indicated proliferation of SER and the development of isolated membranous concentric arrays (Fig. 1) in the livers of all the experimental animals. Most of the Kupffer cells in the livers contained numerous large vacuoles.

Daily levels of tritium excretion over the 7-day test period were similar in all groups, representing less than 1% of the total dose on each day in both the urine and faeces. Total 7-day excretions for the three groups are given in Table 1. The higher excretion levels in the urine and lower excretion levels in the faeces of the infant monkeys were apparently due to the difficulty of separating the two types of excreta from these animals. Total tritium recoveries were similar for the three groups of animals, comprising 58·5 ± 10·5, 71·8 ± 17·0 and 61·6 ± 5·5% of the administered dose for the adult monkeys, infant monkeys and rats, respectively.

A remarkable variation in tissue concentrations of radioactivity between the monkeys and rats was observed, however. Table 2 shows the concentration of tritium in the tissues for the three groups of animals at 7 days. The data for both groups of monkeys were compared with the data for the rats to determine differences of statistical significance. The standard Student's t test was used as indicated. Tissues not listed in the table, particularly the heart, reproductive organs and blood, had low concentrations of tritium not differing significantly between groups.

When the total quantity of tritium in the tissues was evaluated, even more striking differences between the monkeys and rats were observed. Table 3 shows these data as the percentage of the total dose/tissue. Tissues with low tritium levels (< 1·0% for all groups) and showing no significant difference ($P < 0·02$) between groups are not listed.

Table 1. *Total tritium excretion in the 7 days following administration of a single ip dose of [^3H]tetrachlorodibenzo-p-dioxin to three groups of experimental animals*

Route of excretion	^3H excretion (% of dose)* in Adult monkeys	Infant monkeys	Rats
Urine	1·06 ± 0·25	1·96 ± 0·42†	0·51 ± 0·05
Faeces	3·75 ± 0·91	1·26 ± 0·34†	4·96 ± 0·3

*Values are means ± SD for groups of three adult monkeys, four infant monkeys and five rats.

†The difficulty of urinofaecal separation in the case of infant monkeys probably accounts for the higher activity found in the urine.

DISCUSSION

There are distinct differences in the responses of rats and non-human primates to TCDD exposure. In many instances these differences can be equated with the types of lesion developing in the two species. Although both absorption from the gastro-intestinal tract and from ip injections and the rate and routes of excretion of TCDD seem to be similar in monkeys

Table 2. *Tissue concentrations of tritium in three groups of experimental animals 7 days after administration of a single ip dose of [^3H]tetrachlorodibenzo-p-dioxin*

Tissue	^3H level (% of dose/g tissue)† in Adult monkeys	Infant monkeys	Rats
Liver	0·09 ± 0·06***	0·13 ± 0·07***	4·54 ± 0·45
Brain	0·006 ± 0·004**	0·018 ± 0·019**	0·13 ± 0·04
Kidney	0·017 ± —**	0·051 ± 0·037***	0·33 ± 0·07
Lung	0·009 ± 0·003*	0·030 ± 0·014*	0·21 ± 0·09
Spleen	0·008 ± 0·004*	0·031 ± 0·021**	0·83 ± 0·36
Stomach	0·010 ± 0·007*	0·059 ± 0·043*	0·30 ± 0·13
Small intestine	0·015 ± 0·006*	0·059 ± 0·025	0·17 ± 0·08
Large intestine	0·015 ± 0·003*	0·051 ± 0·026	0·18 ± 0·07
Thymus	—	0·15 ± 0·06	0·44 ± 0·15
Adrenals	0·096 ± 0·058	0·19 ± 0·05	—
Muscle	0·004 ± 0·001	0·096 ± 0·05	0·058 ± 0·03
Skin	0·028 ± 0·014**	0·24 ± 0·07**	0·13 ± 0·02
Adipose tissue	0·16 ± 0·06***	0·49 ± 0·12***	3·46 ± 0·21

†Values are means ± SD for groups of three adult monkeys, four infant monkeys and five rats. Those marked with asterisks differ significantly (Student's t test) from the corresponding value for the rats: *$P < 0·02$; **$P < 0·01$; ***$P < 0·001$.

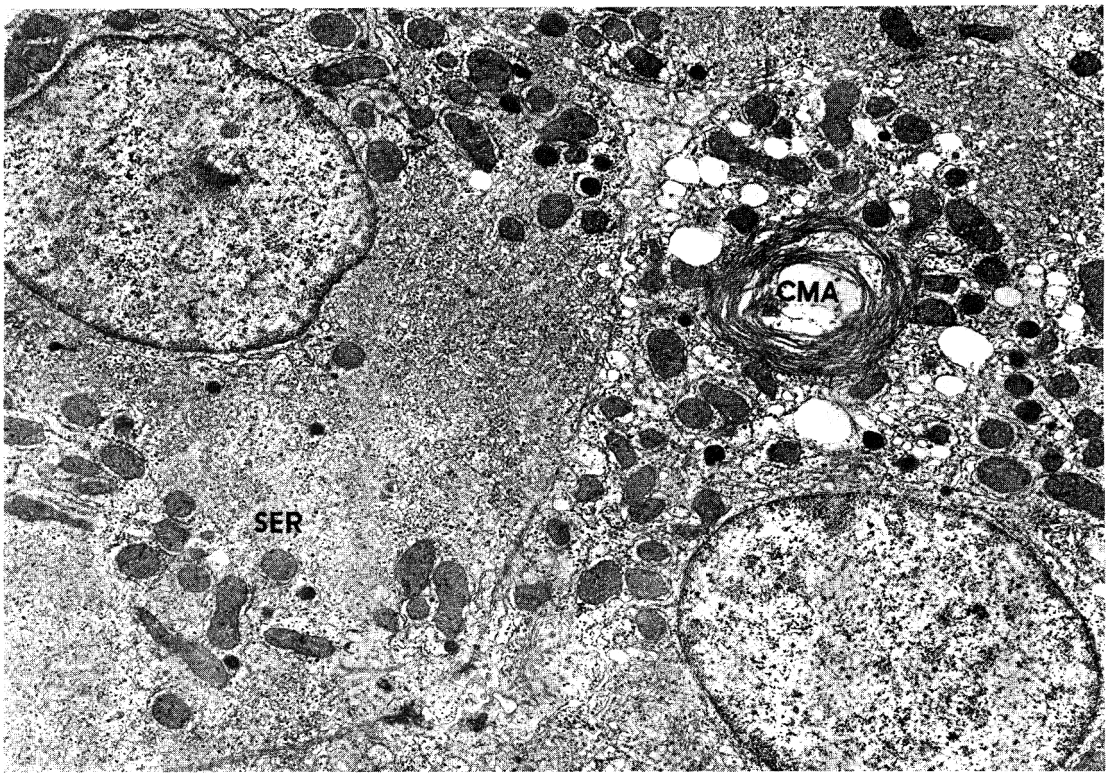

Fig. 1. Proliferated smooth endoplasmic reticulum (SER) and membranous concentric arrays (CMA) present in the cytoplasm of hepatic cells taken from infant monkeys given 400 μg TCDD/kg body weight. $\times$ 7800.

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Table 3. *Tritium retention in tissues of three groups of experimental animals 7 days after administration of a single ip dose of [^3H]tetrachlorodibenzo-p-dioxin*

Tissue	^3H content (% of dose/total weight of tissue)† in		
	Adult monkeys	Infant monkeys	Rats
Liver	10·4 ± 6·9***	4·51 ± 1·60***	43·0 ± 4·7
Brain	0·58 ± 0·34	1·41 ± 1·40	0·21 ± 0·07
Spleen	0·028 ± 0·013*	0·026 ± 0·004**	0·48 ± 0·17
Small intestine	0·87 ± 0·39	1·47 ± 0·64	0·93 ± 0·44
Large intestine	1·29 ± 0·12**	0·64 ± 0·24	0·45 ± 0·21
Muscle‡	8·62 ± 2·39	35·6 ± 14·4**	4·63 ± 2·52
Skin	13·1 ± 4·9*	22·7 ± 8·8**	4·39 ± 0·52
Adipose tissue§	16·2 ± 5·8	—	—

†Values are means ± SD for groups of three adult monkeys, four infant monkeys and five rats. Those marked with asterisks differ significantly (Student's *t* test) from the corresponding value for rats: *$P < 0·02$; **$P < 0·01$; ***$P < 0·001$.

‡Total muscle was taken as 40% of body weight.

§Quantities in infant monkeys and rats were insignificant. For adult monkeys, the calculation was based on an estimate of 300 g mesenteric fat.

and rats, there is a marked difference in the tissue distribution of this toxic compound in the two species. While over 40% of the TCDD becomes localized in the rat liver, the monkey liver contains 10% or less of the total dose. A much greater percentage of the TCDD is present in the skin and muscle of the monkey than of the rat.

One possible explanation for this variation in the tissue localization of TCDD is related to the proliferation of the endoplasmic reticulum (ER) that occurs in the rat liver soon after the administration of TCDD. The proliferation of the SER occurs much more rapidly and to a greater extent in the livers of rats than of monkeys. In fact, it is not uncommon for the rat to show a two- or threefold increase in size within a short period after the administration of chlorinated aromatic hydrocarbons (Allen & Abrahamson, 1972) primarily because of proliferation of the SER. Although a slight liver hypertrophy is associated with SER proliferation in monkeys exposed to chlorinated hydrocarbons (Allen, Abrahamson & Norback, 1973), it never attains the same magnitude as that seen in the rat. The ER of the liver has an affinity for TCDD (Allen *et al.* 1975; Norback, Engblom & Allen, 1975). The extensive proliferation of the SER in the rat liver makes it possible for a large percentage of the TCDD body burden to be localized in these membranes. Even though the hepatic membranes of the monkey show an affinity for TCDD as great as that of the rat (J. P. Van Miller, unpublished observations 1974), the slower rate of proliferation and the resulting smaller quantity of SER in the monkey liver results in the sequestration of a lower percentage of TCDD in this organ. As a result the TCDD moves to other tissues, particularly those of high lipid content, such as the adipose tissue, skin and muscle. Such a concept would be in agreement with that proposed earlier by Norback & Allen (1972) that proliferated SER serves as an area of sequestration for the chlorinated aromatic hydrocarbons.

Acne and alopecia are common signs of TCDD intoxication in the monkey. However, this is not the case in rats. Undoubtedly the large amount of TCDD in the monkey skin compared with the considerably lower levels in the rat skin is responsible for this species variation. Focal necrosis of the liver and splenic atrophy are observed in TCDD-intoxicated rats, but similar changes in the monkey tissues are either not apparent or of a minor nature. These differences may also be related to the greater tissue deposition in the liver and spleen of the rat.

The relatively slow elimination of TCDD from the body of the rat and monkey suggests that these compounds are, at best, not readily metabolized. In addition, it has been shown that only lipid solvents are capable of extracting the radioactivity from the liver (Rose, Ramsey, Wentzler, Hummel & Gehring, 1975) and excreta (Vinopal & Casida, 1973) of rats and mice treated with labelled TCDD. Such data further emphasize the absence of any appreciable metabolism of TCDD. In contrast there are observations suggesting that TCDD may be altered metabolically. It has recently been reported that TCDD had no effect on cell cultures (Beatty, Lembach, Holscher & Neal, 1975), suggesting that the toxic effects of TCDD may be produced by metabolites rather than by unaltered TCDD. Allen *et al.* (1975) found an increased level of radioactivity in the urine of rats 2–3 wk after administration of a single dose of [^{14}C]TCDD, thus posing the possibility of a TCDD metabolite. However, until the chemical nature of TCDD in the tissues and excreta of experimental animals is determined, the presence or absence of TCDD metabolism remains speculative.

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THE ABSENCE OF TETRAHYDROCANNABINOL FROM HOPS

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Abstract—A combined gas chromatograph–mass spectrometer was used in the selected-ion monitoring mode to assay for the presence of tetrahydrocannabinol in seventeen different samples of *Humulus lupulus*, the hop commonly used in brewing. This psychotropic compound could not be detected in any of the hop plants.

INTRODUCTION

Hop plants and marijuana plants are classified as members of the same botanical family on the basis of similar morphological characteristics. Humulus (hops) and Cannabis (marijuana) are both genera of the family Cannabinaceae (Nakamura, 1969; Stevens, 1967). Although tradition and, to a large extent, human experience (Nahas, 1973; Snyder, 1971) suggest that *Cannabis sativa* is the only plant that brings about the psychomimetic effect now considered characteristic of Δ^9-tetrahydrocannabinol, the close botanical relationship between the two plants is continually cited in the lay literature either to promote various uses of hops in the counter-culture (Superweed, 1970; *The New York Times*, 1971) or to discourage the drinking of beer (Bailey, 1964: *ILLCAAP News*, 1969; Prevent Alcohol Problems Association).

The chemical compositions of both plants have been studied extensively (Ashurt, 1967; Mechoulam, 1973) and have been found to contain a number of compounds in common (Hegnauer, 1964; Stevens, 1967). Most of these compounds, such as farnesene, caryophyllene and humulene are widely distributed throughout the plant world. The compounds of greatest interest are, of course, the members of the cannabinoid family, which are responsible for the psychomimetic effects of *Cannabis sativa*.

There are at least two reports in the scientific literature of attempts to detect cannabinoids in *Humulus lupulus*, the hop commonly used in brewing (Nakamura, 1969; Steiner, 1971). In both cases, the assay used was a chemical reaction giving rise to highly coloured products, in solution in one case and on thin-layer chromatoplates in the other. In the studies reported, extracts of the leaves of *Humulus lupulus*, of the pollen (lupulin) and of a commercial extract ('hopfenol') gave negative colour tests. Extracts of flower buds from hop plants and also extracts of a number of other kinds of plant produced purple products with the Duquenois reagent, but the purple material did not exhibit the solubility in chloroform required for a positive Duquenois–Levine test (Butler, 1962). Thus these two investigations provided no evidence for the presence of tetrahydrocannabinol or closely related compounds in hop plants.

Although neither of these authors reported a minimum sensitivity for their assay, other workers suggest that a level of at least 10 μg cannabinols/ml extract is required to produce a positive Duquenois test (Capella & Puig, 1971) and at least 100 ng tetrahydrocannabinol must be present on the thin-layer chromatoplate to give a positive test with Fast Blue Salt B (Willinsky, 1973). Thus one might argue that tetrahydrocannabinol might have been present in concentrations too low to be detected in the hop extracts studied.

The other problem with the classical colour tests is that they give positive reactions with a variety of phenolic compounds besides the cannabinoids (Fochtman & Winek, 1971; Willinsky, 1973). This means that even a positive Duquenois–Levine test is not a firm identification of tetrahydrocannabinol. For this reason, law enforcement agencies are now turning to identification of tetrahydrocannabinol by instrumental methods, especially gas chromatography and mass spectrometry (Fochtman & Winek, 1971; Mule, 1974; Vree, Breimer, Van Ginneken & Van Rossum, 1972).

The combined gas chromatography–mass spectrometer (GCMS) offers both high specificity and high sensitivity for the detection and also for the quantitative estimation of compounds in mixtures (Fenselau, 1974). Reports in the literature suggest that the GCMS used in the selected-ion monitoring mode can detect sub-nanogram amounts of pure tetrahydrocannabinol (Mikes, Hofmann & Waser, 1971; Skinner, 1972). In selected-ion monitoring, the mass spectrometer is set to detect continuously a few highly characteristic ions throughout the chromatographic separation, rather than to scan intermittently a broad mass range in the conventional manner. Selected-ion monitoring provides an identification that is more specific by several orders of magnitude than simple gas chromatography, because the sample must not only exhibit the characteristic chromatographic retention time but also fragment in the mass spectrometer to form the characteristic ions in their expected relative abundances. The identification is less specific than that from a conventionally scanned spectrum (Billets, Carruth, Einolf, Ward & Fenselau, 1973)

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because fewer fragment ions are recorded. However, the continuous monitoring technique offers a greater sensitivity than scanned spectra because the signals are recorded over a longer period of time.

For a previously reported investigation of the possible presence of oestrogenic compounds in *Humulus lupulus* (Fenselau & Talalay, 1973), we assembled a number of samples of hops and commercial hop extracts of known origin. In this paper we report our use of the GCMS in the selected-ion monitoring mode to assay these various hop specimens for tetrahydrocannabinol.

EXPERIMENTAL

Plant materials. Samples of various dried hops from the 1970 harvest were assembled from commercial sources. Dried Cascade hops were obtained from the 1973 harvest. These consisted of leaves, flowers and lupulin (pollen), in which the water content had been reduced to 10% of the total weight. These samples were stored in the dark at 4°C both by the commercial processors and in this laboratory. Under these conditions, tetrahydrocannabinol has been found to decompose in plant material at the rate of 5%/yr (Turner, Hadley, Fetterman, Doorenbos, Quimby & Waller, 1973). Several commercial extracts of hops (complying with Secs 121.1203 and 121.1082 of the US Code of Federal Regulations) were also obtained and stored in the same manner. A sample of confiscated dried marijuana (flowers, leaves and small twigs) was obtained from the US Customs House in Baltimore, Md.

Chemical standards. Δ^9-Tetrahydrocannabinol distributed under the auspices of the National Institute for Mental Health was obtained from the US Customs House in Baltimore. Δ^8-Tetrahydrocannabinol distributed by the National Institute for Mental Health was provided by Dr. S. H. Snyder.

Extraction. Weighed amounts (1–5 g) of plant material were crushed in 100 ml chloroform and allowed to stand at 4°C for 2 hr in the dark, in a modification of the extraction procedure found by Turner, Hadley & Davis (1973) to be optimal for extracting cannabinoids from marijuana. The solutions were filtered and concentrated on a rotary evaporator at 37°C.

Thick-layer chromatography. In most of the experiments, the whole of the concentrated chloroform solution was applied to a thick-layer plate (Silica Gel GF, 2000 μm, Applied Science, State College, Pa.). A standard sample of Δ^9-tetrahydrocannabinol was spotted beside the plant extract. In some cases, plant extract spiked with authentic Δ^9-tetrahydrocannabinol was used as the standard. The chromatogram was developed for 2 hr with benzene and allowed to dry at room temperature. The standard sample was sprayed with caesium sulphate in sulphuric acid (used to detect compounds containing carbon and hydrogen) in order to determine its position on the plate. Silica gel in the same position was removed from the portion of the plate on which the hops extract was chromatographed. This silica was extracted three times with ethanol, the ethanol solutions being combined, filtered and concentrated on the rotary evaporator. Each concentrated ethanol solution was transferred to a 5-ml conical sample vial, in which it was evaporated to dryness under a stream of nitrogen. The residue was redissolved in 4 μl ethanol and stored in the dark at 4°C. In some cases the 4 μl solution was too concentrated to provide good chromatographic resolution or a good base line on the selected ion monitor, and these samples were diluted by the appropriate factor.

Selected-ion monitoring. A DuPont 491 GCMS was used, fitted with a single-stage glass jet interface, a Varian 2700 gas chromatograph and a selected-ion monitoring module. Gas chromatography was carried out on 3% OV-101 on 100/120 mesh Chromosorb WHP (Supelco Inc., or on 3% Dexsil 300 on 80/100 mesh Suplecoport (Supelco Inc., Bellefonte, Pa.) packed in glass columns, 6 ft by 0·25 in. Helium was the carrier gas. The oven temperature was raised from 210 to 280°C at the rate of 6°/min. Under these conditions the two naturally occurring isomers of tetrahydrocannabinol (Δ^8 and Δ^9) could not be separated. The transfer line was maintained above 280°C and the mass spectrometer source was kept at 240°C. Four peaks were monitored, at m/e 314 (M^+ for tetrahydrocannabinol), 299 (M-15), 271 and 231. These are major peaks in the mass spectra of both Δ^8- and Δ^9-tetrahydrocannabinol (Cassen, Fehlhaber & Korte, 1966).

Control experiments. Dried marijuana (1 g) was extracted, chromatoplated and analysed by GCMS using the protocols described above. Tetrahydrocannabinol was detected in abundance, on the basis of profiles of ions of mass 314, 299, 271 and 231 and chromatographic retention time. In another control experiment, 10 ng Δ^9-tetrahydrocannabinol was added to the chloroform extract of 1 g hops and subsequently chromatoplated and analysed as described above. About 35% of the tetrahydrocannabinol was recovered, as demonstrated by comparing the area of the chromatographic peak traced by the selected-ion monitor with areas of peaks resulting from injection of 1, 3 and 10 ng samples into the GCMS.

RESULTS AND DISCUSSION

The 17 different samples analysed are listed in Tables 1 and 2. The weight and complexity of the material extracted into chloroform varied among the many kinds of hop plants. It has been pointed out previously (Rasmussen, Rasmussen & Svendsen, 1972) that organic extracts of plants contain mixtures of compounds, many of which are non-volatile and will contaminate a gas-chromatograph column. For this reason, and also to minimize the number of compounds coming off the column at retention times similar to that of tetrahydrocannabinol, the chloroform extracts were subjected to preliminary purification by thin-layer chromatography. One such chromatoplate is reproduced in Fig. 1. The portion of the thin layer extracted for GCMS is indicated.

Figure 2 shows the selected-ion profiles obtained from a plant extract which had been purified by thin-layer chromatography. Figure 3 contains the selected-ion profiles from that plant extract to which approximately 10 ng of authentic Δ^9-tetrahydrocannabinol had been added. The plant extract contained a number of compounds that fragmented to form one

Table 1. *Assays for tetrahydrocannabinol*

Hops (*H. lupulus*)	Weight extracted (g)	Percentage of final volume injected into GCMS	Level of negative assay (ppb; ng/g)
Oregon Bullion	5	20	3
Oregon Fuggle	5	20	3
Hallertau Mittelfrüh (Germany)	5	20	3
Idaho	3	20	5
Idaho Talisman	3	20	5
Cascade	5	10	6
Hops II Extract	1	20	15
Hopstract-Iso	1	20	15
Oregon Brewer's Gold	5	4	15
Styrians (Yugoslavia)	5	2	30
Hallertau Replants (Belgium)	5	0·2	300
Northern Brewer (Germany)	5	0·2	300
Spalt (Germany)	5	0·2	300
Yakima	5	0·2	300
Oregon Cluster	5	0·2	300
Cosumnes Valley (California)	5	0·1	600

or more of the four ions being monitored. Only two of the components detected formed all four of the ions characteristic of tetrahydrocannabinol, and these compounds had retention times distinct from that of Δ^9-tetrahydrocannabinol. No ions were detected at the retention time of tetrahydrocannabinol (expressed as programmed temperature). This kind of analysis was made for all the compounds listed in Tables 1 and 2.

Our minimum detection limit was determined as 1 ng tetrahydrocannabinol. This minimum was reconfirmed several times throughout a 15-month period by four experimentalists, using OV-17, OV-101 and Dexsil columns, with a new electron multiplier and a freshly cleaned interface line, using chemical ionization as well as electron impact ionization. This was a little disappointing in view of the ease with which we can detect 1 pg methyl stearate on this same instrument. It is probable that the minimum sensitivity to tetrahydrocannabinol is limited by adsorption on the column and in the interface, and that 1 ng is the minimum amount of material required for transmission through the gas chromatograph. Although

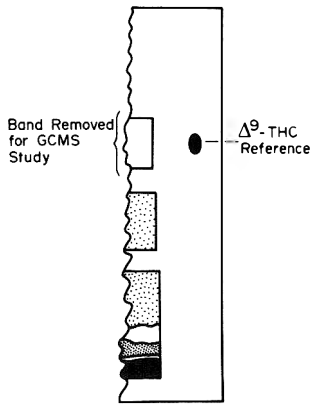

Fig. 1. Thin-layer chromatogram of extract of Cascade hops and Δ^9-tetrahydrocannabinol.

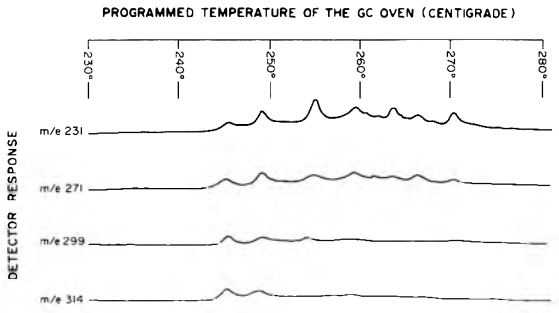

Fig. 2. Selected-ion profiles of material extracted from Cascade hops (partially purified by thin-layer chromatography).

reports have been made of lower sensitivity using the selected-ion monitor (Mikes *et al.* 1971; Skinner, 1972), our experience with chromatographic adsorption of underivatized tetrahydrocannabinol is consistent with that of other laboratories (Agurell, Gustafsson, Holmstedt, Leander, Lindgren, Nilsson, Sandberg & Asberg, 1973; Garrett & Hunt, 1973).

Assuming a 1 ng minimum sensitivity and a 35% recovery from our extraction and separation pro-

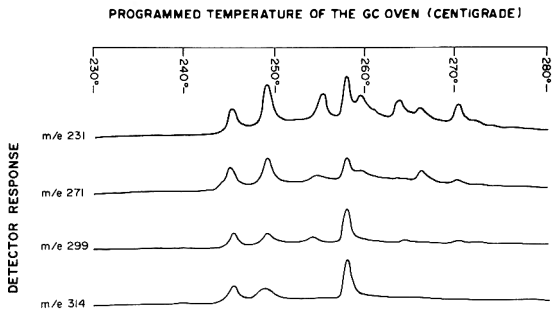

Fig. 3. Selected-ion profiles of material extracted from Cascade hops (partially purified by thin-layer chromatography) to which authentic Δ^9-tetrahydrocannabinol had been added.

Table 2. *Assays for tetrahydrocannabinolic acid, involving GCMS examination without prior thin-layer chromatography*

Hops (*H. lupulus*)	Weight extracted (g)	Percentage of final volume injected into GCMS	Level of negative assay (ppb; ng/g)
Yakima	5	0·1	600
Zatec (Czechoslovakia)	5	0·1	600

cedures (see Experimental section) and allowing for the dilutions necessary to facilitate analysis of the more concentrated samples, the levels down to which our negative results are valid are indicated in Table 1. The wide range of detection levels in Table 1 reflects the range in the amounts of material extracted into chloroform from various plant samples. More concentrated extracts had to be diluted so that less material was injected into the GCMS in order to obtain a good base line in the region of the chromatogram that was of interest. This made the assay less sensitive when calculated on the basis of 1 g plant material.

It has been demonstrated recently that about 95% of the tetrahydrocannabinol in fresh marijuana plants actually exists as the carboxylated biosynthetic precursor tetrahydrocannabinolic acid (Doorenbos, Fetterman, Quimby & Turner, 1971). This acid is very unstable, decarboxylating to tetrahydrocannabinol when the plant material is dried, stored or extracted with solvents. It also decarboxylates spontaneously to tetrahydrocannabinol at the rate of 50%/yr in stored dried marijuana, and rapid and complete decarboxylation is achieved at high temperatures such as those encountered in smoking (Doorenbos *et al.* 1971) or in gas chromatography (Fetterman, Doorenbos, Keith & Quimby, 1971).

It is possible that tetrahydrocannabinol may also exist in the form of this carboxylated analogue in fresh hops. The samples of hops examined here had been dehydrated after harvest by a commercial process involving prolonged exposure to elevated temperatures (Stevens, 1967) and had been stored for more than 3 yr. The commercial extracts had also been prepared at elevated temperatures (according to Secs 121.1203 and 121.1082 of the US Code of Federal Regulations). Thus it seemed likely that any tetrahydrocannabinolic acid present originally would have decomposed to tetrahydrocannabinol and would therefore be detectable by our protocol. Any tetrahydrocannabinolic acid remaining in the plant material would be extracted in chloroform along with tetrahydrocannabinol and the other cannabinoids (Doorenbos *et al.* 1971). However, on the thin-layer chromatoplates used in our procedure, the acid would have been separated from the band of material that we subjected to further analysis (Willinsky, 1973).

In order to eliminate completely the possibility of tetrahydrocannabinolic acid being present in the hops, extracts of two of the plant specimens were examined without the initial separation by thin-layer chromatography. Solutions of the extracts were injected directly into the gas chromatograph, where any tetrahydrocannabinolic acid present would have been decarboxylated immediately to tetrahydrocannabinol. As indicated in Table 2, no tetrahydrocannabinol could be detected in these samples.

Thus, although Doorenbos *et al.* (1971) have extracted tetrahydrocannabinol from dried *Cannabis sativa* (marijuana) at levels between 0·04 and 5% (400 μg/g and 50 mg/g) this compound has now been shown to be absent from *Humulus lupulus* (the hop used in brewing) with levels of detection down to <10 ppb (10 ng/g).

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SHORT PAPERS

METABOLISM AND ELIMINATION OF α-HYDROXYETHANE SULPHONATE BY RATS

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Summary—Potassium α-hydroxyethane [^{35}S]sulphonate was administered in the drinking-water to male albino rats (weighing 100 g) as a single dose equivalent to 50 mg SO_2/kg. After 48 hr, 72–85% of the ^{35}S had been excreted in the urine and 10–15% in the faeces. After 2 wk, less than 1·5% remained in the carcass. Of the urinary ^{35}S, 90–95% was in the form of inorganic sulphate and the rest was not precipitable by barium. No evidence was obtained that unchanged α-hydroxyethane sulphonate was excreted. No signs of toxicity were observed.

Introduction

Acetaldehyde and bisulphite ion combine to form α-hydroxyethane sulphonate, one of the major "bound forms" of dietary sulphites, particularly in wine (Amerine & Joslyn, 1970). The toxicity of potassium α-hydroxyethane sulphonate has been investigated by Lanteaume, Ramel, Jaulmes & Manin (1969), who found the oral LD_{50} value for Wistar rats to be 2·7 g/kg body weight, and by Ramel, Lanteaume, Jaulmes & Bodin (1971), who observed no long-term toxic effects in rats given the compound daily in their drinking-water at doses equivalent to 100–150 mg SO_2/kg. In a continuation of our previous work in this area (Gibson & Strong, 1973 & 1974), we report here a study of the ability of rats to metabolize and eliminate potassium α-hydroxyethane [^{35}S]sulphonate.

Experimental

Preparation of potassium α-hydroxyethane [^{35}S]sulphonate. The procedure of Diemair, Koch & Hess (1960) was followed. Attempts to prepare sodium α-hydroxyethane sulphonate from sodium bisulphite and metabisulphite by this method gave a product containing significant quantities of unbound sulphite, which was not removed by recrystallization from aqueous ethanol. For the preparation of the potassium salt, potassium metabisulphite (1·12 g, 5 mmol), dissolved in 2·6 ml water at room temperature, was chilled in an ice-salt mix and 0·73 mg sodium [^{35}S]sulphite (purchased from New England Nuclear, Boston, Mass.), containing 8 mCi of radioactivity, was added. Freshly distilled acetaldehyde (0·6 ml, 11 mmol) was added dropwise with stirring and the mixture was permitted to stand overnight. In the morning, 1·5 ml 95% ethanol was added dropwise with mixing. The solution was permitted to stand at room temperature for 30 min, then chilled in ice for 5 min and centrifuged for 5 min at full speed in an International Clinical Centrifuge to remove a precipitate, which was discarded. To the clear, colourless supernatant was added 17 ml 95% ethanol dropwise with continuous stirring. The mixture was allowed to stand at room temparature for several days and then overnight in a cold room at 4°C. The next day, the colourless plates were collected by suction filtration through Whatman no. 1 filter paper in an ice-cold funnel. The crystals were redissolved in 1·8 ml water, 95% ethanol was added dropwise to a volume of 9·9 ml, the solution was kept at room temperature overnight and then cooled to 4°C for several hours, and the product was filtered off and rinsed with 95% ethanol. After drying at *c.* 50°C and 0·02 mm Hg over magnesium perchlorate, 0·96 g (5·85 mmol, 58% of theoretical yield) of the colourless, crystalline material was obtained, giving an analysis (Micro-Tech Laboratories, Inc., Skokie, Ill.) of C, 14·52, H, 3·09; S, 19·63 (calculated for $C_2H_5O_4SK$ (164·2): C, 14·65; H, 3·05; S, 19·53).

Animals and dosage. Male albino rats (90–100 g body weight) were obtained from the Holtzman Co., Madison, Wisc. The animals were housed in stainless-steel metabolism cages, described by Gibson & Strong (1973), and fed *ad lib.* with Wayne Lab-Blox, purchased from Allied Mills, Inc., Chicago, Ill. Twelve rats were each given 12·8 mg potassium α-hydroxyethane [^{35}S]sulphonate in 5·0 ml water (equivalent to 50 mg SO_2/kg body weight). The solution was administered in all-glass feeding tubes of approximately 15-ml capacity. Restricting the lower opening of the tubes by fire polishing and adjusting the internal pressure with a syringe inserted through a rubber stopper in the top prevented appreciable loss of solution from the tubes until the lower orifice was licked by the animals. This procedure permitted good recovery of doses administered during this and previous studies (Gibson & Strong, 1973). The animals were dosed at 16·00 hr, and after the dose had been consumed, usually by 22.00 hr, water was provided *ad*

lib. The entire experiment was repeated once using a similar dose.

Sample collection and preparation for radioactivity determination. Groups of three rats were killed 24 and 48 hr after dose administration, urine and faeces having been collected in the intervening periods. The two remaining groups of three rats were killed 1 and 2 wk after dosing. Animals were killed and samples were collected and prepared for scintillation counting as described by Gibson & Strong (1973). Radioactivity was determined in a Packard Model 3003 liquid scintillation spectrophotometer. The counting efficiency was monitored by addition of an internal standard.

Barium-chloride precipitation of rat urine. Urine collected during the first 24 hr after dose administration and a sample of the administered dose were frozen until treatment with barium chloride could be carried out (7–20 days). Aliquots of the test urines, of a sample of control rat urine containing added $Na_2{}^{35}SO_4$, of the original dosing solution and of a freshly prepared solution of potassium α-hydroxyethane sulphonate were acidified in graduated centrifuge tubes to pH 1 with 1 N-HCl. Unlabelled Na_2SO_4 (4 μmol) was added to each tube, the volume was made to 5 ml with water, and a 0·5 ml aliquot was removed for determination of the content of isotope. The remainder was treated with barium chloride either at room temperature or after being heated to boiling in a water-bath. The barium chloride solution (2·0 ml), containing 80 μmol barium, was added dropwise with mixing. With heated samples, the mixture was stirred for 10 min in the boiling water-bath and permitted to cool overnight. In the morning, the mixtures were centrifuged for 10 min at full speed in an International Clinical Centrifuge and the supernatants were sampled for determination of ^{35}S content.

Results and Discussion

The disposition by rats of ^{35}S from a single dose of aqueous potassium α-hydroxyethane [^{35}S]sulphonate is shown in Table 1. Mean values are given for the percentage of the recovered dose found in the urine, faeces and carcass, with the range displayed by individual animals indicated in parentheses. The major part of the ingested radioactivity (72–86%) was excreted in the urine during the first 48 hr. Carcass levels of ^{35}S decreased with increasing time, primarily as a result of urinary excretion. The overall recovery of activity was somewhat low for all the animals in Experiment A (Table 1), apparently because of an erroneously high value for the radioactivity of the original dose. For this reason, the results for urine, faeces and carcass in Experiment A, but not in Experiment B, are expressed as a percentage of the recovered radioactivity, rather than as a percentage of the administered dose. The "overall recovery" (Table 1), however, is expressed as a percentage of the administered dose in both experiments.

The retention and elimination of ^{35}S contained in α-hydroxyethane [^{35}S]sulphonate in these experiments is almost identical to that found with [^{35}S]sulphite (Gibson & Strong, 1973). There is no appreciable accumulation of ^{35}S in the carcass from either free sulphite or α-hydroxyethane sulphonate; the small amount of ^{35}S that does accumulate during the first 24 hr after dosing declines rapidly within 2 days and more slowly thereafter. This residual radioactivity may represent incorporation of some ^{35}S into mammalian sulphur-containing compounds, such as sulphur amino acids or mucopolysaccharides, or into micro-organisms of the intestinal tract.

The percentage of radioactivity not precipitated by hot and cold barium treatment of urine and other samples is shown in Table 2. Boiling prior to adding barium chloride apparently either oxidizes α-hydroxyethane sulphonate to yield sulphate or releases sulphite originally bound to acetaldehyde as free SO_2, which is then volatilized. In either case, ^{35}S is removed from the supernatant solution during this procedure, as shown by the proportion of radioactivity not precipitated from heated, compared with that from unheated, samples containing the sulphonate.

The discrepancy between the results with unheated dosing solution and unheated freshly prepared aqueous α-hydroxyethane [^{35}S]sulphonate was probably due to the fact that these analyses were carried

Table 1. *Disposition of α-hydroxyethane [^{35}S]sulphonate given to rats in a single dose in the drinking-water*

Group no.	No. of rats/group	Time after dosing* (days)	Total radioactivity (%)†‡ in Urine	Faeces	Carcass	Overall recovery (% of administered dose)‡
			Experiment A			
1	3	1	76·70 (72·0–79·9)	13·51 (11·9–15·7)	9·79 (7·2–12·2)	92·6 (90–95)
2	3	2	83·72 (80·9–85·8)	12·37 (10·0–15·4)	3·90 (3·7–4·2)	95·6 (95–98)
3	3	7	—	—	2·39 (1·5–3·9)	—
4	2	14	—	—	1·24 (1·2–1·3)	—
			Experiment B			
1	3	1	80·34 (79·3–81·2)	11·67 (7·9–14·2)	7·99 (6·5–10·9)	96·5 (95–99)
2	3	2	83·83 (81·8–85·3)	11·62 (9·8–14·2)	4·56 (4·0–4·9)	98·0 (96–100)
3	3	7	—	—	1·95 (1·4–2·7)	—
4	3	14	—	—	1·24 (1·1–1·4)	—

*The weight and total activity of the dose administered to each animal were 12·8 mg, $6·5 \times 10^7$ cpm in experiment A and 13·1 mg, $6·2 \times 10^7$ cpm in experiment B.

†Expressed as a percentage of recovered radioactivity for experiment A and as a percentage of the administered dose for experiment B.

‡Values given are means, with the ranges for individual animals in parentheses.

Table 2. *Barium precipitation of components of urine from rats given a single dose of potassium hydroxyethane [^{35}S]sulphonate in the drinking-water*

Sample	Rat or sample no.	Total radioactivity in treated sample (cpm × 10^{-5})		Sample radioactivity not precipitated by barium (% of total)	
		Heated	Unheated	Heated	Unheated
Urine (24 hr collection)*	1	3·22	2·64	2·80	6·52
	2	7·68	5·59	3·01	6·63
	3	8·31	5·77	2·74	6·88
	4	7·83	5·95	2·94	5·49
	5	5·74	3·84	3·36	9·70
Dosing solution	1	2·62	2·41	1·00	80·05
	2	2·48	2·27	1·77	80·39
Freshly prepared aqueous sulphonate	1	3·88	3·81	0·73	88·89
	2	3·83	3·92	1·37	88·33
Control rat urine with added $Na_2{}^{35}SO_4$	1	1·54	1·52	0·48	0·10
	2	1·56	1·56	0·27	0·14

*Different aliquot sizes were used for heated and unheated urine samples from rats 1–5.

out 20 days after preparation of the dosing solution, during which time some oxidation from sulphite to sulphate may have occurred. The 11–12% loss of radioactivity from the supernatant of the unheated freshly prepared sulphonate sample was probably a consequence of the procedure followed for barium-chloride precipitation, which included overnight standing before centrifugation to remove barium sulphate. Any oxidation of sulphite during this time would have resulted in the loss of ^{35}S from the supernatant.

In the case of the test urines, 90–95% of the ^{35}S present appeared to be in the form of sulphate. The major excretion product was certainly not α-hydroxyethane sulphonate, since this would have been expected to result in a much greater difference between supernatant ^{35}S content after hot and cold barium-chloride precipitations. The small (3–6%) difference that was observed may be attributable to the presence in the urine of either a small portion of unchanged α-hydroxyethane sulphonate or a metabolite thereof. The essentially complete precipitation of $Na_2{}^{35}SO_4$ from the control urine eliminates the possibility that the urine may contain sulphate-binding substances that interfere with the precipitation of sulphate and are altered by the heating procedure.

The results presented indicate that the ^{35}S contained in α-hydroxyethane [^{35}S]sulphonate was eliminated after ingestion at virtually the same rate as free sulphite, predominantly in the form of inorganic sulphate. About 5–10%, however, appeared in the urine in a form not precipitable by barium chloride in acid solution. Less than 1·5% of the sulphonate sulphur remained in the carcass of the animals 2 wk after administration of the dose. The test animals exhibited no obvious signs of toxicity during the test period. Hötzel, Muskat & Cremer (1966) found no differences in the toxicity of similar doses of SO_2 fed to rats either in the form of sodium bisulphite or in the "bound form" with glucose or acetaldehyde.

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TOXICITY STUDIES WITH δ-AMINOLAEVULINIC ACID

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Summary—The subacute toxicity of δ-aminolaevulinic acid was determined in mice given ip injections of 0, 10, 50 or 100 mg/kg 3 times/wk for 13 consecutive weeks. A second set of mice was tested for reproductive ability following 13 wk of similar treatment. No toxic effect was noted. The parameters studied, which included growth, haematological profiles, clinical blood chemistry and both gross and microscopic evaluation of tissues, did not differ significantly from control values in animals exposed to ALA. The urinary excretion of ALA among treated animals showed a dose-related increase. Fertility was normal among ALA-treated mice. Females, examined just prior to term, showed no evidence of increased embryotoxicity or reduced numbers of foetuses.

Introduction

Haem synthesis can be disturbed by certain heavy metals and this interference may be demonstrated by lowered numbers of erythrocytes. Certain biochemical alterations can be demonstrated in blood or urine at exposure levels below those that produce gross changes in the haematopoietic system. Lead has been shown to produce measurable biochemical changes in the pathway of haem synthesis.

Gibson, Neuberger & Scott (1955) demonstrated *in vitro* that 10^{-3} M concentrations of copper, mercury and silver completely inhibited δ-aminolaevulinic acid (ALA) dehydrase. At the same concentration, lead and zinc reduced ALA dehydrase activity to 50%, while iron, magnesium, manganese, calcium and strontium had no effect. Investigations (Gibson & Goldberg, 1970; Goldberg, Ashenbrucker, Cartwright & Wintrobe, 1956; Granick, Cumming & Lau, 1966; Morrow, Urata & Goldberg, 1969) have shown that lead stimulates ALA synthetase activity. Lichtman & Feldman (1963), Bonsignore, Calissano & Cartasegna (1965) and Hernberg, Nikkanen, Mellin & Lilius (1970) have demonstrated that in erythrocytes, lead concentrations can be correlated with lowered enzyme activities. These changes produce an increased amount of ALA in the body as indicated by increased urinary excretion of the compound. In view of the increased amounts of ALA in the body, the question arises whether or not ALA itself can produce pharmacotoxic manifestations or pathological changes. This study was undertaken in response to that question.

Experimental

Materials and animals. ALA was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. The material was 99% pure and each 1-g sample was checked for purity using thin-layer chromatographic techniques. The animals used were Charles River strain random-bred albino mice from Charles River Breeding Laboratories, Inc., Wilmington, Mass.

Subacute toxicity study. Four groups each of ten mice of each sex were treated with 0 (control), 10, 50 or 100 mg ALA/kg. Saline solutions of the material were freshly prepared for injection each dosing day. Control animals received 0·1 ml saline ip on Monday, Wednesday and Friday of each of 13 consecutive weeks, and the ALA doses were administered similarly. Food and water were offered *ad lib.* and the mice were weighed at intervals throughout the study. Haematological studies (erythrocyte count, total and differential leucocyte counts, reticulocyte count, haemoglobin concentration and haematocrit determinations) and clinical blood studies (fasting blood-glucose concentration, blood-urea nitrogen concentration and serum activities of alkaline phosphatase and glutamic–pyruvic transaminase) were carried out on five animals of each sex and group during wk 13 of the study. At termination, urine samples were collected for analysis for albumin, glucose, casts, microscopic elements, pH, specific gravity and ALA content (Davis & Andelman, 1967). Gross pathological examinations were conducted on all animals, and histopathology was performed on those in the control and high-dosage ALA groups. The liver, kidneys, spleen and gonads were weighed at autopsy.

Fertility study. Groups of ten males and ten females were treated as described for the subacute toxicity study. Fertility was measured by mating these animals

Table 1. *Mean body weights of mice given 0–100 mg ALA/kg ip for up to 13 wk*

Dose level (mg/kg)	Sex	Mean body weight (g) at wk 0	4	8	13
0	M	21	35	39	41
	F	19	29	32	35
10	M	21	38	39	45*
	F	19	29	31	32
50	M	22	36	41	43
	F	20	29	32	33
100	M	22	39*	42	46*
	F	20	30	32	33

Values are means for groups of ten mice and those marked with an asterisk differ significantly (*$P < 0.05$) from the corresponding control value.

Table 2. *Haematological and clinical chemistry data for groups of mice given 0–100 mg ALA/kg ip for 13 wk*

Dose level (mg/kg)	Sex	RBC (10^6/mm^3)	WBC (10^3/mm^3)	Hb (g/100 ml)	PCV (%)	Retics (% of RBC)	Blood glucose (mg/100 ml)	BUN (mg/100 ml)	SAP (K-A units)	SGPT (Dade units)
0	M	9·75	8·6	17·0	47	1·9	58	29	8·66	61
	F	9·60	11·2	17·2	48	1·7	55	23	10·08	62
10	M	9·61	9·0	16·9	49	2·1	55	18*	7·86	46
	F	9·65	9·2	16·4	47	1·8	51	24	12·04	64
50	M	9·75	8·8	16·4	48	2·6	64	27	7·86	65
	F	9·74	11·0	17·0	47	2·4	48	24	10·31	57
100	M	9·97	8·8	17·2	48	2·3	83*	32*	5·66	73
	F	9·53	15·9*	16·5	47	2·6	82*	22	11·46	65

RBC = Red blood cells WBC = Leucocytes Hb = Haemoglobin PCV = Packed cell volume Retics = Reticulocytes
BUN = Blood urea nitrogen SAP = Serum alkaline phosphatase SGPT = Serum glutamic–pyruvic transaminase

Values are means for groups of five animals and those marked with an asterisk differ significantly ($*P < 0·05$) from the corresponding control value.

Table 4. *Fertility data for mice mated after treatment with 0–100 mg ALA/kg for 13 wk*

ALA dose level (mg/kg)	Treatment of females	No. pregnancies/ females mated	No. (per pregnant female) of		
			Implantations	Resorptions	Foetuses
0	Saline	9/10	11·6	0·4	11·2
	None	10/10	11·4	0·5	10·9
10	ALA	9/10	13·4	0·4	12·9
	None	8/10	14·2	0·6	13·6
50	ALA	9/10	13·8	1·0	12·8
	None	10/10	12·1	0·5	11·7
100	ALA	7/10	12·3	1·1*	11·2
	None	9/10	10·9	0·2	10·7

*One female resorbed seven of nine implantations.

Table 3. *ALA excretion in urine of mice given 0–100 mg ALA/kg ip for 13 wk*

Dose level (mg/kg)	Sex	ALA excretion (mg/100ml)
0	M	1·25 ± 0·42
	F	0·98 ± 0·38
10	M	5·22 ± 2·15
	F	5·00 ± 1·87
50	M	21·42 ± 10·36
	F	26·18 ± 11·08
100	M	39·84 ± 14·29
	F	43·35 ± 12·93

Values are means ± SEM for groups of ten mice.

following the 13-wk exposure period. Each male was caged with two females, one from within the same treatment group and the other untreated. Mating continued for 14 days, during which dosing was continued, and the females were then killed and the numbers of implantations, resorptions and foetuses were determined.

Statistical evaluations. Data obtained in the subacute toxicity experiment were evaluated using analysis of variance followed by Student's *t* test (level of significance 0·05). The data from the fertility study were evaluated using chi-squared analyses, the litter being the experimental unit tested and the confidence level selected again 0·05.

Results and Discussion

In the 13-wk subacute toxicity study, all animals survived the full test period without displaying any reactions that could be related to treatment. Growth, as measured by body weight, was not adversely affected by exposure to ALA (Table 1). Male mice given either 10 or 100, but not 50, mg ALA/kg weighed more than the controls at the termination of the study. The magnitude of this change was not great, however, and probably reflected normal biological variation rather than a true response to the chemical.

Haematological studies (Table 2) revealed no treatment-related changes among the parameters investigated. Blood-glucose concentrations in the mice treated with 100 mg ALA/kg were slightly higher than the corresponding control values, but were within the normal range for the strain of mouse used. All other clinical and pathological parameters investigated were normal in the ALA-treated animals. The apparent increase in leucocyte count among females treated with 100 mg/kg was due to a value of $22{\cdot}0 \times 10^3/mm^3$ in one animal. Elimination of this value from the data yielded a mean value comparing favourably with that of the controls. The urinary excretion of ALA, measured for a 24-hr period at the end of treatment, was increased in a dose-dependent manner (Table 3).

Neither the weights of the liver, kidneys, spleen and gonads nor the corresponding organ-to-body weight ratios differed from those of the control animals. Gross and microscopic evaluation of tissues from ALA-treated mice failed to reveal any compound-related changes. All changes seen in the tissues were those of spontaneous disease commonly observed in the strain of mouse studied.

The reproductive performance of ALA-treated males was equivalent to that of the control males (Table 4). Treated females were fertile and the numbers of implantation sites, resorption sites and foetuses were normal for this strain of mouse and compared favourably with the control data (Table 4).

No deleterious effects were apparent on treatment of mice with ALA at dosages of 10, 50 or 100 mg/kg by ip injection 3 times/wk for 13 wk. The reproductive capacity of ALA-treated animals was also unaffected.

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Review Section

REVIEWS OF RECENT PUBLICATIONS

Report of the Government Chemist 1973. Department of Industry: Laboratory of the Government Chemist. HMSO, London, 1974. pp. iv + 175. £1.80.

Of particular interest in this report is the section devoted to nitrosamines in foodstuffs. Of about 150 samples of fish analysed, some 20% proved to contain dimethylnitrosamine at levels of 1–10 ppb ($b = 10^9$), the higher levels tending to occur in cooked fish. One sample of Stilton cheese contained as much as 13 ppb (although this had fallen below 1 ppb after storage for 22 days at room temperature) and levels of 1–5 ppb were found in 10 of 41 other cheese samples. In meat and meat products, levels of 1–10 ppb occurred in 24 of 52 samples, including 11 samples of fried bacon. Fried bacon also contained up to 30 ppb *N*-nitrosopyrrolidine, while *N*-nitrosopiperidine and diethylnitrosamine (in the 1–5 ppb range) were found in one sample. The last compound was also found in one sample of canned chopped pork and one of fresh minced beef. In addition the presence of *N*-nitrosopiperidine in spice and curing-salt mixtures, first reported in the United States, was confirmed in some British products. A number of other commodities, including bread, flour, soya-bean oil, used frying oil and fat, coffee, spinach, baked beans and cigarettes contained no detectable nitrosamines. However, six of 12 samples of laboratory rat diet contained up to 30 ppb dimethylnitrosamine, and some *N*-nitrosopyrrolidine was also detected.

Other work has been devoted to an investigation of consumer hazards, including the problem of lead leaching from household utensils. A great improvement was noted in imported glazed casseroles, which were found to yield less than 2 mg lead/litre to a hot 4% acetic acid solution, compared with an average value of 58 mg/litre a year previously. Of studio pottery articles, only one released a significant amount of lead into a cold acetic acid solution. White vitreous enamelware did not liberate significant amounts of toxic metals, and, although red, orange and yellow surfaces were found to release significant quantities of cadmium, it appeared that manufacturers were beginning to restrict the use of these colours in cooking ware to external surfaces. Of imported coloured glassware and lead-crystal articles only the latter released lead, at up to 1 mg/litre. It was feared that coloured plastics bins and buckets used for home wine- and beer-making might lead to significant cadmium contamination, but the amount extracted into 0·25% hydrochloric acid and 10% ethanol was found to be negligible even when the plastics contained as much as 0·2% total cadmium. Work was also conducted on paints, including a determination of the ratio of total to soluble lead, and the extractable metal content of plastics toys was studied in connexion with a proposed European safety standard. In toothpastes, the highest lead level found was 5·5 ppm and most samples contained below 1 ppm, a finding which enabled the Home Office to issue a public reassurance as to the absence of hazard from this source.

Analyses of wild birds of prey for pesticide residues suggested that some aldrin- or dieldrin-treated grain must still be available to the seed-eating birds or to the small mammals on which they feed, and similar high levels were occasionally found in fish-eating birds. Some heptachlor epoxide was also detected, although its origin was something of a mystery. The major organochlorine pesticide residue was *p,p'*-DDE, which, although originally derived from *p,p'*-DDT, may well have been largely ingested as such. Polychlorinated biphenyl levels were also high, often approaching or even exceeding those of *p,p'*-DDE; they tended to be most conspicuous in the fish-eating birds. In addition the fungicide hexachlorobenzene occurred occasionally in the liver and eggs of predatory birds, at levels up to 7 ppm.

The above are but a few examples selected from the topics dealt with in this report which, because of its broad coverage of analytical matters related to foods, drugs and environmental problems, should be of wide interest.

Guidelines for Evaluation of Drugs for Use in Man. Report of a WHO Scientific Group. *Tech. Rep. Ser. Wld Hlth Org.* 1975, **563**, pp. 59. WHO, Geneva. Sw.fr. 7 (available in UK from HMSO, London, £1.05).

This Scientific Group was convened in Geneva during October 1974 to consider "all aspects of the evaluation and testing of drugs in the light of increasing knowledge and to formulate proposals and guidelines for present and future research in this field". The Group considered it undesirable to lay down formal requirements for pre-clinical evaluation of drugs, but internationally acceptable guidelines were regarded as helpful, reducing the wasteful use of personnel and resources at present occasioned by the large differences between guidelines in individual countries.

The report reviews the usual steps in the development of a new drug, from its synthesis through biological screening and acute toxicity tests, pharmacodynamic and pharmacokinetic studies and initiation of long-term toxicity tests. While the long-term tests are in progress, early studies in man may begin, and special investigations in animals relating to teratogenic and reproductive effects, carcinogenicity, mutagenicity and liability to induce dependence may be conducted, if indicated by the intended use of the drug and/or its relationship to other compounds of known activity. Controlled therapeutic trials and,

finally, post-registration surveillance complete the programme.

The ethical and legal aspects of testing in man are discussed in the report, as are specific problems such as pharmacogenetics and bioavailability. Throughout, frequent reference is made to earlier WHO reports on different aspects of the testing of drugs and other chemicals, and relevant sections from these reports are often quoted in detail. In conclusion the Expert Group outlines 13 areas in which further research is needed, with emphasis on improving the predictive value of animal tests in terms of the probable human response, and proposes five programmes of work on specific subjects that should be undertaken in the future by WHO. Short-term animal or *in vitro* tests for carcinogenicity and the possible existence of a threshold level for the effects of chemical carcinogens and mutagens are notable inclusions in the first category, while one of the five proposals for WHO work involves a consideration of differences in the degree of importance attached to effects observed at the highest dose levels in carcinogenicity studies as compared with general toxicity studies, to determine whether research is needed to establish valid reasons for this difference. Such studies would be of significance in the evaluation not only of drugs but also of many other chemicals, and it is to be hoped that these recommendations can speedily be implemented.

Fd Cosmet. Toxicol. Vol. 14, pp. 51–56. Pergamon Press 1976. Printed in Great Britain

BOOK REVIEWS

Modern Trends in Toxicology—2. Edited by E. Boyland and R. Goulding. Butterworths, London. 1974. pp. vii + 263. £7.95.

Contributions to the first volume in this series, published in 1968 (*Cited in F.C.T.* 1969, **7**, 341), centred around the experimental approach to toxicology and various techniques used in evaluating the hazards posed by the innumerable xenobiotic chemicals now present in the environment. There is no such unifying theme in the contributions to the present volume. Instead, its nine chapters contain discussions by an equally distinguished collection of authors on a wide range of topics currently of importance in experimental toxicology.

The first of these, by R. Goulding, deals with the major causes of acute poisoning, their diagnosis and the management of poisoned patients. Although enlivened by some lurid historical anecdotes, the chapter conveys the sober message that accidental and deliberately self-inflicted poisonings now account for more than 10% of all general medical and paediatric hospital admissions, compared with 3·6% a decade or so ago.

Allergic reactions to drugs present important therapeutic problems and need to be distinguished from other forms of drug reaction. However, determination of the cause of most drug allergies is hindered by a lack of specific testing procedures. These points are emphasized in a chapter on drug hypersensitivity by J. Pepys, who goes on to discuss the four major categories of allergic reaction, some factors influencing sensitization and some of the clinical manifestations of drug allergy.

Turning from the adverse effects of drugs, there follow chapters on environmental chemical hazards to wildlife (by Barbara M. Brown and D. S. Papworth) and on industrial toxicology (by Jean W. Cuthbert). The former contains a useful critical review of relevant laboratory and field techniques and rightly emphasizes the difficulties of collecting the type of data on which a meaningful assessment of hazard can be based. Industrial toxicology is approached from a clinical standpoint. Particular consideration is given here to the concept of threshold limit values in the light of the availability of increasingly sensitive techniques for the measurement of toxic substances in the working atmosphere and for the detection of adverse effects in exposed populations. Unfortunately the range of topics tackled in this chapter is so broad that the treatment of many aspects is inevitably shallow.

Recent advances in our understanding of the mechanisms by which xenobiotics exert their toxic actions are reviewed by D. V. Parke. In addition to a consideration of the influence of pharmaco-kinetic factors, inhibition and induction of drug-metabolizing enzymes and protein binding on drug toxicity, attention is directed towards toxic reactions to drugs in patients with certain diseases, in the foetus and neonate and in the geriatric patient. The biochemical approach is pursued further by W. N. Aldridge who, with reference to several well-chosen examples, discusses theoretical bases for selective toxicity.

The possibility that the progeny of animals exposed to carcinogens during pregnancy may be predisposed to the development of tumours has been suggested by a number of experimental studies. This experimental evidence is appraised by L. Tomatis, who considers that epidemiological studies of the consequences of prenatal exposure to X-rays, stilboestrol and viral infections provide ample evidence that the same is true for man.

The two remaining chapters contain an interesting, if in places heavy-going, discussion by A. M. Brues on the toxicology of ionizing radiations and the development of radiation exposure standards and a well-balanced evaluation by A. N. Worden of the role of tissue culture in the assessment of toxicity.

A book of this nature cannot hope, and indeed does not aim, to provide complete coverage. Nevertheless, each chapter is followed by a fairly extensive list of references and any search for specific information will be aided by the subject index. In providing an insight into current toxicological thinking, this book should prove as valuable to all interested biologists as did its predecessor.

Residue Reviews. Residues of Pesticides and Other Contaminants in the Total Environment. Vol. 52. Edited by F. A. Gunther. Springer-Verlag, Berlin, 1974. pp. ix + 156. DM 38.80.

One of the aims of *Residue Reviews* is to collate, on an international level, current knowledge on specific pesticide residues or other chemical contaminants in the environment. That this aim is succeeding is well illustrated by chapters included in Volume 52 of the series.

Diquat and paraquat, the most active members of the bipyridylium group of compounds, are used primarily as non-selective contact herbicides, as desiccants and as a means of controlling aquatic weeds. In the opening chapter of this volume, a Canadian author reviews the literature relating to the adsorption of bipyridylium herbicides by humic substances. The effect of such factors as equilibrium time, temperature and the presence of inorganic cations in the humic material is discussed and the mechanism of adsorption of the bipyridylium compounds by humic substances is examined, evidence being presented for the involvement of ion-exchange and charge-transfer mechanisms. Complementing this contribution is the final chapter in the book. In this, UK authors provide an extensive review of the literature relating to the physico-chemical factors involved in the adsorption of paraquat by humic materials in the soil.

The persistence of pesticides in the environment depends not only on the chemical structure of the

pesticide but also on a multitude of interacting environmental factors. In a chapter entitled "The presence and cycling of pesticides in the ecosphere", this subject is surveyed by Greek workers, while a contribution from the United States provides further insight into the behaviour of pesticides in the environment. The findings of the authors, as well as of others in this field, are presented in this review of the physicochemical properties responsible for the behaviour of pesticides in air, water, soil and living organisms (the lipid binding of DDT for example). The authors express the hope that this review may help to provide a foundation for the emerging field of 'environmental chemodynamics'.

Returning from the general to the specific, an interesting chapter is included on residues of dithiocarbamate fungicides and their metabolites on plant foods. This makes particular reference to the ethylenebis-(dithiocarbamates). A short introduction to the chemistry and toxicology of these compounds is followed by a consideration of their degradation and metabolism, and the use of thin-layer chromatography and of paper-chromatographic, photometric, polarographic and gas-chromatographic methods for determining residues of the undecomposed fungicides and their degradation products is reviewed.

Finally, from the Institute of Medical Plants in Poznań comes a chapter entitled "Pesticide residues in medicinal plants in Poland". The Institute promotes research into the production of drugs from plants, and deals with such problems as the choice of pesticides for use in the cultivation of medicinal plants, the determination of optimal conditions for their use and the establishment of a high plant yield with minimal residue contamination of the raw material and of the final product. Medicinal plants tend generally to be given relatively little consideration because they account for only a small acreage in comparison with agricultural plants, but the authors hope that the investigations carried out at the Institute will contribute towards establishing international guidelines applicable to this field.

Chlorinated Insecticides. Vol. II. Biological and Environmental Aspects. By G. T. Brooks. CRC Press, Inc., Cleveland, Ohio. 1974. pp. vii + 197. $44.50.

It is widely agreed that genetic selection and other evolutionary processes have operated throughout the history of every living species to minimize the harmful effects of naturally-occurring chemicals. One of the most important questions arising from the large-scale production of man-made chemicals over the last century concerns the rate of evolution of tolerance to any biological effects of these substances, and the ethic acceptability of such a process for man. It is thus appropriate that the main text of this recent monograph from the CRC Press should begin with a review of the way in which insects develop resistance to chlorinated pesticides, before considering the biological effects of these substances in their wider context. Coverage of their environmental impact is about equally divided between an account of their biochemical/toxicological effects and their metabolic fate in insects, soils, plants, micro-organisms and vertebrates.

Inevitably in so short a volume, only a small fraction of the wealth of scientific literature on these subjects has been cited, a degree of selection typified by the 3-page account of the toxicity of "DDT group" insecticides to non-target organisms. Nevertheless, the author has taken great care to include those advances that have been fundamental to our understanding of the environmental impact of these persistent chemicals. No light is thrown on the biological significance of acquired tissue residues of DDT and its relatives in man, but a detailed discussion of the mode and mechanism of action of these pesticides represents a step in the right direction. It is not without reason that the author has devoted a major part of the book to the biological effects and selective toxicity of chlorinated pesticides in insects, since this must assist on several counts a better appreciation of the possible health effects of these chemicals in man.

Like so many CRC publications before it, this volume is not only an impressive work in its own right, but is equally valuable as a source of over 700 selected references, many of which belong to the post-1970 period.

Foreign Compound Metabolism in Mammals. Vol. 3. Senior Reporter D. E. Hathway. The Chemical Society, London, 1975. pp. xvi + 727. £26.

The aim of this series of monographs is to collate literature published in the field of mammalian xenobiotic metabolism. Volumes 1 and 2 of this series, previously reviewed (*Cited in F.C.T.* 1972, **10**, 693; *ibid* 1973, **11**, 1116), covered the literature published up to 1971. The current volume reviews that published in the 2-year period 1972/1973 and consists of six chapters in addition to a general introduction. There is an author and a compound index but unfortunately, as in previous volumes, a subject index has not been considered essential.

The introduction is divided into three sections. In the first, written by D. L. Gunn, the uses and abuses of the pesticides DDT and dieldrin are discussed. While this section provides an excellent evaluation of the economic, political, toxicological and environmental factors resulting from the widespread use of these two agents, the last two sections are of more general interest. These are devoted to the role of metabolic studies in drug and biocide development and to chronic toxicity testing and metabolic considerations. Both sections stress the importance of metabolic data in the design and safety assessment of new drugs and biocides.

Chapter 1 is written by P. G. Welling and deals with drug kinetics. Many examples are given of the absorption, distribution and elimination of different types of therapeutic agents. In the second chapter, literature concerned with the biotransformation of many types of drugs, food additives and contaminants, agricultural and industrial chemicals, carcinogens and toxins is considered by the Senior Reporter. Like the rest of the book, this chapter is amply illustrated with chemical structures. In Chapter 3, D. H. Hutson deals with the more theoretical aspects of xenobiotic biotransformation and conjugation processes and reviews the properties of the hepatic microsomal mixed-function oxidase complex.

which is responsible for most xenobiotic biotransformations.

Chapters 4, 5 and 6 (by J. D. Baty, S. S. Brown and D. E. Hathway, respectively) deal with important considerations regarding the safety-in-use of both natural and synthetic xenobiotics. Firstly, metabolic and toxicological data derived from animal experiments have to be assessed in terms of the human situation, in which context possible species, strain and sex differences in metabolism must be taken into account. Secondly, since man is exposed to an enormous range of xenobiotics, the problems of possible metabolic interaction between two or more of these agents must be evaluated. Finally, as the majority of xenobiotics may pass from the maternal to the foetal blood stream, teratogenic effects must be considered a possibility.

An additional factor here is that the xenobiotic may be more toxic to the foetus than to the pregnant female as the levels of xenobiotic-metabolizing enzymes in both the placenta and the foetus are very low.

In all, this book lists some 3500 references and thus should serve as a valuable, if expensive, guide to the literature.

Experimental Lung Cancer. Carcinogenesis and Bioassays. International Symposium held at the Battelle Seattle Research Centre, Seattle, WA, USA, June 23–26, 1974. Edited by E. Karbe and J. F. Park. Springer-Verlag, Berlin, 1974. pp. xiii + 611. DM 85.00.

One of the aims of the experimental pathologist is to construct models of human disease in experimental animals so that by patient thought and experiment he may understand some of the underlying factors concerned in the disease process. The ultimate aim, of course, is to apply such understanding to the human situation.

There have been very few human diseases that have captured the headlines, both in the scientific and lay press, as much as has lung cancer. Its notoriety is due not only to its association with a widely prevalent social habit but also to its increasing incidence, particularly in females, who appeared up to some 10 years ago to be reasonably free of this scourge.

Experimental models for the study of lung cancer are not readily available, and much time and energy have been expended in the past 10–15 years in the search for a suitable one. These efforts were reviewed by the various contributors to an International Symposium held at the Battelle Seattle Research Center in June 1974, and their contributions have now been collected together in book form.

The contributions to Session I dealt principally with the more theoretical aspects of pulmonary tumorigenesis in experimental animals, such as the significance of enzyme induction in pulmonary tissues, host (and particularly hereditary) factors in the determination of the response to carcinogens, and the dynamics of particle flow in relation to smoking. Sessions II and III were mainly concerned with bioassay systems. The papers on pulmonary bioassays covered a range of methods particularly suitable for use in the hamster and included both basically short-term studies, such as the method for locating labelled smoke particles in the respiratory tract and internal organs, and studies involving life-time inhalation. A particularly interesting study in the latter category involved the use of the carcinogen methylnitrosourea for the pretreatment of the hamster lung prior to life-time inhalation studies. This method resulted in a substantial increase in the sensitivity of the model, but one doubts whether this has a counterpart in the human situation.

Use of the mouse skin as a bioassay system was not neglected, and the potential of the sebaceous-gland suppression test for predicting the carcinogenicity of cigarette-smoke condensate also received attention. The novelty in this paper lay in the evaluation of a hyperplasia index along with the sebaceous-gland suppression test. Mathematical treatment of the results has produced a test claimed by the authors to be of good predictive value, at least for tobacco-smoke condensates.

Session IV, the last section, deals basically with the efforts spent in finding suitable models for the study of radiation-induced carcinogenesis.

Overall the book will be useful to those engaged in studying the reactions of the pulmonary tissues of experimental animals to cigarette smoke, model irritants and carcinogens. It will be of particular interest to those involved in developing or applying bioassay systems suitable for comparing the effects of conventional cigarettes with those of cigarettes containing new smoking materials.

Carcinogenesis as a Biological Problem. By I. Berenblum. North-Holland Publishing Company, Amsterdam, 1974. pp. xxvii + 376. Dfl. 90.00.

For many years, the author of this book has been well known to biologists in the field of cancer research, many of whom probably associate him most with the fundamental experimental work on mouse-skin cancer involved in the development of the so-called two-stage theory of carcinogenesis. His interest, however, encompass a much broader field than skin cancer, and this book is to some extent a reflection of these wider interests. It covers the more important concepts developed in the search for some understanding of the phenomena involved in the induction of cancer by chemical agents in experimental animals and attempts to provide an indication of the relevance of these concepts to cancer induction in man.

It is perhaps to be expected that, to many newcomers in the field, much of the work cited and discussed in depth will appear dated. Nevertheless, such studies have many implications which have not been given enough attention, and new recruits to the field of cancer research may well benefit from a reappraisal of old conclusions and deductions. In drawing attention to the work carried out in the first three decades of chemical carcinogenesis studies and to the ideas and concepts that flourished in that period, the author has rendered a great service to the scientific community by providing some degree of continuity. So much attention is being paid nowadays to molecular interactions that we seem to be in danger of missing some important message that the target tissue or the animal as a whole may impart.

The more recent concepts and findings are not neglected, however. Perhaps the chapters dealing with this aspect of cancer research may appear jejune or even trivial to the advanced molecular biologist, geneticist or virologist, but one has to consider that many workers in cancer research, while having an interest in these methods of approach, do not have sufficient depth of knowledge to enable them to make a critical assessment of the impact that such specialized studies may have on the field. To this group, this text will serve as a welcome introduction.

Although any criticism of the efforts of such an outstanding figure in cancer research may appear somewhat carping, one cannot help feeling puzzled at the author's apparent reluctance to accept as a general rule that intervening factors may play a predominant role in the induction of cancer in animals by chemical agents. His acceptance of the case for hormonal influences in mammary tumorigenesis does not seem to extend to parallel arguments in connexion with other cancers, such as subcutaneous sarcomas induced by plastics films or cancers of the urinary bladder induced by calculi. The former situation is given very fragmentary consideration, while the latter is hardly mentioned. The scant attention paid to these questions by so eminent a biologist may give the impression that they are of little importance as factors in tumour induction. Yet over a number of years, pathologists and clinicians have accumulated a wealth of experience indicating that repeated tissue damage plays a significant role in the development of cancer.

Drug Interactions. Edited by P. L. Morselli, S. N. Cohen and S. Garattini. Raven Press, New York, 1974. pp. x + 406. $28.00.

It is becoming recognized to an increasing extent that an understanding of the mechanisms underlying the modifying effects of such factors as nutritional status, genetic differences and drug interactions on the pharmacological response of man to drugs is an essential step in placing chemotherapy on a sound rational basis. This volume, one of a series of monographs emanating from the Mario Negri Institute for Pharmacological Research, Milan, and based on an international and interdisciplinary symposium, provides a great deal of valuable information on this complex subject.

The book contains 38 papers by nearly 100 authors on 11 related aspects of the problem and opens with an excellent article by J. J. Burns and A. H. Conney entitled "Drug interactions: Historical aspects and perspectives", which sets the tone for the rest of the volume. Specific instances where gastro-intestinal absorption and renal excretion affect drug action are dealt with in four papers, and other contributions relate to the important role of microsomal enzymes in determining the pharmaco-activity and metabolism of drugs. The effects of protein binding, oestrogen interactions and calcium on drug action are discussed in five papers and views on the importance of genetic factors in determining pharmacological effects are presented.

Other contributions are concerned with multiple mechanisms involving drug interactions, with the importance of monitoring drug levels in therapy and with the design of experimental protocols for evaluating the interactions of drugs. The potential value of computer-based systems for collecting, collating and interpreting information derived from clinical situations is also described.

This book will doubtless be of considerable value to experimentalists in the various disciplines involved in the unravelling of the complex problems concerned with the design and application of drugs.

Mass Spectrometry Volume 3. Senior Reporter R. A. W. Johnstone. The Chemical Society, London, 1975. pp. xiii + 402. £13.50.

This book reviews the literature published between July 1972 and June 1974 in the field of mass spectrometry. It is the third in this particular series of Specialist Periodical Reports produced by The Chemical Society, but this volume has been produced by a largely new team of reporters, 13 in all, with Dr. R. A. W. Johnstone of the University of Liverpool as Senior Reporter.

The ten chapters cover, in turn, questions of theory, methods and results. Chapter 1, on the theory and energetics of mass spectra, is one of the most extensive and is subdivided into major sections on the calculation of ion structures and energies, ionization processes and energy deposition functions, unimolecular decay processes, experimental methods and appearance potential measurements. It will probably be of purely academic interest to the majority of users of the mass spectrometer. The other chapters are concerned with structure and mechanisms in mass spectrometry, alternative methods of ionization and analysis, computerized data acquisition and interpretation, organometallic, co-ordination and inorganic compounds, natural products, reactions of organic functional groups (positive and negative ions), gas chromatography–mass spectrometry, drug metabolism, and protein and carbohydrate sequence analysis, the latter being the only chapter presenting a critical appraisal of the literature.

The main criticism that may be directed at this book is that although there is an author index, there is no subject or compound index. The Senior Reporter comments in his foreword that Volume 4 will contain a cumulative index covering the first four volumes, but this does not really obviate the need for a subject index in individual issues. Its absence means that investigating the literature available on a given compound necessitates reading the whole book, since some compounds are covered in more than one chapter. This is clearly a serious disadvantage in an otherwise useful review book.

Practical Methods in Electron Microscopy. Vol. 3. Edited by Audrey M. Glauert. North-Holland Publishing Company, Amsterdam, 1974. pp. xvi + 353. Dfl. 90.00.

Volume 3 of this series is available either in a single clothbound edition or in two separate parts published

in paperback, part I, by Audrey Glauert, being entitled *Fixation, Dehydration and Embedding of Biological Specimens*, while part II, by Norma Reid, is concerned with *Ultramicrotomy*.

"Good fixation is the cornerstone to good histology.....", a remark attributed to a classical pathologist, may well be extended to the preparation of specimens for electron microscopy, in which fixation is of paramount importance. The fact that the first 70 pages of this book are devoted to fixatives and the next 40 pages to fixation methods emphasizes the importance placed by the author on the initial process of fixation of the specimen. Each fixative is described in detail, and particularly helpful are the step-by-step instructions on the preparation of simple and compound fixatives. Information on the storage, osmolarity and mode of use of each fixative and in the preparation of buffer solutions is well covered. Techniques are described for tissue-culture cells and botanical and marine specimens as well as for perfusion fixation.

Dehydration and embedding are considered equally comprehensively, the techniques covered ranging from those for traditional resins and water-soluble resins to those applicable to the newer urea/aldehyde and protein/aldehyde mixtures.

The second part of the book deals with ultramicrotomy. This is well endowed with photographs of ultramicrotomes and there are many drawings and diagrams in the section dealing with knives and glass-breaking. A very useful chapter on "problems" deals with most of the difficulties one can encounter with this technique. The final chapter is concerned with cryo-ultramicrotomy.

The problem with a standard text book is generally the question not of what to put in but of what to leave out! This is particularly the case in connexion with fixation and embedding, which have given rise to so many variations and modifications that a volume of this size could be written about osmium fixation alone. Audrey Glauert has successfully sorted through this maze and has produced an excellent volume which is easy to read and is suitable not only as an introduction to the subject for the beginner but also as a reference book for those already deeply involved.

On the other hand, the part devoted to ultramicrotomy is essentially a text for beginners and intermediates; the experienced microtomist is often so set in his ways that no book on cutting would be found near his bench! This part is very well written, overcoming the inherent problem of potentially tedious descriptions of the mechanics of moving parts or the scoring of a strip of glass.

The complete version of this book may be thoroughly recommended both to the trainee technician and to the 'classical pathologist', but if funds will not run to that, the paperback edition of Part I should be purchased. It is a pity that having led the reader through the possibilities and problems of fixation and cutting, the authors and publishers did not complete this treatise with a further part covering staining techniques. A combined text on these three stages would be a clear choice for every EM laboratory, and it is to be hoped that this major omission will be rectified in time.

The Structure of Mitochondria. By E. A. Munn. Academic Press, London, 1974. pp. xiii + 465. £9.80.

It is both unusual and refreshing these days to find a book priced at under £10 and as profusely illustrated and well presented as the one named above.

The author, from the ARC Institute of Animal Physiology at Cambridge, has managed to achieve an encyclopaedic range in his first chapter. With examples from unicellular algae, protozoa and fungi, through higher plants and insects to man and other mammals, chapter 1 examines the great variety of normal mitochondrial ultrastructure that has been reported in the literature. In 56 figures, many of them subdivided, the description progresses from the overall shape of mitochondria *in situ* to the organization of cristae, and especially the relationship of cristae number to metabolic demand. A consideration of the arrangement of mitochondria in the cell and a long section on mitochondrial inclusions complete the chapter.

Mitochondria are not static but dynamic objects, as anyone who has witnessed the beautiful cinemicrographic films of T. Hongladarom and S. I. Honda will agree. Chapter 2 discusses the alterations in mitochondrial structure that occur *in situ* in response to physiological, dietary or developmental changes and clinical or pathological disorders. Chapter 3 begins with an outline of the rationale and the methods that may be used to isolate mitochondria and examine their structure in the normal state or after they have been subjected to deliberate manipulations. The latter include variations in the osmolarity of the suspending media, oxygen tension, the presence of 'swelling agents' and procedures causing fragmentation of inner membranes.

Lest biochemists should feel left out, the next two chapters cover the chemical composition of mitochondria. Chapter 4, nearly 80 pages in length, deals with proteins quantitatively, qualitatively and structurally. The lipids, metal ions, nucleotides and other anions occupy chapter 5. The section on mitochondrial nucleic acids gives scope for displaying some of the familiar electron micrographs of shadowed circular DNA and straight RNA which have resulted from the elegant protein monolayer techniques developed by A. K. Kleinschmidt.

The three chapters that follow are concerned with the functional organization of mitochondria. Chapter 6 contains a mixture of concrete evidence and speculation about the structural arrangement of submitochondrial membranes. Particular attention is paid here to enzymes as structural components, while a short seventh chapter considers the large-scale organization of groups of enzymes constituting metabolic pathways. Dr. Munn stresses the compartmental arrangement of enzymes involved in the tricarboxylic acid cycle, amino acid and fatty acid metabolism and hydroxylation of steroids. The problem of transport across the inner mitochondrial membrane is examined in some detail, though the author admits that a great deal more information exists than has been presented in this chapter.

No book on mitochondria would be complete without some treatment of the short-term size fluctuations first observed in isolated mitochondria by B. Chance

and L. Packer and studied in the electron microscope by C. R. Hackenbrock and others. The energy state of the cristae membranes seems to be the critical factor that determines the morphological appearance. Since the author has obviously been closely involved in this field, the chapter is particularly complete and well organized.

After such exhaustive (though not exhausting) chapters, the final one flies off into the little-explored territory of the origin of mitochondria—are they (or were they once) chloroplasts or bacteria? In each case, structural and biochemical similarities are offered, though the author favours an episomal rather than an endosymbiont origin.

Dr. Munn is obviously an enthusiastic electron microscopist and uses every opportunity to slip in an appropriate electron micrograph where it will illustrate his point. Fortunately, the publishers have been liberal, both in allowing him the space to include 186 figures and 58 tables, and in providing excellent reproduction of micrographs and diagrams. It is said that one can judge the excellence of a book by the thoroughness of the indexing. For a volume with 371 pages of text to have a 46-page bibliography, 30 pages of author indexing and 20 pages of subject index speaks highly of the author's industry.

This book is up-to-date and sticks to the scope of its title. It represents good value and will appeal to morphologists and biochemists alike.

Environmental Medicine. Edited by G. M. Howe and J. A. Loraine. William Heinemann Medical Books Ltd., London, 1973. pp. xii + 271. £6.00.

This is an interesting book and is generally very readable, except in a few parts where a multiplicity of headings tends to break up the text. The joint editors have brought the combined approaches and experience of the physician and geographer to the task of assembling chapters from some twenty contributors, who in turn are drawn from various specialist branches of medicine, ecology, geography and the social sciences. The result is a wide-ranging demonstration of the possible interactions between environmental conditions and the health and diseases of individuals and communities.

The first chapter is a general introduction to the problems of environmental influences on health. Some of this may seem a statement of the obvious, but it is probably worth repeating, if only to set the scene for what follows. Subsequent chapters consider individual questions, including the health hazards of radiation, geographical variations in the trace elements in soils and food supplies, and the relationship of weather and climate to health and disease. A chapter on water demonstrates the book's breadth of approach, ranging as it does from 'myths to molecules' and touching on such diverse aspects as the importance of surface waters as social, industrial and tourist resources and as factors in regional and urban development, the effects of water deprivation and the contamination of water by chemicals, viruses, microorganisms and other disease-carrying organisms.

Problems for which man is more directly responsible are represented *inter alia* by the effects of air pollution, the ecology of pesticides and the relationship between modern economic and environmental development and a variety of disease states and gynaecological and obstetric conditions. Particular problems of our time are represented by sections on alcoholism, mental health, drug dependence and venereal disease, and some understandably depressing comments are made on the effectiveness of health education programmes. The development of resistance to antibiotics and the arguments about the possible association between water hardness and the incidence of cardiovascular disease are also considered, together with the more established connexion between the environment and various thyroid disorders. A final chapter on documentation makes the interesting observation that the first journal providing some form of abstracting service was published as early as 1665! It also reviews the range of currently available abstracting journals relevant to the fields covered in this book.

A single publication that casts its net so widely cannot deal with its subject in depth, and in that sense is clearly not a book for the specialist. Nevertheless for the specialist, as for other readers, it may serve as a salutary reminder that, in this context, a problem can seldom be viewed in isolation but must be considered as one part of a complex of interrelating factors.

BOOKS RECEIVED FOR REVIEW

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Food Processing Hygiene. By D. J. Cook and R. Binsted. Food Trade Press Ltd., London, 1975. pp. 71. £2.50.

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Peptide Transport in Protein Nutrition. Frontiers of Biology Vol. 37. Edited by D. M. Matthews and J. W. Payne. North-Holland Publishing Company, Amsterdam, The Netherlands, 1975. pp. xxii + 503. $54.25.

Dangerous Properties of Industrial Materials. By N. I. Sax. 4th ed. Van Nostrand Reinhold Company, New York, 1975. pp. vii + 1258. £21.25.

Fd Cosmet. Toxicol. Vol. 14, pp. 57–65. Pergamon Press 1976. Printed in Great Britain

Information Section

ARTICLES OF GENERAL INTEREST

CARBON DISULPHIDE TOXICOLOGY: THE PRESENT PICTURE

Current questions regarding the results of industrial exposure to carbon disulphide (CS_2) were presented at the Third International Symposium on Toxicology of Carbon Disulphide, held in Cairo and Alexandria in May 1974 (Lieben, *Archs envir. Hlth* 1974, **29,** 173). The main interest centred on the cardiovascular effects of CS_2, and its effects on blood, liver, kidneys and gonads, and on the value of its metabolites as possible early indicators of significant exposure. Threshold limit values (TLVs), which differ widely between East and West, came in for scrutiny, with several authorities supporting a case for reducing the TLV from 20 to 10 ppm CS_2 despite the difficulty apparently experienced by some companies in maintaining even the higher value. The determination of two CS_2 metabolites, 5-mercaptothiazolidone and thiourea, was suggested as a possible improvement on the merely qualitative urinary iodine-azide test for detecting significant exposure. No agreement was reached during the symposium on whether women should be permitted to work at all under conditions involving exposure to CS_2. East Germany and the USSR are not at present prepared to restrict the exposure of women to CS_2, but several other countries have prohibited such exposure on the strength of demonstrations of teratogenesis induced in experimental animals (Lieben, *loc. cit.*).

A number of new advances in CS_2 toxicology were discussed in the rather tardily reported 1972 Yant Memorial Lecture (Teisinger, *Am. ind. Hyg. Ass. J.* 1974, **35,** 55). Rats exposed to 1·2–2·4 mg CS_2/litre for 6 hours daily for 10–50 weeks developed a disturbance in pyridoxine metabolism which could be counteracted by giving pyridoxine supplements, but the neuropathy that developed improved only slowly, and it seems possible that CS_2 may also upset nicotinic acid metabolism. CS_2 has been reported to inhibit serotonin activity in the brain by reducing monoamine-oxidase activity. A diminution in the tissue levels of copper and zinc available for enzyme systems occurs in CS_2 exposure and may be involved in the toxic action, although whether CS_2 itself or one of its metabolites (mercaptothiazolidone or thiourea) is primarily responsible for CS_2 toxicity is so far unknown. A relationship between exposure to CS_2 and coronary heart disease probably exists, but studies of the lipid disturbances involved have given contradictory results, and there is no more than a strong suspicion that exposure to CS_2 aggravates the atherosclerotic process (Teisinger, *loc. cit.*). Other reports claim that CS_2 has a toxic effect on the optic nerve and produces changes in the retina suggestive of diabetic retinopathy, and that it decreases secretion of testosterone and inhibits spermatogenesis (Teisinger, *loc. cit.*). Some of these aspects of CS_2 toxicity may now be examined in more detail.

Nervous system

Brüderl & Benini (*Schweiz. med. Wschr.* 1974, **104,** 15) report five instances in which moderate chronic industrial intoxication by CS_2 resulted in polyneuropathy as well as the vaguer symptoms of insomnia, irritability, gastric disorders, circulatory disorders, loss of weight, loss of libido and loss of potency. The patients came from a group of six men aged 41–63 years, who had been exposed in a viscose plant to CS_2 concentrations ranging up to 65 mg/m^3 for several years. Sleep disturbances, fatigue, nausea and headache were encountered after periods of exposure ranging from 1 to 26 years, and these effects were complicated by the polyneuritic symptoms of paraesthesia, muscle weakness and cramping pains in the extremities. There were signs of improvement after termination of exposure to CS_2, but these features persisted to some extent in three patients. Among the sequelae to CS_2 intoxication were intermittent claudication, loss of detectable pulse in the foot, atrophy of the skin of the lower thigh, giddy spells and premature senility.

The tisssue half-life of CS_2 in rats in relation to its effect on brain-catecholamine concentrations was studied by Magos *et al.* (*Int. Arch. Arbeitsmed.* 1974, **32,** 289), who exposed rats to 2 mg CS_2/litre either for 1 hour or for two 4-hour periods on consecutive days. The whole-body burden of CS_2 after the 1-hour exposure was 160 nmol/g and it fell exponentially from this level with a half-life of 35 minutes. In blood and liver, CS_2 concentrations were similar to the whole-body concentration, but their rates of decrease showed initially a rapid phase, in which the half-life was 10 minutes, followed by a slower phase with a half-life approaching 35 minutes. Immediately after the two 4-hour periods of exposure, brain concentrations of noradrenaline and dopamine were below control values; after 20 hours the level of noradrenaline was still depressed, while that of dopamine had returned to the control level. Sensitivity to amphetamine-induced stereotypy was still enhanced after 20 hours. Thus, in spite of its relatively rapid elimination from the body, CS_2 can have a cumulative depressant effect on catecholamines. These results support the hypothesis that disorders of catecholamine metabolism may be a factor in the development of ischaemic heart disease in persons occupationally exposed to CS_2 (*Cited in F.C.T.* 1974, **12,** 260). In rats exposed to 2·5 mg CS_2/litre for 15 hours, glutamate

decarboxylase activity in the brain was diminished (Tarkowski, *Int. Arch. Arbeitsmed.* 1974, **33,** 79). This effect may explain the reduced γ-aminobutyrate levels observed in the brain after CS_2 exposure (*Cited in F.C.T.* 1973, **11,** 918). Pyridoxal phosphate failed to protect rats against this enzyme inhibition.

Adrenals

The adrenal glands of rats exposed to 2 mg CS_2/litre for 4 hours on two successive days showed a doubling of their dopamine content (Jarvis & Magos, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* 1973, **278,** 207) and the increase in dopamine concentration was significantly enhanced by pretreatment with phenobarbitone or by starvation before exposure. The highest adrenal dopamine content observed was in CS_2-treated animals that were both starved and pretreated with phenobarbitone, but no increase occurred in the absence of CS_2 exposure.

Liver

Freundt *et al.* (*Int. Arch. Arbeitsmed.* 1974, **32,** 297) reported that pretreatment of rats with three successive daily doses of 80 mg phenobarbitone/kg, their subsequent exposure for 8 hours to 20 or 200 ppm CS_2 and finally the injection of 100 mg hexobarbitone/kg failed to cause any significant fat accumulation in liver cells or to alter serum levels of glutamic–oxalacetic and glutamic–pyruvic transaminase (SGOT and SGPT). Pretreatment with hexobarbitone followed by exposure to 200 ppm CS_2 for 8 hours on two successive days also failed to produce hepatic fatty change. In contrast, pretreatment with phenobarbitone followed 24 hours later by oral intubation of 1 ml CS_2/kg in olive oil (1 ml/kg) resulted in extensive fatty deposits in the Kupffer cells and in the centrilobular, intermediate and acino-peripheral areas of the liver, and in a rise in esterified fatty acids and SGOT. Without phenobarbitone pretreatment, orally administered CS_2 produced less extensive fatty deposition, and olive oil alone had no effect. Thus it appears that occupational exposure to the TLV of 20 ppm CS_2 with occasional peaks of 200 ppm is probably unlikely to produce degenerative liver damage in persons with a healthy liver who are being treated therapeutically with a barbiturate drug.

In rat-liver microsomal preparations incubated with CS_2 labelled with ^{14}C or ^{35}S, carbonyl sulphide (COS) was produced in the presence of NADPH but not in its absence (Dalvi *et al. Life Sci.* 1974, **14,** 1785). The production of COS was inhibited in an atmosphere of CO. It is therefore suggested that (by analogy with the metabolism of parathion to paraoxon) CS_2 may be metabolized to COS with the release of reactive sulphur and its covalent binding to the microsomal membrane. This second reaction may be responsible for the liver necrosis produced by CS_2 in phenobarbitone-pretreated rats (Dalvi *et al. loc. cit.*). de Matteis & Seawright (*Chemico-Biol. Interactions* 1973, **7,** 375) reported that whereas the pretreatment of rats with phenobarbitone and starvation enhanced the liver toxicity of CS_2, the administration of a small prior dose of CS_2 diminished the liver toxicity produced by the standard dose. A study of the excretion of $^{14}CO_2$ after an ip dose of $^{14}CS_2$ showed that the amount of $^{14}CO_2$ exhaled was linearly related to the amount of cytochrome *P*-450 present in the liver at the time of dosing, and was also related, but in a more complex way, to the severity of the toxic changes induced in the liver. Incubation of CS_2 with rat-liver microsomes produced a marked loss of cytochrome *P*-450 only when NADPH was present, and the addition of EDTA, which prevents malonaldehyde formation, did not influence this reaction. Thus, the oxidative metabolism of CS_2 appears to be an essential step in its production of liver toxicity.

In phenobarbitone-treated rats, a rapid increase in the water content of the liver, maximal at 12–16 hours and returning to control values by 48 hours, was apparent after exposure to 4 mg CS_2/litre for 4 hours (Butler *et al. J. Path.* 1974, **113,** 53). At the same time, the concentration of Na^+ and K^+ in the liver increased when calculated on the dry weight. One effect of CS_2 may be to cause dissolution of the smooth endoplasmic reticulum in the liver, and an increased water uptake by osmosis; meanwhile an intact Na^+ pump maintains normal liver concentrations of Na^+ and K^+, and during the recovery phase the damaged membrane, included in membrane-bound autophagic vacuoles, does not impede the return of cytoplasmic hydration to normal. The same authors (*idem, ibid* 1974, **113,** 79) have reported that the treatment described above resulted in an early and consistent loss of glucose-6-phosphatase and aniline hydroxylase from the endoplasmic reticulum of the centrilobular areas of the liver. Loss of acid phosphatase also occurred during the phase of hydropic degeneration, while during recovery large acid phosphatase-positive bodies were seen within the hydropic areas. There was no detectable loss of adenosine triphosphatase from the plasma membrane. These findings indicate that CS_2 damages only the smooth endoplasmic reticulum.

Butler *et al.* (*ibid* 1974, **112,** 147) failed to find any ultrastructural changes in the hepatic parenchymal cells of rats exposed to 4 mg CS_2/litre for 4 hours without phenobarbitone pretreatment. Phenobarbitone alone induced a proliferation of the smooth endoplasmic reticulum; this was most prominent in the centrilobular cells but also appeared in the periportal cells, Exposure of rats to CS_2 following starvation and phenobarbitone treatment resulted in dilatation of the cisternae of the rough endoplasmic reticulum of the centrilobular hepatocytes, followed by an increase in size of the vesicles for 12–16 hours, the mitochondria and other organelles remaining normal. During the recovery phase the vesicles collapsed, the cytoplasm reverted to normal and large autophagic vacuoles appeared. The CS_2-induced lesions in the liver appeared, therefore, to depend on induction of the smooth endoplasmic reticulum by phenobarbitone.

Circulatory effects on the eye

Teisinger (*loc. cit.*) has referred to the toxic effect of CS_2 on the optic nerve, and to retinal changes resembling those of diabetes mellitus. A study of the microcirculation of the ocular fundus of workers exposed to CS_2 has been described by Raitta *et al.* (*Albrecht v. Graefes Arch. klin. exp. Ophthal.* 1974, **191,** 151). Ophthalmological examination was carried out on 100 men who had been exposed to CS_2 in a viscose-

rayon plant for 1–27 years (mean 15 years) and of 97 controls who worked in a paper mill. The only significant differences observed were a delay in peripapillary filling, either circumferential or segmental or both, and an increase in the mean calibre of the retinal arterioles in 68 of the exposed men compared with 38 of the controls. These changes could not be related to ageing, smoking habits, serum-cholesterol levels or glucose tolerance, and intraocular tension was within normal limits. Thus, no retinopathy directly attributable to CS_2 exposure was detected. However, it is possible that the widening of the retinal arterioles reflected the release or the increased appearance of a circulating mediator affecting the vasculature, and this may be related to other suspected but not confirmed cardiovascular effects of CS_2.

Detection of excessive exposure

The necessity for more specific and quantitative indices of early exposure to CS_2 was stressed during the Third International Symposium (Lieben, *loc. cit.*). At present the qualitative method of urine analysis by the iodine-azide reaction, which depends upon the presence of the CS_2 metabolite, thiourea, is used. Mack *et al.* (*Biochem. Pharmac.* 1974, **23,** 607) have reported that in 19 healthy subjects exposed for 6 hours to 10–80 ppm CS_2, there was first a marked depression in the excretion of a dose of 7 mg amidopyrine/kg and subsequently an increase. At 10 ppm, CS_2 markedly reduced the urinary excretion of the amidopyrine metabolite, 4-aminoantipyrine, but the early deficit was compensated almost completely during the later excretion phase. Inhibition of the drug excretion was reversible, and increased with increasing degree of exposure to CS_2; it was no longer detectable 18 hours after exposure to 20 ppm CS_2. Daily exposure to 20 ppm CS_2 for 6 hours on 5 successive days produced a delay in amidopyrine excretion similar to that seen after a single 6-hour exposure to 40 ppm CS_2, so that some cumulative effect was indicated. The authors suggest that inhibition of drug metabolism of this type might prove valuable as a sensitive test for exposure of workers to CS_2.

The prediction of an individual worker's susceptibility to CS_2 was the subject of a study by Djuric *et al.* (*Archs envir. Hlth* 1973, **26,** 287), involving the use of disulfiram. This drug and CS_2 are apparently metabolized by the same enzyme system, or by related systems, and therefore may be competitive. The metabolite from disulfiram is diethyldithiocarbamate (DDC). A single dose of 500 mg disulfiram was given to 33 viscose workers known to be susceptible to CS_2 since they had developed polyneuritis as a result of exposure in the past, to 21 workers who had been exposed to high concentrations of CS_2 for 7–14 years without developing signs of intoxication and to 18 workers who had been exposed to sub-TLV concentrations for 11–15 years without developing signs of intoxication. A urine specimen was taken from each subject 5 hours after the disulfiram administration and assayed photometrically for DDC. Increased CS_2 exposure was associated with a decrease in urinary excretion of DDC, the lowest excretion rate of DDC being in those workers who had once developed signs of chronic CS_2 intoxication. The authors suggest that a potential for greater metabolism of compounds containing sulphur may go with a higher resistance to CS_2 intoxication and *vice versa*. It is therefore possible that a worker's degree of susceptibility to chronic CS_2 exposure could be predicted from the knowledge of how efficiently he can excrete a single dose of disulfiram.

[P. Cooper—BIBRA]

PATTERNS OF CHLOROFORM METABOLISM

We recently reported the results of teratology studies on chloroform administered orally and by inhalation (*Cited in F.C.T.* 1975. **13,** 402). Although these and numerous other fairly sophisticated toxicological and biochemical studies have been carried out on chloroform, its metabolism appears to have received only limited attention in the past, most of the investigations reported having been in connexion with carbon tetrachloride metabolism, in which chloroform is apparently formed as an intermediate (Hathway, *Arzneimittel-Forsch.* 1974, **24,** 173; Uehleke *et al. Xenobiotica* 1973, **3,** 1). Its metabolic fate has recently received more systematic consideration, however, having been investigated in the mouse, rat and squirrel monkey (Brown *et al. ibid* 1974, **4,** 151).

The animals were given ^{14}C-labelled chloroform, with a specific activity of about 5 μCi/kg, by gavage in a dose of 60 mg/kg and were kept in glass metabolism cages. The exhaled air was passed through a series of traps containing, first, anhydrous magnesium perchlorate and silica gel to remove water, then toluene to remove toluene-soluble metabolites and chloroform, and finally a mixture of cellosolve and ethanolamine to trap carbon dioxide (CO_2), and the contents of each trap was assayed for retained radioactivity. Blood samples were taken from mice and squirrel monkeys, toluene extracts of bile samples from frozen monkey sections were analysed by gas-liquid chromatography, and the faeces, urine and carcasses of treated animals were examined for radioactivity. Whole-body autoradiography was carried out on the rat and monkey.

Distinct species differences were found in the patterns of chloroform excretion, but three mouse strains all gave similar results. After administration of a single oral dose, a peak level of chloroform (about 3 μg/ml) appeared in the blood of the mice within 1 hour. Within 24 hours of dosing, some 80% of the radioactivity appeared in the ethanolamine traps as $^{14}CO_2$ while about 13% was present in the urine as bicarbonate and/or carbonate. Approximately 6% was found in the toluene traps and about 2·5% remained in the carcass 48 hours after treatment. Excretion in the rat followed a similar pattern, although about 66% of the radioactivity appeared as $^{14}CO_2$ and 20% as exhaled chloroform or its toluene-soluble metabolites in the first 48 hours. A small amount (about 8%) appeared in the urine and faeces over the 96

hours of the study. In monkeys, only about 18% of the administered dose appeared as $^{14}CO_2$, while 79% appeared as unchanged chloroform or its metabolites in the exhaled air.

In some treated mice the collection of expired air was extended over a period of 5 days, with ethanol replacing toluene in the middle traps. The ethanol was then bulked and fractionated and examination of the distillates for chloroform and its metabolites indicated that unchanged chloroform was the only ethanol-soluble radioactive product excreted through the lungs. Thin-layer chromatography of urinary metabolites excreted by mice and rats showed the presence of [^{14}C]urea and two unidentified substances. A similar pattern is anticipated in the monkey, in which the total percentage of the radioactivity excreted in urine and faeces was similar to that in the mouse, but the urinary metabolites in the monkey could not be examined because urine and faeces had been collected together.

Autoradiography studies showed similar patterns of chloroform distribution in the rat and monkey, except for particularly high concentrations of chloroform in monkey bile, the level reaching a peak 6 hours after dosing. Excretion by the biliary route is unusual for such a small molecule, although not unprecedented.

It is interesting to compare these findings with the results of a study of the pulmonary excretion of chloroform and its metabolites in man (Fry *et al. Archs int. Pharmacodyn. Ther.* 1972, **196**, 98). Twelve healthy men and women weighing 60–80 kg were given 500 mg chloroform in a capsule, and for the following 8 hours the expired gases were monitored for CO_2 and chloroform content. Blood was analysed for chloroform during this period, and urine was collected for 28 hours after dosing. The proportion of the dose exhaled during the 8-hour period ranged from 17·8 to 66·6%, with a time lag of 40–120 minutes between dosing and the first appearance of chloroform in the exhaled air. Examination of the differences between males and females, their body weights and the biphasic pattern of chloroform levels in the blood, suggested a direct relationship between obesity and pulmonary excretion of unchanged chloroform, with greater uptake of chloroform by the adipose tissue leading to an increase in biotransformation to CO_2. A study of exhaled CO_2 derived from chloroform was carried out in two volunteers using [^{13}C]chloroform. About 50% of the dose appeared in the exhaled air as $^{13}CO_2$, maximum concentrations being recorded 75–210 minutes after administration. No chlorinated intermediates were found using sensitive gas–liquid chromatography procedures and it seems, therefore, that chloroform is transformed directly to CO_2 without the formation of free chlorine radicals by the splitting of a C–Cl bond.

Following the three-species study of chloroform metabolism, Taylor *et al.* (*Xenobiotica* 1974, **4**, 165) compared the metabolic picture in male and female mice. Chloroform has been found to exert a nephrotoxic effect in adult male mice but not in females. Male castrates were not susceptible to the effect either, unless they were pretreated with testosterone. Taylor *et al.* (*loc. cit.*) therefore carried out metabolic studies on males and females of three mouse strains given 60 mg [^{14}C]chloroform/kg in olive oil using methods similar to those described for the multi-species study. Autoradiography was performed on pairs of male and female mice killed 3, 5, 7 and 24 hours after dosing. After contact with X-ray film for 9 days, the sections were heated at 60°C for 2 hours to eliminate any unbound volatile material before being exposed to a second film for a further 9 days. Quantitative studies in mice killed 5 hours after dosing were used to assess the level of [^{14}C]chloroform in various organs.

As observed previously, some 80% of the dose of chloroform administered was expired as CO_2, the difference between males and females in this respect being negligible. However, a significantly smaller amount of chloroform or toluene-soluble metabolites was exhaled by male than by female mice over a 48-hour period. Furthermore, early autoradiography sections showed much greater levels of bound radioactivity in the renal cortex and medulla of the males than of the females. Although the overall radioactivity had diminished after 24 hours, a similar pattern of distribution was still evident. In females most of the activity appeared in the liver, intestine and bladder. No significant difference was found between the exposure patterns before and after heating of the sections, indicating that most of the radioactivity was bound to tissue constituents and was not due to free chloroform. Substantially similar results were obtained with all three strains of mouse.

The quantitative studies reinforced the autoradiographic findings. The ^{14}C levels in the kidneys were significantly higher in males than in females in all three strains. Female livers contained more radioactivity than those of males, but the difference was not statistically significant within individual strains. Administration of testosterone to female mice increased the level of radioactivity in the renal cortex compared with normal females similarly treated with [^{14}C]chloroform, but stilboestrol had little effect on levels in the kidneys of male animals. Castration reduced the kidney radioactivity, but while normal levels were restored by treatment of castrated mice with testosterone, stilboestrol did not enhance the effect of castration.

This sex-linked difference in the ability to metabolize chloroform appears to be peculiar to the mouse. No such effect was seen in rats or nonhuman primates (Brown *et al. loc. cit.*), nor was it reported in studies in man (Fry *et al. loc. cit.*). Moreover, man appears to excrete a large amount of an orally administered dose of chloroform unchanged in the exhaled air, while mice metabolize a large proportion of such a dose. Taylor *et al.* (*loc. cit.*) conclude that the mouse is an unsuitable species for investigations on the toxic effects of chloroform carried out with a view to extrapolating the results to man.

[F. A. Charlesworth—BIBRA]

ETHYLENE OXIDE RESIDUES IN STERILIZED MEDICAL DEVICES

The safety of ethylene oxide (EO) used as a sterilizing agent and the acceptability of the residues remaining after such use have attracted attention fairly frequently over the past few years. Indeed, the FDA currently appears to be involved in an evaluation of the use of EO in the sterilization of foods, cosmetics and medical devices. The effects of the products arising from EO, particularly ethylene chlorohydrin, ethylene glycol and 2-mercaptoethanol have been investigated (*Cited in F.C.T.* 1972, **10,** 589, 592 & 719; *ibid* 1973, **11,** 330; *ibid* 1974, **12,** 275) and last year saw the appearance of two reports on a study of changes in biocompatibility as a function of the post-exposure aeration time of EO-sterilized materials (Gunther, *Am. J. Hosp. Pharm.* 1974, **31,** 558 & 684).

The five materials used for the first part of the study were a thin silicone rubber from an anaesthesia face mask, a gum rubber from a catheter, and three different brands of PVC tubing of the kind often used in heart-lung machines, blood sets and catheters. Materials for single-sterilization tests were placed in individual glassine envelopes in Kraft paper bags and were sterilized for 1 cycle (421 mg EO/litre at 55°C for 3 hours). Samples for repeated sterilization were treated in a similar manner after they had been preconditioned by daily sterilization (exposure for 7·5 hours) for 5 days. EO and its residues were determined by the distillation and titration of approximately 2-g samples, and the haemolytic activity of sterilized and unsterilized samples was studied using human blood and a colorimetric procedure. Toxicity was assessed by iv injection of extracts into mice and sc injection into rabbits. Samples were aerated after sterilization either at ambient temperature for 24 hours or by forced, heated ventilation at 50°C for 8 hours.

Significant quantities of residual EO (0·5–2·4 mg/g) were found in all the PVC products after use of the former aeration method, but heated aeration left a measurable residue (0·5 mg/g) in only one. A permanent residue found in the gum rubber rose from 1 to 7 mg/g after repeated EO treatment followed by the 8-hour heated aeration, but this effect was not observed with the other polymers tested. Silicone rubber desorbed the most rapidly, and produced no haemolysis. This was also the only sample that caused no haemolysis in the unsterilized state; it was closely followed by one PVC sample, which caused 2% haemolysis, the other unsterilized products causing 10–20% haemolysis. In general, the degree of haemolysis induced by the sterilized products paralleled the levels of EO residues retained, ranging from 0 to 10% in samples subjected to the 8-hour heated aeration. With the exception of gum rubber, which after repeated sterilization produced a slight local erythema, no products gave positive reactions in the animal tests, and the erythema was considered to be of doubtful significance. The author suggests that red-cell haemolysis is a responsive and reproducible index of the EO content of sterilized equipment following aeration.

In his second paper, Gunther (*ibid* 1974, **31,** 684) concentrates on the EO sterilization of finished products, those investigated being two different kinds of rubber endotracheal tube, a gum-rubber Foley catheter and two different PVC endotracheal tubes. Samples were tested for haemolysis following sterilization and aeration by procedures similar to those described in the first report, but the haemolysis test was slightly modified in that all the haemoglobin released by cell haemolysis was acidified to haematin prior to its estimation so that all forms of haemoglobin could be detected on one wavelength (520 nm). Measurements had previously been taken at the isosbestic point for oxyhaemoglobin and reduced haemoglobin (548·5 nm) and were not sensitive to the presence of methaemoglobin.

Again, significant EO residues were found after the 24-hour ambient aeration of all sterilized products. After the 8-hour heated aeration only the gum rubber catheter was free of residues, although the remaining levels were minor in one of the PVC endotracheal tubes and in one of the rubber ones. A permanent residue was found in all the products, except one type of PVC tube, after repeated sterilization. It is noteworthy that the rubber products were inherently incompatible with red blood cells even before the sterilization procedure. Control studies with ethylene glycol and ethyl alcohol showed no haemolysis at concentrations up to 15 mg/20 ml, while Freon-12 was without effect up to 32 mg/20 ml. It was also found that the degree of haemolysis caused by 15 mg ethylene chlorohydrin/20 ml was similar to that produced by 2 mg EO/20 ml.

This second study thus confirmed that while there is a considerable variation in residue content after EO treatment even in similar polymers, the 8-hour heated aeration consistently produces better results than longer aeration at room temperature. There was, however, some discrepancy between the results obtained in the two parts of the study with regard to the gum-rubber catheters. From the comparative investigation of the various EO derivatives, it seems that EO is the most potent inducer of haemolysis and it is thus particularly important that EO be desorbed from sterilized products.

[F. A. Charlesworth—BIBRA]

ENZYMES FOR THE WASH

It is now 10 years since enzymes were introduced into washing powders to produce biological whiter-than-whiteness. Since that time, many critics have berated the manufacturers of enzyme detergents about the adverse effects of their products compared with those of conventional washing powders. Not only have extremely washday-red hands been reported, but also washday rhinorrhoea, sneezing, cough and other res-

piratory difficulties, while the workers involved in the manufacture of enzyme preparations have had an even harder time of it.

In 1971, a group of American scientists expressed their concern over the possible long-term subtle effects of enzyme detergents (*Cited in F.C.T.* 1971. **9**, 891). Later in the same year, an FDA-commissioned committee found that "the average enzyme detergent laundry product in normal use by consumers has not produced more primary irritation of the skin than have similar products that contain no enzymes" (*Nature, London* 1971, **234**, 245). The committee suggested that consumers should not experience adverse respiratory effects, although it was aware that such effects sometimes resulted from exposure in industrial situations. The mechanism of reactions to enzyme detergents is by no means straightforward, as both allergic and primary irritant components appear to be involved, and several attempts have been made to establish a relationship between positive skin-test reponses, a history of atopy and reaction to enzyme detergents, with varying degrees of success (*Cited in F.C.T.* 1971, **9**, 889; *ibid* 1972, **10**, 120 & 608).

In an exhaustive survey of workers in a factory where atmospheric levels of enzyme were quite high (4 $\mu g/m^3$ rising to as much as 128 $\mu g/m^3$ after spillage), little correlation could be found between any of these factors (Mitchell & Gandevia, *Am. Rev. resp. Dis.* 1971. **104**, 1). Of the 98 subjects studied, 62 developed nasal symptoms on exposure and 38 showed signs of disturbance in the lower respiratory tract. Although there was a tendency for a higher percentage of the symptomatic groups to have a history of respiratory disease and to react positively to prick tests and intradermal tests, the only significant association was that between the occurrence of nasal symptoms and immediate reactions to the prick test. These authors reported cases in which respiratory symptoms persisted for some months after cessation of exposure, although pulmonary function tests did not reveal any permanent impairment of function. The levels of exposure in this factory have been reduced since this study, and it remains to be seen whether the numbers of reactions will decrease.

Impairment of pulmonary function was found elsewhere in 17 of 19 men suffering from respiratory symptoms following occupational exposure to enzyme detergents (Shore *et al. Envir. Res.* 1971, **4**, 512). These men worked in three different plants producing enzyme detergents for one manufacturer. All had experienced a gradual onset of respiratory symptoms, generally cough, wheezing and dyspnoea on exertion, with rhinorrhoea, conjunctivitis and/or skin rash in some cases. Exposure periods varied from 6 months to 2 years. The level of exposure was similar in the 19 men, and was admitted to be heavy. There was evidence of impairment of pulmonary gas exchange in 17 of the men and air-flow rates in seven were indicative of early obstructive disease of the airways, while another six showed premature airway closure. There was no apparent correlation between the duration of exposure or smoking and the severity of the lesion. It was suggested that the observed persistence of lung damage could have been due to the proteolytic properties of the enzyme as well as to an allergic effect, in the same way that papain has been observed to cause emphysema when given intratracheally to dogs.

Other researchers have not found such a clear indication of persistent lung changes. Weill *et al.* (*J. Am. med. Ass.* 1971, **217**, 425) evaluated groups of workers from two plants, designated A and B, in terms of clinical, radiological, functional and immunological evidence of sensitization or respiratory-tract disease. Fifty out of 125 men in plant A and 60 of 486 in plant B were selected and classified as having been exposed to high, moderate or low levels of enzyme in their working environment. Plant A had been open for a shorter time and atmospheric levels of enzyme were lower than in plant B, where the high-exposure level ranged from 3–30 to a peak of 1000 $\mu g/m^3$. Average exposure levels of the high-exposure group of workers in plant A were 3–18 $\mu g/m^3$ with a peak of 60 $\mu g/m^3$. Minor signs of rhinitis were common but were not related to exposure in either plant. Workers in plant A showed no disorders of the lower respiratory tract, but those in plant B showed a characteristic picture of wheezing, cough and dyspnoea although no radiological abnormalities were apparent. The few cases of airways obstruction did not seem to be related to enzyme exposure. Pulmonary diffusing capacity showed no changes compared with pre-employment figures and did not differ significantly in the two groups giving negative and positive skin-test results. Positive reactions to skin tests were obtained in approximately 50% of the men in both plants. The most significant finding appeared to be a reduction in the forced expiratory flow during expiration of the middle 50% of vital capacity in the plant B workers compared with those in the corresponding exposure groups in plant A. At this stage in their study, the authors felt that the pulmonary-function data did not indicate any irreversible damage comparable with that normally associated with organic-dust reactions.

In a follow-up study 3 years later (1970–72), however, these findings were not totally confirmed (*idem, Ann. N.Y. Acad. Sci.* 1974, **221**, 76). Skin-test responses showed a peak in the sensitization rate in plant A in 1971, after which they started to decline, and the incidence in plant B had presumably already passed its peak rate before regular testing was initiated, as it was falling each year. Positive skin-test results were more prevalent in atopic subjects, but this relationship was not statistically significant. A peak for job-associated symptoms correlated with evidence of sensitization appeared in plant A workers, and again had probably already been passed in plant B workers with the subsequent establishment of a plateau.

However, there was an excess reduction in lung volumes—total lung capacity, vital capacity and residual volume—compared with the expected value. This downward trend appeared to be greater in plant A workers, although it was levelling off in 1971–1972. No relationship could be found between changes in pulmonary function and skin-test results, although there was a tendency for mean values of pulmonary volume to be lower in sensitized subjects. No consistent pattern was found in the relationship between changes in pulmonary function and a history of atopy or levels of exposure. Smokers generally showed a slightly greater decline in lung-function values. Dust

levels in both plants had fallen progressively, reaching levels of 1 $\mu g/m^3$ or less by 1972, except for occasional peaks. These occasionally high exposures may be the cause of the continuing appearance of symptoms in some workers.

The pathology of this impairment in lung-function values is unknown, for obvious reasons. No radiological signs were found. The authors attribute the reductions in lung-function values, particularly those of lung volume and maximum expiratory flow rates, to airways obstruction, although there is no evidence of an organic-dust hypersensitivity such as occurs in farmers' lung. The excess impairment of lung function has been reduced by improvements in working conditions. However, the question of persistent changes in pulmonary function due to industrial exposure to enzyme detergents can only be resolved fully by further follow-up studies. Investigations on changes in the immunoglobulins of enzyme-sensitized subjects may enable identification of those individuals who could develop respiratory disorders following industrial exposure to the enzymes. It remains apparent, however, that the best way to reduce symptoms is to reduce the level of enzymes in the working atmosphere.

[F. A. Charlesworth—BIBRA]

FACTORS AFFECTING THE NATURAL INCIDENCE OF HEPATOMA IN MICE*

Introduction

Hepatic nodular lesions are induced by a variety of chemicals (Tomatis *et al. Int. J. Cancer* 1973, **12**, 1) and their induction is widely regarded as an index of chemical carcinogenicity. Unfortunately, it is doubtful whether this view is supported by reliable scientific evidence.

In the first place, there is considerable dispute about the diagnosis of these nodular lesions. Some pathologists regard them all as neoplastic and call them 'hepatomas' without distinguishing between benign and malignant lesions, while others recognize that not all proliferative lesions are necessarily neoplastic and consider that some of these nodules may represent a regenerative process, presumably following some functional deterioration such as may result through loss of cells from necrosis or accelerated apoptosis or from the impairment of some metabolic process (Grasso & Hardy, in *Mouse Hepatic Neoplasia*, edited by W. M. Butler and P. M. Newberne, ASP Biological and Medical Press BV, Amsterdam, 1975, in press). This type of nodule is often called 'hyperplastic'. Although this distinction is theoretically sound, it introduces diagnostic difficulties, since the histologist is asked to distinguish not only between malignant and other nodular lesions in the mouse liver, but also between benign neoplasia and hyperplasia. At the moment, the histological criteria for effecting this subdivision are in dispute, and until more experience is available one cannot regard the histologist's opinion as an adequate basis for judging the carcinogenic potential of a chemical.

Secondly, the hepatoma incidence varies widely in different strains of mice. In some it is very low indeed and in others quite high. This state of affairs presents two problems; if the incidence is high, it may be difficult or impossible to obtain a statistically significant difference in tumour induction between test groups and controls in carcinogenicity tests, but if the strain used has a low natural incidence one cannot be sure that it has the appropriate sensitivity to carcinogenic agents, particularly those of low potential.

Thirdly, there are indications that genetic, dietary and environmental factors may influence the natural incidence of these hepatic lesions. Furthermore there is evidence that the natural incidence fluctuates from time to time without any identifiable cause.

In this brief article, all hepatic nodular lesions are grouped together as hepatomas, since in most of the papers cited no further indication of the nature of the lesion is given. The variation in hepatoma incidence between different mouse strains is outlined and evidence is presented that dietary, genetic and environmental factors are involved in determining the apparently natural incidence of these mouse tumours.

Strain differences

The number of different strains used in the experimental induction of cancer is probably greater in the case of the mouse than in that of any other laboratory species. This is true both in the general sense and in the particular area of liver cancer. Unfortunately, however, studies of this kind have been conducted until recently on relatively small groups of animals, so that although a wide variety of species has been used, the information obtained on the natural incidence of tumours is not as reliable as might have been expected.

A look through the papers concerned with the experimental induction of hepatic tumours in mice reveals immediately that most of the early experimentalists used only males. The reason for this choice is not obvious, but one suspects that males were thought to be more appropriate for this purpose than females because of the generally accepted view that hepatic nodular lesions occurred more frequently in males, the assumption being that this indicated a greater sensitivity to hepatocarcinogens.

Among the strains used, the C3H is conspicuous for the highest incidence of hepatic nodular lesions. The incidence quoted by various authors varies between 18% (Silverstone, *Cancer Res.* 1948, **8**, 309) and 100% (Heston & Vlahakis, *J. natn. Cancer Inst.* 1961, **26**, 969). A similar range has been found in the substrains, some of which have an exceptionally high incidence. This is particularly true of substrains Y/A^yA, Y/*Aa* and eB/De, in which incidences of 100, 88 and 90%, respectively, have been reported (Heston & Vlahakis, *loc. cit.*; Murphy in *Biology of the Labora-*

*Based on a paper presented by Dr. P. Grasso to a symposium on "Evaluation of the Hepatoma in the Rodent in Carcinogenesis Bioassay" held in Jefferson, Arkansas, USA, in February 1975.

tory Mouse, edited by E. L. Green, 2nd ed., p. 521. McGraw-Hill Book Co., New York, 1966). Perhaps because of this high incidence of hepatomas, as well as of other tumours, the C3H mice have not been used to a great extent in recent years in the screening of compounds for carcinogenic activity. Thus it is interesting to note that the C3H strain and its substrains were absent from a compilation of data derived from 56 long-term experiments (lasting 80 weeks or more) in various mouse strains (Sher, *Toxic appl. Pharmac.* 1974, **30,** 337).

Other strains do not appear to have this high spontaneous incidence of hepatomas. In a review carried out in 1974, Grasso & Hardy (*loc. cit.*) found the incidence of hepatomas in BALB/c, Charles River, strains A, Y and C, CFW, C57BL, C57/IF, STS, DBA/2eBDe, ICR, RF and ASH-CS1 mice to be 5% or less. Moreover, an incidence of the same order in CD-1, Swiss ICR/Ha, Swiss IMR and "albino" mice was reported by Sher (*loc. cit.*). Other strains and substrains of mice show a higher incidence, but never one reaching the levels seen in the C3H strain or its substrains. The strain showing the highest incidence apart from the C3H was the CBA strain, in 285 males and 229 females of which Pybus & Miller (*Rep. Br. Emp. Cancer Campn* 1942, p. 42) found an incidence of 41 and 27%, respectively.

Sex differences

In most of the studies conducted in the 1940s and early 1950s, male mice were used far more than females, so no satisfactory comparison of the hepatoma incidence in the two sexes could be made. In recent years, however, comparative data have been derived from larger numbers of mice of various strains following the fairly extensive use of the species for carcinogenicity testing. In the C3H strain and substrains the incidence in female mice, as in males, tends to be high, sometimes reaching nearly 60% according to Murphy (*loc. cit.*). In the commonly used strains, the incidence in females tends to be very low and is usually of the order of 2% or less, such levels having been reported in BALB/c, CFW, C57, various Swiss-derived strains and CTM females (Grasso & Hardy, *loc. cit*; Sher, *loc. cit.*). In the males of these strains the incidence is usually at least twice as high.

This sex difference raises one important question. How sensitive are female mice to chemical carcinogens? Unfortunately the data available do not allow a categorical answer, but it would seem that there is no great difference between males and females in susceptibility to tumour induction. Thus the response of BALB/c females to tumour induction by dimethylnitrosamine was the same as that in the males (Toth *et al. Cancer Res.* 1964, **24,** 1712). Similar responses were obtained in males and females of the C57/IF stock treated with 2-aminodiphenylene oxide or 4-aminodiphenyl (Clayson *et al. Br. J. Cancer* 1967, **21,** 755) and in those of the CBA strain given isoniazid or hydrazine sulphate (Severi & Biancifiori, *J. natn. Cancer Inst.* 1968, **41,** 331). In the testing of an extensive series of chemical carcinogens in hybrid mice (Innes *et al. ibid* 1969, **42,** 1101), the response of the females tended in a few instances to be lower than that of the males, but when account was taken of the lower incidence of hepatomas in the controls the statistical analysis was as meaningful for the females as for the males.

Fluctuation in incidence

The probability that a variety of factors may influence the spontaneous incidence of hepatomas is indicated not only by the wide differences between various strains and substrains but also by differences within the same substrain (Grasso & Hardy, *loc. cit.*; Sher, *loc. cit.*). Observation of the same strain for several years has sometimes revealed that the tumour incidence does not maintain a steady level. Thus Andervont (*J. natn. Cancer Inst.* 1950, **11,** 581) reported that the incidence of hepatomas rose approximately threefold between 1942 and 1945 in both the males and females of the C3H strain. A similar experience has been reported by Mary J. Tucker (personal communication 1974), in whose strain of mice the incidence of hepatoma increased from about 3% in 1965–66 to 10% in 1968 and 15% in 1970, climbing further to 20% by 1972.

Various factors have been studied in relation to their effect on hepatoma incidence. Some of these factors have received more attention than others but all require further study.

Hormonal factors

The undoubted, and in some strains, striking difference between males and females led to an investigation of the role of hormonal factors over 30 years ago. Miller & Pybus (*J. Path. Bact.* 1942, **54,** 155) found that injection of oestrone in olive oil to gonadectomized and intact CBA mice of both sexes led to an increased incidence of hepatoma in the intact male mice, but decreased the incidence in intact females and in gonadectomized males and females. Shimkin & Wyman (*J. natn. Cancer Inst.* 1946, **7,** 71) reported a reduction in hepatoma incidence in male C3H mice after the implantation of pellets containing diethylstilboestrol or oestradiol, while Andervont (*loc. cit.*) demonstrated that gonadectomy decreased the natural incidence of hepatomas in C3H mice.

Effects of diet

Both the amount of protein and the amount of fat in the mouse diet have been implicated as contributory factors in relation to the natural incidence of mouse hepatoma. The importance of the protein content was demonstrated clearly in the extensive investigations of Tannenbaum & Silverstone (*Cancer Res.* 1949, **9,** 162), who found that increasing the casein content from 9 to 36% at the expense of cornstarch (the other major constituent of the diet), without altering the other essential dietary constituents, increased the hepatoma incidence from 10 to 50%. In this experiment the authors used female C3H mice. When they repeated the same experiment with males of the same strain the results were less clearcut owing to the higher spontaneous incidence of hepatomas in this sex.

The influence of the fat content of the diet on the incidence of hepatomas in C57BL mice has been discussed by Gellatly (in *Mouse Hepatic Neoplasia*, edited by W. M. Butler and P. M. Newberne, p. 77, ASP Biological and Medical Press BV, Amsterdam, 1975). Data accumulated from 276 males and 260

females revealed an incidence of 12 and 13%, respectively, when the animals were maintained on stock pelleted diet. This diet contained 26·3% protein and 4·5% fat. Mice maintained on a purified diet containing the same amount of protein but approximately twice the amount of fat (groundnut oil) had a hepatoma incidence of 39 and 64%, respectively, in males and females. It is interesting, however, that this increase over the incidence of mice kept on stock diet was accounted for mainly by an increase in the incidence of hepatomas of 5 mm or less, the larger lesions actually showing a decrease from an average incidence of 18% in mice on the pelleted diet to one of 11% in those on purified diet containing 10% groundnut oil.

Successful attempts have been made to reduce hepatoma incidence by caloric and dietary restriction. Tannenbaum & Silverstone (*Cancer Res.* 1949, **9,** 724) found that severe caloric restriction abolished the incidence of hepatomas, while food restriction had a similar effect.

These experiences have been duplicated recently in the Pharmaceutical Division of ICI Ltd. at Alderley Park, Cheshire, by Mary J. Tucker, who found that restricting food intake to about 5·5 g/day from the average of 6·0 g reduced the incidence of hepatoma substantially (personal communication 1974).

Genetic factors

The work of Heston & Vlahakis (*loc. cit.*) clearly established a relationship between the presence of the A^y gene and hepatoma incidence. They studied the C3H/*Aa* strain which has a high hepatoma incidence (88% in the 94 autopsied males included in this report) and by suitable cross-breeding produced the C3H/A^yA strain, in which they found that all 63 males observed and autopsied had hepatomas.

The importance of the A^y gene in determining hepatoma incidence has since been confirmed by Wolff (*Cancer Res.* 1970, **30,** 1722), and by Wolff & Pitot (*ibid* 1972, **32,** 1861) who went somewhat further. They compared the incidence of hepatomas in two YS substrains of C3H mice, one with and the other without the A^y gene. Of 106 mice without the A^y gene only three had hepatomas, while 12 out of 108 mice with this gene had liver tumours. In a second experiment, Wolff & Pitot (*loc. cit.*) compared two other substrains of C3H/VY mice. One substrain had the A^{vy} gene, while the other did not. Observing the hepatoma incidence, they found that 16 out of 123 mice without the gene had tumours, while in the group with the A^{vy} gene, 30 out of 127 had developed hepatomas.

It seems, however, that these two genes affect the liver in other ways as well. At 4 weeks of age, the livers of mice possessing these genes were larger than those of comparable animals without the genes and this difference persisted at least until week 16 (Wolff, *loc. cit.*). Furthermore, the enlarged livers apppeared to have a greater activity of at least one enzyme, malic dehydrogenase (Wolff & Pitot, *loc. cit.*).

Environmental factors

If one excludes the question of food, there is little knowledge of the way in which environmental factors may affect hepatoma incidence. It is important, however, not to overlook possible environmental causes when considering any unexpectedly high incidence of hepatomas. It is possible that some environmental cause or causes may be responsible for the fluctuating incidence of these tumours mentioned earlier. In this context it may be relevant to mention that, in his observations on the natural incidence of hepatomas in C3H and CBA mice over the course of 4–5 years, Andervont (*loc. cit.*) mentioned that the transfer of one group of animals to a different animal house was associated with a considerable increase in the incidence of hepatomas. One wonders to what adverse conditions these animals had been exposed.

There is some stronger evidence, however, that environmental factors can play a decisive role in hepatoma induction. Sabine *et al.* (*J. natn. Cancer Inst.* 1973, **50,** 1237) reported that the very high incidence of hepatic and mammary tumours (approaching 100%) in their C3H/A^{vy} and C3H/A^{vy}fB strains dropped dramatically (to below 10%) in the progeny when the strain was transferred from the USA to Australia. Close examination of the conditions in Australia revealed that the only significant difference was in the bedding. When the bedding used in the USA (cedar shavings) was imported into Australia, the incidence of mammary tumours in the mice for which it was used rose to near the levels observed in the colony reared in the USA. This careful observation makes it difficult to avoid the implication that some factor in the bedding might have been responsible and emphasizes the need for attention to such details in the evaluation of changes in tumour incidence.

Conclusions

The incidence of hepatoma in the mouse varies considerably between different strains. In any one strain, however, irrespective of whether the natural incidence is high or low, the males always have a higher incidence than the females. Of the factors known to influence hepatoma induction, dietary protein appears to be the most important, followed by dietary fat and genetic factors. Hormonal factors are probably responsible for the sex difference, but changes in the hormonal pattern of the adult animal have to be drastic in order to effect a significant change in the natural incidence. In some situations, unspecified or ill-defined environmental factors may play an important role in increasing the spontaneous incidence of tumours.

Although the evidence on which these conclusions are based is often fragmentary, it is sufficient to indicate that some degree of caution is required in the interpretation of the induction of hepatic nodular lesions in carcinogenicity studies in the mouse.

[P. Grasso—BIBRA]

Fd Cosmet. Toxicol. Vol. 14, pp. 67–80. Pergamon Press 1976. Printed in Great Britain

TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS

ANTIOXIDANTS

2950. A cyclic metabolic pathway for BHT

Chen, C. & Shaw, Y.-S. (1974). Cyclic metabolic pathway of a butylated hydroxytoluene by rat liver microsomal fractions. *Biochem. J.* **144,** 497.

It has been reported (*Cited in F.C.T.* 1973, **11,** 1141) that incubation of butylated hydroxytoluene (BHT) with rat-liver microsomal preparations yielded a variety of products, including 4-hydroxy-4-methyl-2,6-di-*tert*-butylcyclohexa-2,5-dienone (BHT-3°OH) and its presumed precursor, 4-hydroperoxy-4-methyl-2,6-di-*tert*-butylcyclohexa-2,5-dienone (BHT-OOH), as well as 2,6-di-*tert*-butyl-4-hydroxymethylphenol (BHT-alcohol; BHT-1°OH). An extension of this study has now demonstrated that these three BHT derivatives are all part of a cyclic metabolic pathway and can be reconverted to BHT.

When BHT was incubated as before with a rat-liver microsomal preparation, formation of BHT-OOH, BHT-3°OH and BHT-1°OH was confirmed. When BHT-OOH was used as the substrate, BHT-3°OH, BHT and BHT-1°OH were obtained, whereas when BHT-3°OH was the substrate BHT, BHT-OOH and BHT-1°OH resulted. In all cases, increases in the microsomal proteins or substrate concentrations led to increases in product formation. No activity was observed when the microsomal fraction was omitted or when it was added after boiling, except in the conversion of BHT-OOH to BHT-3°OH, which could have been mediated by metal ions or chelates. Glutathione greatly increased the reduction of BHT-OOH to BHT-3°OH, perhaps by acting as a hydrogen donor. Anaerobic incubation of BHT-OOH or BHT-3°OH increased the yield of BHT, the yield of oxygenated compounds being correspondingly reduced. SKF-525A, a common oxygenase inhibitor, also lowered the yield of oxygenation products, but EDTA had no effect on BHT hyperoxidation, suggesting that this was mediated by cytochrome *P*-450.

On the basis of these findings, the authors propose that BHT is converted to BHT-OOH and subsequently to BHT-3°OH, which is either reconverted to BHT or transformed to BHT-1°H; BHT-1°OH can also be formed directly from BHT. However, the precise mechanism of each stage in this cyclic reaction still remains to be clarified.

2951. Ethoxyquin and the liver—adaptive or toxic response?

Parke, D. V., Rahim, A. & Walker, R. (1974). Reversibility of hepatic changes caused by ethoxyquin. *Biochem. Pharmac.* **23,** 1871.

Ethoxyquin is permitted in the UK for use in the prevention of scald on apples and pears (The Antioxidant in Food Regulations 1974, Statutory Instrument 1974 no. 1120), and is also used as an antioxidant in animal feedstuffs (*Cited in F.C.T.* 1964, **2,** 254). It has been observed (Cawthorne *et al. Br. J. Nutr.* 1970, **24,** 357; Parke *et al. Biochem. J.* 1972, **130,** 84P) that, on administration to rats, ethoxyquin causes liver enlargement and induction of some hepatic drug-metabolizing enzymes. The present study was undertaken to ascertain whether these hepatic changes were adaptive and reversible or were manifestations of a toxic response.

In rats fed ethoxyquin at a level of 0·5% in the diet for 14 days, the liver weight increased by 90% compared with pair-fed controls, while body weights were not changed significantly. There was a decrease of 35% in DNA concentration compared with a 25% increase in total hepatic DNA, and from the results of the DNA analyses it was calculated that the increase in liver mass was due to a 25% increase in cell number and a 50% increase in cell mass. There was an accompanying increase both in concentration and in the total liver content of microsomal protein, cytochrome *P*-450, cytochrome b_5 and biphenyl-4-hydroxylase. The specific activity of ethylmorphine-*N*-demethylase in the microsomes was depressed by about 13%; because of the increased liver mass, however, total activity in the liver increased by 65% relative to the pair-fed controls.

After treatment, recovery from cell hypertrophy was rapid and DNA concentration was normal by day 3, while microsomal-protein concentration and enzyme concentrations other than that of cytochrome b_5 were not significantly different from control values by day 7. Recovery from hyperplasia was slower and the total amounts of microsomal protein and the enzymes studied did not fall to control levels until day 30, although in most cases the difference was not statistically significant after 14 days. Cytochrome b_5 was slow to recover, the specific activity remaining elevated even after a 14-day recovery period, when the microsomal-protein concentration had returned to normal. The authors suggest that this may have been due to the slower turnover rate of this cytochrome compared with that of cytochrome *P*-450 and other microsomal enzymes.

In the authors' view, the complete reversibility of the hepatic changes produced in rats by dietary ethoxyquin suggests that the observed hepatomegaly and enzyme induction should be considered a truly adaptive response. It is suggested, however, that the effects of ethoxyquin on the activity of known hepatic carcinogens, such as acetamidofluorene, should be investigated.

PRESERVATIVES

2952. Testing nitrofurans for mutagenicity

Yahagi, T., Nagao, M., Hara, K., Matsushima, T., Sugimura, T. & Bryan, G. T. (1974). Relationships between the carcinogenic and mutagenic or DNA-modifying effects of nitrofuran derivatives, including 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide, a food additive. *Cancer Res.* **34,** 2266.

Nitrofuran derivatives, some of which are used as food additives in Japan, include compounds which have been shown to be neurotoxic to man when given in therapeutic doses and to induce tumours in rats (*Cited in F.C.T.* 1967 **5.** 426). This paper describes several rapid microbial assay systems for detecting mutagenic and DNA-modifying effects in nitrofurans.

In tests using cultures of *Escherichia coli* strains B/r WP2 *try*$^-$ and WP2 *try*$^-$, *hcr*$^-$ and *Salmonella typhimurium* strains TA 1535, TA 1536, TA 1537 and TA 1538, 26 of the 27 nitrofuran derivatives tested were mutagenic for *E. coli* but no positive results were obtained with *S. typhimurium.* In repair tests using the *E. coli* strains *rec*$^+$, *recA13, and recB21*, and *S. typhimurium* TA 1978 (*uvr*$^+$) and TA 1538 (*uvrB*), 25 test compounds showed DNA-modifying effects on *E. coli* and 24 on *S. typhimurium.* Of the 15 known carcinogens in the series, 14 were mutagenic for *E. coli* and exerted a DNA-modifying effect in both organisms. Nitrofurazone showed positive results in both mutagenicity and repair tests with *E. coli*, but gave negative results with *S. typhimurium.* Positive results for both mutagenicity and DNA-modifying effects were obtained with three other derivatives previously reported to be non-carcinogenic. These were nitrofurantoin, 5-nitro-2-furamidoxime, and 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (furylfuramide; AF-2). The last compound has been used in Japan as a food additive and toxicity studies have been reported (*ibid* 1972, **10,** 717). Although it has not been shown to be carcinogenic, it has been found to be a potent mutagen in several test systems.

The main lesson to be derived from this paper is that tests of this kind designed to detect potential mutagens and carcinogens cannot be considered to yield useful results unless they utilize several different microbial test systems.

AGRICULTURAL CHEMICALS

2953. More mutations from DDT

Larsen, K. D. & Jalal, S. M. (1974). DDT induced chromosome mutations in mice—further testing. *Can. J. Genet. Cytol.* **16,** 491.

Chromosomal mutations have been produced in mice by the ip injection of DDT (*Cited in F.C.T.* 1975, **13,** 476) and a further study of this activity has now been reported. When mice were injected ip with 25, 50, 100 or 250 mg DDT/kg in peanut oil and killed 48 hr later, karyotypes from bone-marrow cells from the femur revealed a significant increase in deletions (clear breaks) at 50 and 100 ppm, and the increase in gaps (discontinuities) plus deletions was also significant when subjected to regression analysis. Gaps alone, stickiness and mitotic indices all showed increases at some levels, but these were not statistically significant. The authors speculate that because DDT residues of this order occur in many "secondary consumers" of DDT, such as predatory animals, filter feeders and soil organisms, the insecticide may have been responsible for increases in mutation rates in some species.

[It is dubious whether DDT concentrations given ip can be compared with those attained in body tissues after its continued ingestion. In short-term oral studies, it is noteworthy that dose levels of 100 mg/kg or more have been required to produce any direct indications of mutagenicity (Palmer *et al. Fd Cosmet. Toxicol.* 1973, **11,** 53; *Cited in F.C.T.* 1974, **12,** 272; *ibid* 1975. **13,** 476). Whether the liver and lung tumours reported in mice fed lower levels of DDT for prolonged periods (*ibid* 1974, **12,** 764) can be regarded as indicative of a genetic hazard is still open to question. However, studies of human occupational exposure have so far provided no evidence that DDT is carcinogenic in man, and the results of carcinogenicity studies in animal species other than the mouse have been dismissed as inconclusive (*IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Man: Some Organochlorine Pesticides*; Volume 5, p. 109; International Agency for Research on Cancer, Lyon, 1974, pp. 241).]

2954. Methanearsonate and the rabbit liver

Exon, J. H., Harr, J. R. & Claeys, R. R. (1974). The effects of long term feeding of monosodium acid methanearsenate (MSMA) to rabbits. *Nutr. Rep. Int.* **9,** 351.

Monosodium acid methanearsonate (MSMA) has been used as a silvicide in the precommercial thinning of forests. A study of its toxicity in cattle has shown that it produces severe diarrhoea, haemorrhagic gastritis, liver necrosis and renal tubular degeneration when fed at 10 mg/kg daily for 10 days (*Cited in F.C.T.* 1973, **11,** 895).

Adult rabbits fed 50 ppm MSMA in the feed for 2–52 wk developed toxic hepatitis after 7–12 wk. The daily doses which produced this effect were of the order of 1·5 mg As/kg. Reduction of the daily intake to 0·74 mg A/kg by restriction of food intake prevented the development of hepatitis, although at this dose level the As concentration in the liver tissues increased. The toxicity of MSMA to rabbits therefore appears to depend more upon the quantity ingested

during a given period than upon the tissue concentration of As. A dose-related initiation of *in vivo* conversion of the pentavalent arsenical to a more toxic metabolite may offer a partial explanation for this observation. A rapid increase in the As content of the liver and kidney during the first 2 wk of exposure was followed by a plateau effect until wk 17, when these levels again increased. This plateau may have been a result of the blocking of the mechanism for As accumulation in the liver by the early changes associated with hepatitis. A lack of As residues in the stomach contents and tissues of offspring of females fed 50 ppm MSMA for 12–24 wk indicated that MSMA metabolites did not cross the placenta or reach the milk.

2955. Anaemia from MCPA

Palva, H. L. A., Koivisto, O. & Palva, I. P. (1975). Aplastic anaemia after exposure to a weed killer, 2-methyl-4-chlorphenoxyacetic acid. *Acta haemat.* **53,** 105.

Rats fed for long periods on high levels of 2-methyl-4-chlorophenoxyacetic acid (MCPA) showed increases in relative liver and kidney weights and anaemia, the latter being ascribed in the absence of any detectable bone-marrow abnormalities simply to an increased rate of erythrocyte breakdown (Gurd *et al. Fd Cosmet. Toxicol.* 1965, **3,** 883). Acute effects associated with suicidal ingestion of MCPA and the related compound 2,4-D (2,4-dichlorophenoxyacetic acid) have included inflammation, ulceration and necrosis of the digestive tract, liver necrosis and renal tubular degeneration (Geldmacher-von Mallinckrodt & Lautenbach, *Arch. Tox.* 1966, **21,** 261).

The present report concerns a 64-yr-old farmer, who used a leaking container to spray weeds with an MCPA preparation. The container was borne on his back, and consequently his clothing became saturated with the chemical. After an interval of 2 wk, he developed skin haematomas and began to feel lethargic, and on admission to hospital 2 months later he was found to have marked capillary fragility and low blood levels of haemoglobin, platelets, reticulocytes and iron. In addition, his leucocyte count was above normal, with an unusually low percentage of neutrophils. However, bone-marrow morphology was normal. Serum levels of bilirubin and liver enzymes were unaffected, but results of the thymol turbidity test suggested some slight liver damage. Haemorrhagic gastritis was also found, but urine analysis provided no evidence of kidney damage. The anaemia increased during subsequent weeks, but drug treatment eventually led to complete recovery after several months.

2956. Organophosphates and neurobehavioural effects

Rodnitzky, R. L., Levin, H. S. & Mick, D. L. (1975). Occupational exposure to organophosphate pesticides. A neurobehavioral study. *Archs envir. Hlth* **30,** 98.

Occupational exposure to organophosphate pesticides may lead to impaired nerve and muscle function even in the absence of a depression in blood cholinesterase activity (*Cited in F.C.T.* 1972, **10,** 414). Neurological and behavioural abnormalities described in over-exposed workers have included ataxia, tremor, vertigo, convulsions, drowsiness, coma, anxiety, confusion, depression, impaired concentration, defective memory, impaired language function and inability to perform simple calculations. However, such effects have normally been readily apparent on clinical examination, and the present study was designed to determine whether similar abnormalities might exist in milder form in asymptomatic individuals.

Twelve farmers and 11 commercial pesticide sprayers who had used organophosphate pesticides regularly to within 2 wk of the study were compared with a comparable but unexposed control group in tests for memory (verbal recall), vigilance (release of push button in response to a test stimulus), signal processing time (release of specified push buttons in response to different light signals), linguistic competence (ability to repeat sentences of increasing length and speed) and proprioception (maintaining a constant amount of pressure on a spring-loaded button with the help of auditory but not visual feedback). No significant differences in responses were found between the two groups, despite the fact that those involved commercially in pesticide application (but not the farmers) had lower plasma-cholinesterase levels than the control subjects. However, these levels were still within normal limits, and no significant differences were found in the cholinesterase levels in the red blood cells.

The study does not rule out such possibilities as abnormalities of neuromuscular transmission in the peripheral nervous system, depressions in brain cholinesterase or other neurophysiological brain changes, all of which have been reported in asymptomatic workers or animals exposed to organophosphates, but it does suggest that such exposure is unlikely to result in widespread neurobehavioural dysfunction of practical importance.

2957. Ruelene not teratogenic for heifers

Rumsey, T. S., Samuelson, G., Bond, J. & Daniels, F. L. (1974). Teratogenicity to 35-day fetuses, excretion patterns and placental transfer in beef heifers administered 4-*tert*-butyl-2-chlorophenyl methyl methylphosphoramidate (Ruelene^{R}). *J. Anim. Sci.* **39,** 386.

Ruelene (4-*tert*-butyl-2-chlorophenyl methyl methylphosphoramidate) is a systemic organophosphorus insecticide which has been given to cattle for grub control. A study of its possible adverse effects on foetal development was carried out by injecting it into the amniotic sac of pregnant beef heifers, and by injecting Ruelene labelled with ^{14}C at the *tert*-butyl carbon atom into the jugular vein to determine maternal excretion and placental transfer. The excretion pattern of [^{14}C]Ruelene after oral administration was also studied.

There were no weight or size anomalies and no structural abnormalities in foetuses taken at day 180 of gestation from cows in which 250 mg Ruelene had been injected into the amniotic sac on day 35 or in others injected iv with a Ruelene dose of 19·4 mg/kg on the same day of gestation. In heifers injected iv with 100 μCi [^{14}C]Ruelene on day 180 of gestation, radioactivity appeared in the fatty tissues of both dam and foetus within 30 min, reaching a peak concentration in foetal liver and fatty tissue at 8 hr. Little activity reached the amniotic fluid after iv injection.

After the oral administration of 200 μCi [^{14}C]Ruelene, the highest activity appeared in blood taken at 8 hr, and 62% of the administered activity was excreted in the urine within 24 hr. After 3 days, the latter figure had risen to 74% and a further 9·8% had appeared in the faeces, while a small quantity (0·12%) had been identified in the expired air.

Thus it may be concluded that Ruelene and/or its metabolites are able to cross the placental barrier. Nevertheless, the rapidity of maternal metabolism and excretion of this insecticide appears to offer the foetus a fair degree of protection from exposure to toxic concentrations.

2958. Toxaphene metabolism

Ohsawa, T., Knox, J. R., Khalifa, S. & Casida, J. E. (1975). Metabolic dechlorination of toxaphene in rats. *J. agric. Fd Chem.* **23,** 98.

Toxaphene is a complex mixture of at least 177 related C_{10} polychloro compounds, only one of which (2,2,5-*endo*-6-*exo*-8,9,10-heptachlorobornane) has been identified. Toxicological investigations (*Cited in F.C.T.* 1974, **12,** 570) have thrown little light on the metabolic fate of this insecticide, though toxaphene components appear to be less persistent in mammals than many other chlorinated insecticides since many of the constituents undergo extensive metabolic dechlorination. A preliminary picture of the variations in distribution, metabolism and persistence among toxaphene components was obtained in the present study, which involved administration of radiolabelled toxaphene fractions to rats.

Rats were treated orally either with [^{36}Cl]toxaphene, or with one of seven [^{36}Cl]toxaphene fractions of equal chlorine content, these fractions being complex mixtures of at least 26–47 compounds. Analysis of the isotope distribution in various solvent extracts revealed that approximately 50–60% of the ^{36}Cl was excreted in the urine and 30–40% in the faeces, in all cases within 14 days. For each fraction examined, about half of the carbon–chlorine bonds were cleaved, the excreted Cl^- being determined as phenylmercuric chloride. Comparable studies with ^{14}C-labelled preparations of toxaphene and either or both of two components known to display high toxicity in mammalian systems (designated toxicants A and B) established that the faeces contained a small proportion of the dose in the unchanged state and that the metabolites (in urine or faeces) probably included acidic materials, partial or complete dechlorination products and $^{14}CO_2$.

Analysis of the tissue distribution of ^{14}C in treated rats revealed very low levels (< 1 ppm) of toxaphene and toxicant B or their metabolites 14 days after administration of a [^{14}C]toxaphene dose of 8·5 mg/kg, indicating that these materials were effectively non-persistent. Data on tissue levels of [^{36}Cl]toxaphene and its fractions were not meaningful in view of the low specific activity of the administered material.

The authors point out that, although there are large toxicity differences between the [^{36}Cl]toxaphene fractions, the facility for metabolic dechlorination is probably independent of the structural features that confer high toxicity. The components of highest toxicity are considered to be those that combine appropriate configurations for the disruption of nerve activity with some degree of resistence to metabolism. Further studies are required to define the chemical structures of residues resulting from administration of toxaphene and its components, using the suggested gas chromatography–chemical ionization–mass spectroscopy technique.

FEED ADDITIVES

2959. An organoarsenical hazard in turkey rearing

Wise, D. R., Hartley, W. J. & Fowler, N. G. (1974). The pathology of 3-nitro-4-hydroxyphenylarsonic acid toxicity in turkeys. *Res. vet. Sci.* **16,** 336.

In spite of the suggestion that roxarsone (3-nitro-4-hydroxyphenylarsonic acid) and other organoarsenicals are suitable supplements for improving weight gain in turkeys, the feeding of turkey poults with roxarsone at 0·01–0·04% of the diet has led to a decrease in body weight and an increase in mortality (*Cited in F.C.T.* 1973, **11,** 896). The paper cited above reports in the same vein, and presents evidence that there is only a narrow margin between the alleged growth-promoting levels and the toxic concentrations of this compound in turkey feeds.

An outbreak of leg weakness among turkeys aged 5–16 wk resulted in unwillingness to walk, an unsteady gait and sometimes increasing disability to a point at which the bird could progress only by using its wings. Certain mildly ataxic birds became worse, and trembled markedly when handled. More than half grew crooked and incurled toes, a condition which failed to improve when vitamins of the B group were administered. It was eventually discovered that twice the recommended concentration of roxarsone (100 instead of 50 ppm) had been included in their feed. It was established experimentally that the growth of turkey poults was depressed by feeds containing

50–400 ppm roxarsone and all birds fed 200 or 400 ppm died within 15 days. Those fed 100 ppm showed a high mortality by wk 5. In all groups except those fed 50 ppm or the control diet, neurological disturbances developed. Histopathological examination showed no lesions in the brain or spinal cord, but slight to marked degeneration of peripheral nerve fibres, more marked distally than proximally, was seen in all the birds that had developed mild to severe neurological signs.

PROCESSING AND PACKAGING CONTAMINANTS

2960. Phthalate esters in human tissues

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

Roll, D. B., Douglas, J. D. & Petersen, R. V. (1974). GLC analysis of bis(2-ethylhexyl) phthalate plasticizer in tissue and plasma. *J. pharm. Sci.* **63,** 1628.

Di-(2-ethylhexyl) phthalate (DEHP) is used as a plasticizer in concentrations up to 40% in vinyl-based medical equipment. It has been detected in the tissues of patients who have received transfusions of blood stored in PVC packs (*Cited in F.C.T.* 1971, **9,** 910; *ibid* 1973, **11,** 914), and experiments have suggested that injected DEHP has a significant retention time in the tissues (*ibid* 1975, **13,** 146).

The first paper cited above describes the use of gas chromatography and mass spectrometry to determine DEHP residues in heart and gastro-intestinal tissues taken from infants who died shortly after umbilical catheterization or administration of a blood product. The lower limit of detection of DEHP attributed to the method used was 0·02 μg/g tissue. Control tissues from ten infants and three older subjects not exposed to plasticized materials showed a mean DEHP content of less than 0·07 μg/g, or 0·04 μg/g when samples from one stillborn infant giving unaccountably high results were excluded. In 17 infants subjected to intravascular catheterization with or without administration of blood from PVC bags, the heart tissue residue after expression yielded a mean of 1·27 μg DEHP/g, and the expressed heart extract a mean of 0·66 μg/g. A history of particularly extensive use of catheters and blood, and of early death, was associated with higher tissue levels of DEHP. DEHP residues (up to 0·63 μg/g) in the gastro-intestinal tissue of catheterized infants who developed necrotizing enterocolitis were significantly higher than those in the same tissue from infants who did not develop the disease, indicating that DEHP accumulates in the affected tissues of critically ill infants.

The second paper describes GLC analysis of DEHP residues in tissues and plasma, using di-(*n*-octyl) phthalate as an internal standard. The method was considered satisfactory for tissues of low lipid content (liver, lung and spleen), but when applied to lipid-rich tissues, such as brain or fat, it was subject to interference from substances with retention times similar to those of DEHP or the standard. The lower limit of detection of DEHP in lung, liver, spleen and plasma was stated to be about 10 μg/g but the presence of phthalates in solvents and equipment was claimed to make it difficult to deal with samples containing less than 50 μg/g.

THE CHEMICAL ENVIRONMENT

2961. Sodium chromate nephrotoxicity

Evan, A. P. & Dail, W. G., Jr. (1974). The effects of sodium chromate on the proximal tubules of the rat kidney. Fine structural damage and lysozymuria. *Lab. Invest.* **30,** 704.

Although renal toxicity has not been a major concern in experimental animals fed low levels of chromates for prolonged periods, renal failure has occasionally featured among the effects of acute chromium poisoning in animals and man. After acute dosage of rats with chromate, light-microscopic studies have demonstrated a selective necrosis of the proximal tubule (Biber *et al. Am. J. Med. 1968*, **44,** 664). In the study cited above, light- and electron-microscopic examination of kidney tissue from rats treated with chromate was designed to throw further light on the nephrotoxic action of heavy metals.

Wistar rats, injected ip with a single dose of 10 or 20 mg sodium chromate/kg body weight in aqueous solution (20 mg/ml) were killed 1, 6, 24 or 48 hr later. The major renal change observed after chromate treatment was in the convoluted portion of the proximal tubule. Under the light microscope, an increase in the number of apical vacuoles was visible 6 hr after the higher dose and 24 hr after the lower dose of sodium chromate. Within 1 or 6 hr of treatment, according to dose, electron microscopy revealed swelling of the microvilli of the convoluted portion. Disappearance of some microvilli was accompanied by abnormal vacuolation of degenerating cells 6 and 24 hr after administration of the upper and lower doses, respectively. After 24 and 48 hr, respectively, swelling of the mitochondria was apparent and this was followed by their degeneration, which was marked by virtually complete loss of matrical material, rupture of the outer membrane and an apparent loss of cristae. Liquefaction of the cytoplasmic ground material accompanied the mitochondrial swelling, and desquamation indicated the final stage of cell death.

There was no evidence of any structural changes in the glomerulus. The amount of urinary lysozyme increased as damage to the proximal tubule became more marked, suggesting that this enzyme may provide a good indication of severe proximal tubular damage.

The authors suggest that the known ability of heavy metals to augment pinocytosis and to change the selective permeability of tubular-cell membranes (to allow movement of sodium and water into the cell) may explain the vacuole formation. They also speculate that since microvilli are rich in adenosine triphosphatase and chromate damages or eliminates these structures, the expected accumulation of adenosine triphosphate could well trigger the observed transformation of mitochondrial configuration. Lysozyme in the urine is considered to indicate a change in the reabsorptive capacity of the cells of the renal tubule.

2962. The perils of inhaled cobalt

Kerfoot, E. J., Fredrick, W. G. & Domeier, E. (1975). Cobalt metal inhalation studies on miniature swine. *Am. ind. Hyg. Ass. J.* **36,** 17.

Tungsten carbide workers may suffer from asthma and related allergic symptoms, and sometimes also from a disabling and progressive interstitial fibrosis of the lungs (*Cited in F.C.T.* 1972, **10,** 269; Coates & Watson, *Ann. intern. Med.* 1971, **75,** 709). Animal experiments have suggested that the offending agent in the tungsten carbide is cobalt, which is used as a binder during the fabrication of the carbide, and the present study was undertaken to verify this hypothesis and to assess the validity of the present threshold limit value (TLV) for cobalt of 0·1 mg/m^3.

Miniature pigs were exposed to an atmosphere containing pure cobalt-metal powder in concentrations of 0·1 or 1·0 mg/m^3 for 6 hr daily on 5 days/wk for 3 months. Because pulmonary reactions to cobalt are thought to be mediated through a hypersensitivity mechanism, the pigs were also exposed to cobalt at the test concentration for 1 wk, followed by a 10-day rest period, before the start of the main experiment. The particle size ranged from 0·4 to 3·6 μm, the optimum range for lung penetration and retention. The animals gained weight normally, but lethargy and wheezing began to develop after 4 wk, and after 3 months both groups showed decreases in pulmonary compliance (a measure of lung elasticity) and tidal volume, effects which were reversible, however, on cessation of exposure. In addition, there were increases at both exposure levels in the total serum proteins and α-globulins and a decrease was observed in the albumin/globulin ratio. These changes are thought to be early manifestations of lung damage. Other findings indicative of cobalt poisoning were a transient increase in red and white blood cells during wk 3 of the high-level exposure and electrocardiographic (ECG) findings (decreases in QRS and T-wave amplitudes), which were suggestive of cardiomyopathy. Surprisingly, however, the ECG changes were more marked in the group on the lower exposure. The amount of cobalt appearing in the urine increased from a control level of 18 μg/litre to 29 μg/litre in the low-cobalt group and to as much as 220 μg/litre in the high-cobalt group.

When X-rayed, the lungs appeared normal, and no pathological changes were detected in any organ by light microscopy. However, electron microscopy of the lungs exposed to the 1·0 mg/m^3 level revealed a thin and attenuated epithelium and septa markedly thickened by masses of collagen, elastic tissue and fibroblasts. Similar but less severe damage was apparent at the 0·1 mg/m^3 level. These findings were similar to those reported in workers exposed to tungsten carbide, confirming the aetiological role of cobalt in this connexion. The study also suggested that the TLV for cobalt is too high, and further work is required to define a lower acceptable level.

2963. Methylation of lead in the environment

Wong, P. T. S., Chau, Y. K. & Luxon, P. L. (1975). Methylation of lead in the environment. *Nature, Lond.* **253,** 263.

Jarvie, A. W. P., Markall, R. N. & Potter, H. R. (1975). Chemical alkylation of lead. *Nature, Lond.* **255,** 217.

Inorganic mercury can be methylated by micro-organisms common in water and soil (*Cited in F.C.T.* 1974, **12,** 581) and arsenic can undergo a similar process of conversion (Wood, *Science, N.Y.* 1974, **183,** 1049). That lead, too, falls into this category has now been demonstrated.

When sediment and water from three Canadian lakes were incubated in an anaerobic nutrient broth with added glucose for 2 wk at 20°C, the presence of tetramethyllead (Me_4Pb) in the gas phase was identified and confirmed by gas chromatography and mass spectrometry (Wong *et al.* cited above). The yield of Me_4Pb was greatly increased by addition of trimethyllead (Me_3Pb^+) as the acetate and in some cases also by addition of inorganic lead as the nitrate or chloride, but lead hydroxide, cyanide, oxide, bromide or palmitate had no such effect. None of the inorganic lead salts yielded detectable levels of the probable intermediate, Me_3Pb^+. This was the case even with those that were transformed to Me_4Pb, and the mechanism of their methylation thus remained obscure.

In the case of Me_3Pb^+, conversion to Me_4Pb was not effected by ultraviolet light in the absence of micro-organisms, ruling out the possibility of light-activated chemical disproportionation reactions, and it was prevented by autoclaving. However, bacterial isolates from Lake Ontario were able to convert Me_3Pb^+ to Me_4Pb even in the absence of sediment, although no Me_4Pb was produced by these isolates from inorganic lead in chemically-defined media. The yield of Me_4Pb from Me_3Pb^+ rose to a maximum of 6% in wk 2 and declined thereafter, a phenomenon attributed to the exhaustion of nutrients, accumulation of toxic metabolic products or pH changes. Me_4Pb was found to be about twice as toxic to algae as Me_3Pb^+, and twenty times as toxic as lead nitrate.

In the second study cited above, the probable mechanism of the conversion of Me_3Pb^+ to Me_4Pb was elucidated further. Following incubation of 15 mg Me_3Pb^+, as the acetate or chloride, with an anaerobic lake sediment in a nutrient broth, the concentration of Me_4Pb above the solution built up gradually to 6 μg/ml after 2 wk. However, this concentration was attained in only 2 days when Me_3Pb^+ was incubated with sodium sulphide in distilled water, although no Me_4Pb was produced from Me_3Pb^+ in distilled water alone. Smaller amounts of tetraethyllead resulted from incubating triethyllead chloride with lake sediment or with sulphide, the latter again producing a more rapid yield, but no methyltriethyllead could be detected. Methylation by one of the major routes described for mercury (Bertilsson & Neujahr, *Biochemistry, N.Y.* 1971, **10,** 2805) was ruled out experimentally for Me_3Pb^+ (as the acetate or chloride), for dimethyllead dichloride and for lead nitrate. The findings suggest that trialkyllead salts in the environment may react with hydrogen sulphide, produced by many organisms under anaerobic conditions, and the sulphides thus formed may then decompose to yield tetraalkyllead. Autoclaving, which in the first study prevented Me_4Pb formation, would have converted any hydrogen sulphide present to insoluble inorganic sulphides and thus rendered it unavailable for further reaction.

2964. Showing up the mercury cycle

Edwards, T. & McBride, B. C. (1975). Biosynthesis and degradation of methylmercury in human faeces. *Nature, Lond.* **253,** 462.

The addition of ^{203}Hg-labelled mercuric chloride ($^{203}HgCl_2$) to human faeces followed by incubation under anaerobic conditions produced significant amounts of methylmercury (CH_3Hg), which was identified by benzene extraction and thin-layer chromatography. With the maximum concentration tested (7×10^{-6} M-$HgCl_2$), a peak concentration of nearly 5 ng CH_3Hg/g appeared after 2 days, rapidly declining thereafter. Incubation of $^{14}CH_3Hg$ with faeces showed that this compound disappeared at a constant rate over 7 days without the production of $^{14}CH_4$, so that reductive demethylation was not the mechanism of removal.

These results indicate that the intestinal flora of man can transform inorganic to organic mercury and *vice versa*, and the authors suggest that the transformation may contribute substantially to the CH_3Hg burden of the body and may thus add to the risk of alkylmercury poisoning or increase its severity.

[Other workers have reported that the methylation of mercury by *Escherichia coli* and *Aerobacter aerogenes* occurs less readily in anaerobic than in aerobic cultures, since the precipitation of sulphide in the former case may remove part of the available mercury (*Cited in F.C.T.* 1974, **12,** 581). The reversible nature of the transformation and the possibility that other organisms of the human gut are involved indicate the need for further work on this important topic.]

2965. Carbon monoxide and the brain

Ginsberg, M. D. & Myers, R. E. (1974). Experimental carbon monoxide encephalopathy in the primate. I. Physiologic and metabolic aspects. *Archs Neurol., Chicago* **30,** 202.

Ginsberg, M. D., Myers, R. E. & McDonagh, B. F. (1974). Experimental carbon monoxide encephalopathy in the primate. II. Clinical aspects, neuropathology, and physiologic correlation. *Archs Neurol., Chicago* **30,** 209.

Anaesthetized rhesus monkeys breathing 0·1–0·3% carbon monoxide (CO) were studied in the first experiment described in these papers. Spontaneous inhalation of 0·1% CO for 325 min produced stable plateau levels of 78% carboxyhaemoglobin (COHb) within 90–120 min, while mechanical ventilation with 0·1% CO for 150 min produced a level of 62% COHb within a similar period. In both groups, modest reductions in peripheral blood pressure occurred, central venous pressure remained unchanged, arterial blood pH remained above 7·28 and the ECG pattern did not change. Occasional slow waves developed in the frontal EEG. Spontaneous inhalation of 0·2% CO for 100–215 min produced a rapid rise in blood COHb to a plateau value of 76%, declining to 28% within 90 min of withdrawal. Ventilator-administration of 0·3% CO for 85–165 min produced blood COHb concentrations of 81%, falling to 36% within 90 min of withdrawal, while spontaneous inhalation of 0·3% CO produced 84% COHb after 45 min. Arterial hypotension and metabolic acidosis were variable in degree but were consistently recorded.

Premature ventricular contractions appeared within 45–75 min of exposure to 0·2 or 0·3% CO and tended to increase in frequency. Serious ventricular arrhythmias were usually but not always prevented by injection of lignocaine (lidocaine). One of nine monkeys died with progressive hypotension, bradycardia and respiratory depression after exposure to 0·2% CO for 164 min. Two of six died of ventricular fibrillation after breathing 0·3% CO for 60 and 85 min respectively, in the absence of lignocaine treatment. Another died after exposure for 150 min, having received an unusually large cumulative dose of lignocaine.

The second paper describes the examination of the 14 survivors 14 days–5 months after exposure to these levels of CO for periods of 75–325 min. Three showed mild and three severe neurological abnormalities following exposure to 0·2% CO (or 0·3% in one case). The severe sequelae included limb paralysis, altered muscle tone, blindness and deafness. The monkeys mildly affected exhibited motor disturbances. The brain surface from these animals had a normal appearance, but coronal sections revealed bilateral white-matter lesions, which were most extensive in the frontal and posterior parietal regions. Two monkeys had lesions affecting the globus pallidus and hippocampus. The extent of the white-matter lesions correlated with the degree of metabolic acidosis and systolic hypotension observed during CO exposure. Hypoxia alone did not appear to be a determining factor in the brain damage, since no grey-matter

lesions were observed. The two monkeys that had been exposed to only 0·1% CO showed no brain lesions whatever.

2966. Acrylonitrile metabolism

Gut, I., Nerudová, J., Kopecký, J. & Holeček, V. (1975). Acrylonitrile biotransformation in rats, mice, and Chinese hamsters as influenced by the route of administration and by phenobarbital, SKF 525-A, cysteine, dimercaprol, or thiosulfate. *Arch. Tox.* **33**, 151.

Acrylonitrile is metabolized in animals to cyanide, which is converted to thiocyanate prior to its excretion in the urine, but it is doubtful whether this route of decomposition is of sufficient importance to account for the toxic effects of acrylonitrile on nerve tissues (*Cited in F.C.T.* 1963, **1**, 299). The evidence available suggests rather that the toxicity of acrylonitrile is related to its ability to react with tissue sulphydryls involved in pyruvate metabolism (*ibid* 1973, **11**, 154). In view of indications of possible species differences in acrylonitrile metabolism, the authors cited above studied, in rats, mice and hamsters, the fate of acrylonitrile (26·5 or 40 mg/kg) administered by different routes, the urinary elimination of thiocyanate being monitored under various conditions.

Over 20% of a single dose of acrylonitrile given orally to rats was excreted in the urine as thiocyanate within 48 hr, but this proportion was reduced to 2–5% after ip or sc administration and to less than 1% after iv injection. A similar pattern of excretion following dosage by the different routes was seen in the mouse and hamster, except that there was little difference in the mouse between the results after ip and iv injection. For each route of administration, however, the proportion of the dose converted to thiocyanate was greater in the mouse and hamster than in the rat. In addition, elimination of thiocyanate after oral dosing with acrylonitrile began more rapidly in the mouse and hamster than in the rat.

The amount of thiocyanate excreted was not significantly affected by pretreatment with a microsomal-enzyme inducer (phenobarbitone) or with a potent inhibitor of drug metabolism (SKF 525-A), a finding which supports the view that in a given species the effect of the route of administration reflected the distribution of acrylonitrile rather than the rate of its metabolism in the liver. In experiments with potential antidotes to acrylonitrile toxicity, no decrease in thiocyanate excretion resulted from pretreatment with cysteine or dimercaptopropanol, both of which contain thiol groups, but simultaneous administration of thiosulphate significantly increased the proportion of acrylonitrile metabolized after ip injection into rats. This effect was even more marked in mice and indicates that under normal conditions the cyanide formed is not effectively metabolized to thiocyanate. It also suggests, in conjunction with the relative acute toxicities of acrylonitrile in rats and mice, that while cyanide-mediated toxicity is of doubtful importance in rats, it is probably a more effective factor in mice.

2967. Sub-mammalian mutagenicity of 2-haloethanols and haloalkanes

Rosenkranz, S., Carr, H. S. & Rosenkranz, H. S. (1974). 2-Haloethanols: Mutagenicity and reactivity with DNA. *Mutation Res.* **26**, 367.

Brem, H., Stein, A. B. & Rosenkranz, H. S. (1974). The mutagenicity and DNA-modifying effect of haloalkanes. *Cancer Res.* **34**, 2576.

The discovery of the formation of ethylene chlorohydrin (2-chloroethanol) in foods sterilized with ethylene oxide stimulated interest in the toxicology of this group of compounds. The chlorohydrins and various haloalkanes widely used in industry and agriculture have been screened for possible effects on genetic material by a number of techniques. These tests are still at the developmental stage and hence must be interpreted with caution, but results obtained include demonstrations of the mutagenicity of iodo-, bromo- and chloroethanols in *Klebsiella pneumoniae* (Voogdt, *Mutation Res.* 1973, **21**, 52). This area of study has now been extended to include the activity of these compounds in other bacteria (first paper, cited above) and to investigate the mutagenic activity of a series of haloalkanes (second paper, cited above).

The assay methods used for all of these studies involved applying the test compound to agar plates carrying the test organism, either by incorporating the compound in the agar overlay or by depositing it on to sterile filter discs placed on the surface of the agar. The organisms used were *Escherichia coli* pol A^+ and its DNA polymerase-deficient derivative *E. coli* pol A_1^- and three strains of *Salmonella typhimurium*. After incubation of the *E. coli* test plates at 37°C for 8 hr, measurement of the inhibition zones revealed that the 2-haloethanols as well as 1,1- and 1,2-dibromoethane, 1,2-dichloroethane, 1-bromo-2-chloroethane, 1,5-dibromopentane, 1,2-dibromo-2-methylpropane, 1,1,2,2-tetrabromoethane and 1,1,2,2-tetrachloroethane all inhibited preferentially the growth of the pol A_1^- strain. The activity of the 2-haloethanols decreased in the order bromo- > iodo- > chloroethanol. These three compounds were also mutagenic for *S. typhimurium* TA 1530 and TA 1535 but not for the TA 1538 strain, an indication that they induced base-substitution but not frameshift mutations. The same was true for the series of haloalkanes, with the exception of 1,1,2,2-tetrabromoethane, which did not induce mutations in any of the three strains. Exposure of calf-thymus DNA to ethylene halohydrins increased its buoyant density, suggesting a direct reaction of the compounds with a DNA component and providing a possible chemical basis for the effects on bacterial growth.

[The relative mutagenic activities of the 2-haloethanols in terms of their ability to inhibit the growth of *Klebsiella pneumoniae*, *Salmonella typhimurium* and *Escherichia coli* Pol A_1^- showed little consistency. This reinforces the argument that data from sub-mammalian test systems are of greatest value in setting priorities for more definitive testing.]

2968. Another index of foreign compound metabolism

Notten, W. R. F. & Henderson, P.Th. (1975). The influence of *n*-hexane treatment on the glucuronic acid pathway and activity of some drug-metabolizing enzymes in guinea-pig. *Biochem. Pharmac.* **24,** 127.

Stimulation of the activity of microsomal-enzyme systems in the liver by foreign compounds is often accompanied by synthesis of D-glucaric acid, with intermediate formation of D-glucuronic acid in the liver cell. It follows that enhanced urinary D-glucaric acid excretion may be a useful indicator of adaptive acceleration of hepatic xenobiotic metabolism, which in turn reflects the intake of foreign compounds. To investigate these concepts, the authors cited above examined the effect of *n*-hexane on drug metabolism and the glucuronic acid system in the liver. Prolonged inhalation exposure to high concentrations of this solvent can cause muscle weakness and sensory loss in man (*Cited in F.C.T.* 1973, **11,** 157), although there is no evidence that any metabolic steps contribute to this toxic effect.

Intraperitoneal injection of guinea-pigs with *n*-hexane (in sesame oil) at a rate of 3 or 60 mg/kg/day for 8 days enhanced the *in vitro* glucuronyl conjugation of *p*-nitrophenol, an induction which was accompanied by an enhanced microsomal-protein content. In contrast, *in vitro* rates of aniline *p*-hydroxylation and aminopyrine *N*-demethylation were, respectively, slightly decreased and unchanged after *n*-hexane treatment. Treatment with 60 mg *n*-hexane/kg/day increased urinary excretion of D-glucuronic acid within 24 hr of the first dose and of D-glucaric acid after about 5 days. With the lower dose of *n*-hexane, given for 35 days, the time lag for increased D-glucaric acid excretion was about 21 days. These changes were paralleled by induction of the microsomal enzyme, UDPglucuronosyltransferase.

The authors conclude that *n*-hexane may cause a direct stimulation of glucuronide formation and that, during subacute or more prolonged administration, the hydrocarbon may be considered as a specific inducer of the glucuronidation system. It has not yet been established, however, at which step in the sequence of enzymic reactions the stimulation is initiated.

2969. Industrial exposure to sub-threshold levels of methylchloroform

Seki, Y., Urashima, Y., Aikawa, H., Matsumura, H., Ichikawa, Y., Hiratsuka, F., Yoshioka, Y., Shimbo, S. & Ikeda, M. (1975). Trichloro-compounds in the urine of humans exposed to methyl chloroform at sub-threshold levels. *Int. Arch. Arbeitsmed.* **34,** 39.

Although methylchloroform (1,1,1-trichloroethane; MC) is known to have some neurological effects in man, limited exposure to concentrations up to 500 ppm has been found to have no particularly adverse effect on psychophysiological functions (*Cited in F.C.T.* 1972, **10,** 273). In fact, MC has generally been regarded as a relatively safe member of the group of chlorinated solvents of importance in industrial and household applications. More recent studies have indicated that acute exposure to high levels of this solvent may affect cardiovascular function (*ibid* 1975, **13,** 675) and have raised the question of whether MC accumulating in the tissues as a result of repeated exposure to lower concentrations might have similar effects. The paper cited above is concerned with the fate of MC in man following prolonged low-level exposure under industrial conditions.

The study was carried out in Japan on 196 male workers, aged between 23 and 53 years, employed in four similar printing plants owned by one company. The men had been exposed to MC at average concentrations of 4, 25, 28 or 53 ppm for 8 hr/day, 5·5 days/wk for at least 5 yr. Routine medical examinations, tests for sense of vibration and clinical tests, which included peripheral haemograms, determination of the specific gravity of the blood and urine analyses for urobilinogen and protein, were carried out. No dose-related adverse effects attributable to MC were found in any of the workers. The urine content of total trichloro compounds (TTC) was found to increase linearly with the level of MC exposure.

Individual urine samples were collected from seven workers during the period between termination of exposure in the afternoon and resumption of work the next morning. From the decrease in the urinary TTC, the biological half-life of MC was estimated to be 8·7 hr.

In a worker exposed to concentrations of 100–2400 ppm MC on Monday–Friday and a lower concentration (20–33% of the weekday values) on Saturday, a steady increase in the level of urinary metabolites was noted towards the end of the week. This increase was attributable primarily to trichloroethanol, the level of trichloroacetic acid showing no significant increase. Significant amounts of metabolite continued to be excreted for over 24 hr after exposure. The increased level of metabolites excreted towards the end of the week, together with the estimated biological half-life for MC of 8·7 hr, is in line with previous indications of storage of MC in the tissues after repeated exposure to the vapour.

The authors consider that the MC data currently available support the German threshold limit value of 200 ppm as an acceptable, if tentative, limit for industrial exposure to the solvent vapour.

[The current threshold limit value for methylchloroform in the UK and USA is 350 ppm.]

2970. Persistence of nitrosamines in the environment

Tate, R. L., III & Alexander, M. (1975). Stability of nitrosamines in samples of lake water, soil, and sewage. *J. natn. Cancer Inst.* **54,** 327.

Previous studies on nitrosamines have concentrated on their presence and formation in foods or their metabolism and toxicity in man and laboratory animals. The authors of the above paper, however, have investigated the equally important topic of the stability of nitrosamines in samples of natural ecosystems.

Dimethyl-, diethyl- and di-*n*-propylnitrosamines were incubated at 30°C with Williamson silt loam

(pH 6·8), littoral water (pH 8·2) from Cayuga Lake, New York, or raw municipal sewage at pH 6·0 or 7·2. Nitrosamine concentrations were estimated colorimetrically and by gas-liquid chromatography.

When incubated with soil, the nitrosamines (initial concentrations 13–22 ppm nitroso-nitrogen) were not metabolized for several days, but subsequently the nitroso-nitrogen slowly disappeared, so that after 70 days (when the rate of breakdown had slowed almost to zero) only about 50% of the added nitrosamine remained. When nitrosamines were incubated with samples of lake water, no loss or breakdown occurred over 3 months. However, slow degradation of nitrosamines did occur in sewage, the average weekly loss over a 42-day period being 8·9 ppm at pH 7·2. Sterilization of the sewage by autoclaving had little effect on the rate of breakdown at pH 6·0 and only slightly reduced the rate at pH 7·2, suggesting only a minor involvement of micro-organisms. Attempts to select for bacteria capable of metabolizing nitrosamines proved unsuccessful.

The results suggest, therefore, that nitrosamines are likely to be relatively persistent in the environment, especially in lakes and rivers.

2971. Succinonitrile metabolism in the mouse

Curry, S. H. (1975). Cumulative excretion of succinonitrile in mice. *Biochem. Pharmac.* **24,** 351.

The excretion kinetics of succinonitrile and its metabolites have been studied in mice given single or multiple ip injections of the ^{14}C-labelled compound in doses of 25 mg/kg. Urine and faeces were collected at intervals up to 72 hr after the single dose and 24-hr urines were collected after administration of the radioactive doses in the multiple-dose studies.

After injection of a single dose of [^{14}C]succinonitrile, nearly 80% of the radioactivity appeared in the urine within 72 hr and 6·5% was found in the faeces within 24 hr. Excretion was rapid initially, with 60% of the dose appearing in urine and faeces in the first 24 hr, but later it was relatively slow. The proportion of radioactivity excreted as unmetabolized succinonitrile was high in the first 6 hr and maximal between 0 and 2 hr. Excretion of cyanide (as thiocyanate) and of unidentified material soluble in amyl alcohol fluctuated somewhat but was high between 2 and 48 hr, after which excretion of other water-soluble metabolites was most prominent.

After dosing of the rats with three daily doses of unlabelled succinonitrile followed by a final dose of labelled material, the activity excreted in the urine within 24 hr (52%) was similar to that after a single succinonitrile dose (56%). In a further study, in which all four daily doses were labelled, about 50% of the total body content of radioactivity was excreted in each 24-hr period, indicating some accumulation of metabolites rather than of succinonitrile, since the proportion of the latter fell steadily as dosing progressed. Mild accumulation of cyanide occurred, but there was no evidence of any enzyme induction. One probable metabolite was cyanoacetic acid, but neither this suggestion nor the identity of any other metabolites has yet been confirmed.

2972. The burning question of plastics again

Blandford, T. B., Seamon, P. J., Hughes, R., Pattison, M. & Wilderspin, M. P. (1975). A case of polytetrafluoroethylene poisoning in cockatiels accompanied by polymer fume fever in the owner. *Vet. Rec.* **96,** 175.

Petajan, J. H., Voorhees, K. J., Packham, S. C., Baldwin, R. C., Einhorn, I. N., Grunnet, M. L., Dinger, B. G. & Birky, M. M. (1975). Extreme toxicity from combustion products of a fire-retarded polyurethane foam. *Science, N.Y.* **187,** 742.

For some years, the high temperatures generated in polytetrafluoroethylene (PTFE) non-stick linings of domestic utensils when they boil dry have been known to release fever-inducing toxic pyrolysis products (*Cited in F.C.T.* 1969, **7,** 368). The temperature at which pyrolysis occurs determines the toxicity for different animal species, birds being particularly susceptible (*ibid* 1974, **12,** 794).

The first paper cited above describes the curious case of five cockatiels (*Nymphicus hollandicus*) and their owner. The birds were kept in a lounge adjoining the kitchen where a PTFE milk-pan containing water boiled dry over an electric stove, and they died within 30 min of exposure to the fumes. The owner and his wife continued to sit in the lounge, but within a further 30 min the man became breathless, and 20 min later he was shivering, dizzy and nauseated and complaining of tightness of the chest. After a sleep of 8 hr he woke with a headache, which disappeared during the morning, and a tightness of the chest which lasted for the rest of the day. Apart from a possible memory defect regarding the accident, he recovered fully. His wife was unaffected throughout. No leaks of natural gas or build-up of carbon monoxide on the day of the occurrence could be traced. Post-mortem examination of the cockatiels showed severe lung congestion and oedema, with serous and fibrinous exudation into the tertiary bronchioles, and intrabronchiolar oozing of blood into the lung parenchyma. Their livers showed severe congestion of the sinusoids and portal veins. The incident confirms the high susceptibility of parakeets to PTFE pyrolysis products.

In the face of current disquiet concerning the toxic substances produced by pyrolysis of polyurethane foams, the second paper cited above indicates that the main toxic hazard stems from a fire-retarding agent and not from the material itself. Rats were exposed for 20 min to smoke from the non-flaming combustion of Douglas fir or from heated samples of rigid polyurethane foam which was either treated with the fire-retardant, *O,O*-diethyl-*N,N*-bis-(2-hydroxyethyl)aminomethyl phosphonate, or was untreated. Inhalation of wood smoke caused severe respiratory distress and pulmonary oedema indicative of carbon monoxide intoxication. Inhalation of fumes from untreated polyurethane foam also showed reactions characteristic of mild to moderate elevation of carboxyhaemoglobin (28–33%), without respiratory distress or post-mortem evidence of respiratory-tract irritation. Fumes from the fire-retarded foam induced repeated seizures, however, leading to major motor seizures (status epilepticus) within about 1 hr. Behav-

ioural responses were severely impaired. Lid-cornea, ear-flick and righting reflexes were preserved, although the latter was somewhat impaired, but response to painful stimuli was grossly diminished. The dead animals showed serosanguinous froth in the trachea, bronchi and lungs, and mottled haemorrhages of the lung surface, but no changes appeared in other organs. Chemical analysis showed the presence of 4-ethyl-1-phospha-2,6,7-trioxabicyclo[2,2,2]octane-1-oxide in the smoke from the fire-retarded polyurethane. The development of seizures was attributed to this component.

NATURAL PRODUCTS

2973. Garlic to the rescue!

Jain, R. C. (1975). Onion and garlic in experimental atherosclerosis. *Lancet* **I,** 1240.

Claims have been made that both onion and garlic possess hypoglycaemic and hypolipaemic properties and may have an inhibitory effect on the development of atherosclerosis. An active proponent of this theory has now published the results of a study in rabbits, which provides some support for the potential benefit of garlic in this connexion.

Four groups of rabbits were fed a stock diet. Three of these groups were also given 0·5 g cholesterol in 5 ml olive oil, presumably daily, for 16 wk and over the same period one of the cholesterol-treated groups was given 10 ml of juice from raw onion bulbs and another 10 ml of garlic juice, both in distilled water. The cholesterol suspension and the garlic and onion juices were given by gastric intubation. The animals were killed after wk 16 and autopsied.

The control rabbits given the stock diet alone showed no atherosclerosis, while the garlic-treated animals showed less atherosclerotic development than the other cholesterol-treated groups. Higher levels of serum cholesterol accompanied the more severe atherosclerotic lesions seen in the cholesterol-treated rabbits given the onion juice or no extra treatment, while the garlic-treated group had a mean serum-cholesterol level much nearer to the control value as well as a less severe degree of atherosclerosis.

2974. More on the orotic acid problem

Okonkwo, P. O. & Kinsella, J. E. (1974). Fatty livers induction by orotic acid contained in skim milk powder. *Experientia* **30,** 993.

The authors of the paper cited above have previously reported the presence of orotic acid in cows' milk, and have drawn attention to the possibility that the presence of this compound in milk powder may be a possible cause of the liver necrosis seen in rats fed a milk-powder diet (*Cited in F.C.T.* 1970, **8,** 238). Others studies have indicated that the addition of orotic acid to a normal rat diet induces fatty liver, but that this result does not follow if there is simultaneous reduction in the intake of protein and choline (*ibid* 1971, **9,** 278).

Mature rats fed either a diet based on skim-milk powder and containing 0·15% orotic acid or a control diet plus 0·15% orotic acid for 3 or 8 days developed fatty deposits in the liver and a mild but not highly significant increase in liver size. A marked increase was observed in the total liver content of lipid, and this became greater as the feeding of orotic acid progressed. The palmitic acid content of the liver progressively declined, while that of arachidonic acid increased. Orotic acid does not occur in human milk, but milk from ruminants contains relatively high concentrations, especially when in the form of powdered milk or whey concentrates. Dried-milk powders have been reported to contain 100–180 mg/100 g. We have the ironic situation, therefore, where an essentially fat-free product such as dried skim milk contains constituents capable of inducing fatty livers.

2975. Contact allergy to sesame oil

Neering, H., Vitányi, B. E. J., Malten, K. E., van Ketel, W. G. & van Dijk, E. (1975). Allergens in sesame oil contact dermatitis. *Acta derm.-vener., Stockh.* **55,** 31.

Although it is known that the ingestion of sesame seeds can provoke allergic reactions in certain individuals, these reactions have been attributed to the crude sesame-seed extracts, while sesame oil, in common with many other vegetable oils, has been considered free from allergens (*Cited in F.C.T.* 1965, **3,** 539).

In the study cited above, reference is made to about 30 Dutch patients who had leg ulcers, accompanied in most cases by stasis eczema, and in whom frequent and prolonged application of pastes containing sesame oil had resulted in contact dermatitis. As a previous study had suggested that the sensitizing factors could be located in the unsaponifiable portion of the oil, which contains sesamol, sesamolin and sesamin (Dijk *et al. Acta derm.-vener., Stockh.* 1973, **53,** 133), these substances were tested on 13 patients with contact allergy to sesame oil in an attempt to identify the allergen.

Patch tests with sesamolin and sesamin produced a positive reaction in all but one of the 13 patients, eight of whom also reacted to sesamol. Both crude and purified sesame oils were shown to contain sesamol, sesamolin and sesamin. The sesamolin was contaminated with 5–10% sesamin and as the sesamin concentration in the purified oil was about 100 times the sesamolin concentration, it is suggested that sesamin may have been the main inciting allergen. Patch tests with the pure substances on thin-layer sheets were carried out in an effort to resolve this point, but the tests proved inconclusive. Allergy to several substances chemically related to sesamol could not be demonstrated clearly in the group of patients under consideration.

COSMETICS, TOILETRIES AND HOUSEHOLD PRODUCTS

2976. A new cosmetic hazard in imidazolidinyl ureas

Mandy, S. H. (1974). Contact dermatitis to substituted imidazolidinyl urea—a common preservative in cosmetics. *Archs Derm.* **110,** 463.

A new series of antimicrobial preservatives (Germalls) based on substituted imidazolidinyl ureas is reported to be finding favour in cosmetic preparations. What is apparently the first reported case of contact dermatitis to one of these compounds occurred in a middle-aged woman who suffered for about 1 wk from an acute eczematous dermatitis of the face, 2 wk after starting to use a particular moisturizing lotion concentrate. After suppression of the rash with a topical corticosteroid, patch tests with routine allergens and a cosmetic kit were performed. Only the offending moisturizer and a hypoallergenic liquid eye liner gave positive results, which were traced to Germall 115. It was ascertained that this preservative was present at a level of 100 ppm in the moisturizing formulation. With a 1% solution of Germall 115, a +++ vesicular reaction was obtained.

It is suggested that, since Germall 115 is widely used in cosmetics, other undiagnosed cases of sensitivity to it may have occurred.

2977. Acute toxicity of ingested household detergents

Muggenburg, B. A., Mauderly, J. L., Hahn, F. F., Silbaugh, S. A. & Felicetti, Sharon A. (1974). Effects of the ingestion of various commercial detergent products by beagle dogs and pigs. *Toxic. appl. Pharmac.* **30,** 134.

This study was undertaken in connexion with the question of the potential toxicity of accidentally ingested household detergents, a problem that arises particularly with children under the age of 5 yr, as shown by a report published by the US Department of Health, Education, and Welfare (National Clearing House for Poison Control Centers, September/October Bulletin, 1971).

Four commercially available detergents, representing the four main types of builder now used, were purchased through normal retail outlets. Those selected were a phosphate-based Tide and a nitrilotriacetate-based Tide (designated Tide NTA), both from Proctor and Gamble, Sears Heavy Duty Laundry Detergent (based on sodium carbonate; Sears Roebuck and Co.) and Electrosol Dishwashing Detergent (sodium metasilicate; Economics Laboratory, St. Paul, Minn.). Each product was administered in single doses of 0·1, 0·5, 1·0 and 2·5 g/kg to groups of three beagle dogs, an additional group being given 0·25 g/kg in the case of Electrosol. The doses were introduced into the back of the mouth by means of a syringe.

In the 6 days following administration, no deaths were recorded among the dogs ingesting Tide, but at dose levels above 0·1 g/kg, this detergent caused gastric irritation and vomiting. There were no significant gross lesions, but histopathological examination revealed proximal tubular nephrosis in two dogs given 2·5 g/kg and in one given 1·0 g/kg. Tide NTA also caused vomiting, the severity being directly related to the dose. No significant gross or histological lesions occurred and there was no significant effect on the serum-chemistry values examined or on the leucocyte counts. One dog died after the ingestion of 2·5 g Sears/kg. This product caused serious damage to the oral, oesophageal and gastric mucosa. All the dogs given the three highest doses of Electrosol died within 54 hr. Gross lesions of variable severity were found in the oral cavity, pharynx, oesophagus, stomach, larynx, lungs and kidneys of all dogs given doses of 0·25 g/kg or more, but no gross lesions were found after the 6 days in dogs that received 0·1 g/kg. Microscopic examination revealed various changes, including acute ulcerative necrosis of the epithelial lining of the digestive tract, necrosis, ulceration and oedema of the larynges, oedematous lungs and necrosis of the proximal renal tubules. Effects on serum-chemistry values were indicative of alimentary tract and kidney damage.

In a second series of experiments, pigs were given a single dose of each detergent, selected on the basis of the highest dose that did not cause 100% mortality in dogs. Thus 0·25 g/kg was given in the case of Electrosol and 2·5 g/kg in the other cases. The pigs appeared to swallow little of the detergent, much of it being retained in the pharyngeal diverticulum. This possibly accounted for the lower incidence and severity of vomiting compared with that in the dogs, for a lack of significant changes in the haematological and serum-chemistry values and for the absence of kidney lesions. One pig died 47 hr after receiving Sears and another 95 hr after the Electrosol dose. Lesions in the areas directly in contact with the detergent were similar to those found in the dogs. No gross or microscopic lesions were observed in pigs following ingestion of Tide or Tide NTA.

A study with 12-wk-old pups was also carried out. The results obtained were similar to those in the adult dog, suggesting that age did not influence the severity or distribution of the lesions.

On the basis of the results obtained in this study, the authors conclude that the varied findings indicate the importance of evaluating the toxicity of individual detergent formulations so that appropriate therapeutic action can be taken in the event of their ingestion.

2978. Guilty AES contaminants identified

Connor, D. S., Ritz, H. L., Ampulski, R. S., Kowollik, H. G., Lim, P., Thomas, D. W. & Parkhurst, R. (1975). Identification of certain sultones as the sensitizers in an alkyl ethoxy sulfate. *Fette Seifen Anstr-Mittel* **77,** 25.

During 1966, a high incidence of contact hypersensitivity occurred among Norwegian users of a dishwashing formulation containing alkyl ethoxysulphate (AES). Since the demonstration that this outbreak was due to some contaminant in a particular batch

of AES (*Cited in F.C.T.* 1973 **11,** 1156), efforts have been concentrated on identifying the compound(s) responsible.

The authors cited above have now reported the successful outcome of this work. It had already been demonstrated that the sensitizer could be separated from the AES by extraction with petroleum ether (*ibid* 1975, **13,** 594) and could be concentrated by chromatography of this extract on a silica-gel column. The work now described, however, used a hexane extract of the sensitizing batch of AES. Evaporation of the hexane left an oily residue amounting to about 1% of the original material and containing all the biologically detectable activity. Fractionation of this residue was effected by a series of thin-layer chromatographic separations, the sensitizing capacity of each fraction being assayed in guinea-pigs. The separated materials were then identified by gas chromatography and mass spectroscopy.

In this way, 1-dodecene-1,3-sultone and 1-tetradecene-1,3-sultone were identified as the skin-sensitizing agents in the main biologically active fraction of the offending AES. In addition, two other sensitizers isolated from the AES were tentatively identified as 2-chloro-1,3-dodecanesultone and 2-chloro-1,3-tetradecanesultone. The authors postulate that in the processing of this particular batch of AES, the presence of small quantities of dodecene and tetradecene in the reaction mixture undergoing sulphation gave rise to 2-dodecene- and 2-tetradecene-1-sulphonic acids, which reacted with the hypochlorous acid generated in the next stage of the process to yield the chlorosultones. The latter are strong sensitizers and are believed to be present in the material in question. Their partial dehydrochlorination could have produced the unsaturated sultones that have been definitely identified.

CANCER RESEARCH

2979. Carbon tetrachloride and the liver response to nitrosamines

Taylor, H. W., Lijinsky, W., Nettesheim, P. & Snyder, Catherine M. (1974). Alteration of tumor response in rat liver by carbon tetrachloride. *Cancer Res.* **34,** 3391.

The ability of dimethylnitrosamine (DMNA) to cause hepatocellular carcinomas in rats was enhanced by carbon tetrachloride (CCl_4) given in a single hepatotoxic dose of 2·5 ml/kg, a fact attributed to the increase in liver-cell mitosis induced by the latter compound (*Cited in F.C.T.* 1974, **12,** 291). DMNA may be formed by reaction between aminopyrine (AP) and nitrite (Lijinsky & Greenblatt, *Nature, Lond.* 1972, **236,** 177) and rats given AP and nitrite in their drinking-water showed a high incidence of haemangioendothelial (Kupffer cell) sarcomas in the liver (Lijinsky *et al. ibid* 1973, **244,** 176).

In the study cited above, rats were given drinking-water containing both AP and sodium nitrite, each in a concentration of 1000 ppm, on 5 days/wk for 30 wk, and 0·1 ml CCl_4 in 0·1 ml olive oil was administered by stomach tube once every 2 wk. Other groups received drinking-water containing either the same concentrations of AP plus nitrite or 40 ppm DMNA without the CCl_4 treatment or were given the doses of CCl_4 and untreated drinking-water. Rats in the latter group were killed at varying intervals up to 68 wk.

All 18 animals given the AP–nitrite–CCl_4 combination were dead by wk 35, six with hepatocellular tumours and two of these and seven others with haemangioendothelial liver sarcomas; six of the latter also showed metastases to the lungs. In all cases there was a severe degree of post-necrotic liver cirrhosis, and the other five rats in this group died before wk 25 with liver failure but no tumours. A 100% incidence of haemangioendothelial sarcomas, with some lung metastases, was found in rats given AP plus nitrite or DMNA without CCl_4, all the rats in the former group dying by wk 55 and in the latter by wk 35. However, there was no evidence of cirrhosis in these rats, and the absence of any hepatocellular tumours in the DMNA group was at variance with previous studies. With CCl_4 alone, there was a great increase in liver-cell division sometimes accompanied by slight centrilobular fatty change, but no liver lesions were seen even after 68 wk.

The authors speculate that CCl_4 encourages the development of hepatocellular tumours in DMNA-fed rats not only by enhancing cell division but also by affecting liver-cell enzymes and membranes that influence DMNA metabolism. The high DMNA concentration in the portal circulation after intestinal absorption may have been responsible for the preferential response of the Kupffer cells; DMNA may also have been metabolized in nearby hepatocytes to some proximate carcinogen, which was then taken up by the Kupffer cells either by pinocytosis from intact liver cells or by phagocytosis of debris from dead or dying cells.

2980. Carcinogens and the gut flora

Reddy, B. S., Narisawa, T., Wright, P., Vukusich, D., Weisburger, J. H. & Wynder, E. L. (1975). Colon carcinogenesis with azoxymethane and dimethylhydrazine in germ-free rats. *Cancer Res.* **35,** 287.

Certain colon carcinogens, including derivatives of both 3-methyl-4-aminobiphenyl and 1,2-dimethylhydrazine, require activation by the flora of the gut before they become actively carcinogenic (*Cited in F.C.T.* 1972, **10,** 440). In considering human carcinogenesis in relation to diet, not only the carcinogens that may be derived from food but also alterations in the intestinal flora induced by different diets must, therefore, be taken into account. Further evidence of the com-

plexity of the interaction between potential carcinogens and gut floral patterns is provided in the paper cited above.

Female rats, conventional, germ-free and monocontaminated with *Clostridium perfringens*, were given weekly intrarectal instillations of azoxymethane (AOM) in doses of 10 mg/kg/wk for 20 wk. Other conventional and germ-free rats were given 20 weekly sc injections of 1,2-dimethylhydrazine (DMH) in doses of 20 mg/kg/wk. AOM produced a greater incidence of colonic tumours in germ-free and *Cl. perfringens*-monocontaminated rats than in the conventional controls (93 and 100%, respectively, compared with the 60% control figure). No germ-free rats developed tumours of the ear-duct, whereas nearly half of the conventional and monocontaminated rats treated with AOM had squamous-cell carcinomas of the external auditory canal. Tumour incidence in the small intestine was comparable in all groups given AOM, but the germ-free rats had a much lower incidence of kidney tumours (13%) than the others (43–48%). Injections of DMH induced colonic tumours in 21% of germ-free and 93% of conventional rats, and tumours of the ear-duct, kidney and small intestine in 87, 20 and 80%, respectively, of conventional controls, but no tumours in these last three sites followed DMH-treatment of germ-free rats.

This study clearly shows that the gut flora can have a considerable influence on the carcinogenic impact of AOM or DMH on the colonic and other tissues of the rat. The induction of ear-duct tumours in conventional but not germ-free rats treated with DMH or AOM suggests that reactive metabolites produced by the intestinal microflora are absorbed and reach the ear. The increased carcinogenic effect of AOM in rats monocontaminated with *Cl. perfringens*, which has strong β-glucuronidase activity, indicates a possible line of investigation which might be pursued further.

Fd Cosmet. Toxicol Vol. 14, pp. 81–82. Pergamon Press 1976. Printed in Great Britain

LETTERS TO THE EDITOR

SHORT-TERM TOXICITY STUDY OF AMMONIUM SULPHAMATE IN RATS

Sir,—There has been a rapid increase in recent years in the number and type of herbicides used in agriculture. Ammonium sulphamate, introduced as a herbicide in 1945 by E.I. du Pont de Nemours & Co., Inc., under the trade name "Ammate", has been found to be very effective in the manufacture of weed-killing formulations (Bel'kov & Shutov, *Les. Khoz.* 1960, **13**(1), 7) and of fire-retardant compositions for extinguishing forest fires (Krasavina, *ibid* 1965, **18**(6), 52). Its strong phytotoxic activity makes it useful as a non-selective herbicide for the control of woody plants (Leonard & Harvey, *Bull. Calif. agric. Exp. Stn* 1965, no. 812, p. 26) and of poison ivy in fruit orchards (Ahrens & Stoddard, *Circ. Conn. agric. Exp. Stn* 1962, no. 222, p. 1).

Little information is available regarding the toxicity of ammonium sulphamate. The LD_{50} of ammonium sulphamate administered orally is 3·1 and 4·4 g/kg body weight in mice and rats, respectively (Vinokurova & Mal'kova, *Gig. Truda prof. Zabol.* 1963, **7**(5), 56). Concentrations of 0·25–0·50 g/litre in air caused damage to the respiratory system; application of a 20% solution to the skin caused only local changes (Vinokurova & Mal'kova, *loc. cit.*).

Manufacture of ammonium sulphamate on a commercial scale has been started recently in India. The product, supplied by Dharamsi Morarji Chemical Co. Ltd., Bombay, is a white crystalline hygroscopic and odourless solid of 99·0% purity, containing 0·5% moisture and 100·0 ppm (maximum) iron. Its melting point is 130°C and it is extremely soluble in water and moderately soluble in glycerol, glycol and formamide.

We have studied the short-term toxicity of this compound in albino weanling rats of either sex (body weight 30–50 g) and albino adult rats (body weight 170–200 g) of the ITRC strain. Six groups of ten rats were housed individually in wire cages. The experimental rats were given 500 mg ammonium sulphamate/kg in distilled water (100 mg/ml) by gavage on 6 days/wk over a period of 90 days extending from May to July. The control animals, kept under similar conditions, were given equivalent volumes of distilled water. This dose of ammonium sulphamate was approximately 1/9 of the oral LD_{50} (4·4 g/kg) for rats (Vinokurova & Mal'kova, *loc. cit.*). Throughout the experimental period, the animals were maintained on a standard diet (67% pellets from Hindustan Lever, India, Ltd., and 33% green vegetables). Food and water consumption were measured fortnightly. The weight of the animals was recorded twice weekly during the first 2 months and weekly in month 3. The dose of ammonium sulphamate was adjusted to the body weight whenever the animals were weighed.

The appearance and behaviour of the rats were recorded daily and deaths, if any, were noted. Haematological studies were carried out at monthly intervals and included the measurement of haemoglobin and packed cell volume, total red cell counts and total and differential white cell counts. Two animals from each group were killed at the end of 30, 60 and 90 days of treatment and were autopsied. The stomach, intestine, liver, kidney, heart and testis or ovary were fixed in 10% formol-saline solution. Paraffin-embedded sections were cut at 6 μm and stained with haematoxylin and eosin for histological examination.

Throughout the period of observation, the general condition and health of all the rats appeared good. One rat from the group of treated adults and two from the treated group of male weanlings died during the experimental period. These deaths were apparently unrelated to treatment, as three animals among the control adults also died. No cause of death could be assigned at autopsy.

The male and female weanling rats grew well and there were no significant differences between the mean body weights of control or treated animals during or at the end of the observation period. However, although the adults showed no differences in weight gain between the treated and control groups over the first 60-day period, the treated group started to lag behind in weight gain at the end of this period. By the end of the 90 days, the body weights of the treated adults were significantly less than those of the controls.

In general the food intake of all animals, treated or controls, weanlings or adults, gradually reduced as the experiment progressed. Comparison between treated and control groups indicated that while there was no noticeable difference in the case of adults, food intake by the treated weanlings (both males and females) was significantly lower than that in the controls. On the other hand, water intake generally increased in all the animals. The treated weanlings, males as well as females, drank significantly more water than did the controls, but no such difference was noted among the adult rats.

Haematological examination conducted at 30, 60 and 90 days revealed no changes other than a non-significant increase in the neutrophils in the treated adults and in the male-weanling group.

In the histological examination, organs in all the six groups of animals appeared normal. However, the liver of one adult rat treated with ammonium sulphamate showed slight fatty degenerative changes in the cytoplasm.

Short-term administration of ammonium sulphamate to rats produced no toxic effect of consequence at the level of 500 mg/kg body weight. The general health, survival and organ pathology of the test animals remained unaffected by this treatment.

The chemical structure of ammonium sulphamate is such that it is readily dissociated in the body into its biodegradable ammonium and sulphate radicals, which then enter the general metabolic pool of the organism. The significance of the observation that adult rats fed ammonium sulphamate showed a lower weight gain than the controls is not understood. It is noteworthy that there was no difference in the food and water intake of these two groups. Further study either with higher doses or over longer periods could throw some light on this observation. On the other hand, an increase in water intake along with a decrease in food intake without any change in growth rate among the weanlings is also intriguing. In view of the chemical structure of ammonium sulphamate, one might expect a change in the acid–base balance of the organism and this might, in turn, influence the food and water intake of the growing animals.

The authors are grateful to Dr. S. H. Zaidi, Director, Industrial Toxicology Research Centre, Lucknow, India, for his keen interest in this work. They wish also to thank Mr. H. K. Sharma and Mr. G. S. D. Gupta for technical assistance.

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DIETARY TRADITIONS AND TOXIC FOODS

Sir,—According to the Jewish tradition, food sources and food preparation have to comply with strict laws and prescriptions dating from the times of the Bible, when the Jews lived in the Middle East. Religious Jews still observe traditions of kosher food regardless of where they live at present.

Recent scientific evidence indicates that some of these dietary traditions may be sound and may have served to prevent health hazards. This is particularly so as regards consumption of meat from 'unclean' animals, such as pigs (a meat also forbidden by the Muslim religion). The omnivorous pig is often fed the left-overs of human foods, and such feeds are likely to become mouldy, especially during rainy seasons or in the humid heat prevailing in some parts of the Middle East.

It has now been recognized that under appropriate conditions some of the common moulds can produce toxic and/or carcinogenic secondary metabolites, such as the trichothecenes, ochratoxins, sterigmatocystins and aflatoxins, which may present health hazards not only to those who ingest them directly but also to those who are exposed to them 'by proxy' when consuming meat and other products derived from animals that have ingested toxic fungal metabolites in their feeds (Schoental, *Int. J. environ. Stud.* 1975. 8, 49). Instances have been reported of high concentrations of aflatoxins (1000–5000 μg/kg) in some samples of bacon and lard in a cake factory (Hanssen, *Naturwissenschaften* 1969, **56**, 90; Hanssen & Jung, *Pure appl. Chem.* 1973, **35**, 239), and significant amounts of aflatoxins have also been found in the carcasses of pigs, which on gross inspection appeared not unhealthy and which passed inspection after slaughter (Krogh *et al. ibid* 1973, **35**, 275).

Other effective carcinogens are the nitrosamines. These can be formed under appropriate conditions from food constitutents and the nitrite used in the curing of foods. Fried bacon can contain relatively high concentrations of nitrosopyrrolidine (Bills *et al. J. agric. Fd Chem.* 1973, **21**, 876). I have already pointed out that the preparation of kosher meat, which includes salting and soaking the meat in water, is likely to diminish the risk of nitrosamine formation by removing the water-soluble products of protein autolysis and amines (Schoental, *Fd Cosmet. Toxicol.* 1974, **12**, 167).

It makes one wonder how people so many centuries ago could have acquired the insight into problems that only now receive scientific explanation.

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Fd Cosmet. Toxicol. Vol. 14, p. 83. Pergamon Press 1976. Printed in Great Britain

MEETING ANNOUNCEMENTS

ASPET SYMPOSIA FOR 1976 AND 1977

The American Society for Pharmacology and Experimental Therapeutics, Inc., has announced details of its Spring and Fall Meetings for 1976.

At the former, to be held on 12–16 April at Anaheim, Cal., ASPET will present a symposium on "Pediatric Aspects of Environmental Pharmacology" under the Chairmanship of Dr. R. J. Roberts, Toxicology Center, University of Iowa. Speakers will include Dr. A. P. Autor, University of Iowa (who will discuss oxygen toxicity and superoxide dismutase in relation to hyaline membrane disease), Dr. J. J. Chisolm, Jr., Department of Pediatrics, Baltimore City Hospital (heavy metal poisoning in pediatrics), Dr. J. Dancis, Bellevue Medical Center, New York (environmental influences on the foetal environment), and Dr. N. Kretchmer, National Institute of Child Health and Human Development, Bethesda.

The Fall Meeting, to be held in New Orleans, La, on 15–19 August 1976 under the Chairmanship of Dr. M. G. Horning, Institute for Lipid Research, Baylor College of Medicine, Houston, will be a symposium entitled "Toxicological Potential of Clinically-used Polymeric Materials". Topics to be discussed will be the programme and guidelines of the Bureau of Medical Devices, FDA (Dr. K. Bruch), the toxicity of ethylene oxide and ethylene chlorohydrin (Dr. T. Balazs, FDA), the toxicity of methyl methacrylate cement and related materials in man (Dr. J. Autian, University of Tennessee Medical Units, Memphis), the toxicity of adipate and phthalate plasticizers (Dr. R. J. Jaeger, Harvard University School of Public Health, Boston), the pharmacological effects of cyanoacrylates in tissue adhesives (Dr. F. Leonard, George Washington University School of Medicine, Washington) and the tissue response to siloxane polymers in prosthetic devices (Dr. C. A. Homsy, Baylor College of Medicine).

Notice has also been given that symposia on "Metal Contaminants and their Effects" and "Environmental Pharmacology of the Lung" are planned for the 1977 Spring and Fall Meetings, respectively.

DERMATOLOGY: TWO MEETINGS

"Identification of the 50 Common Dermatoses" will be reviewed at a 2-day meeting to be held at the University of California School of Medicine, San Francisco, on 14 and 15 January 1977. This course is planned to cover the practical identification and treatment of the 50 common dermatoses that account for 95% of dermatological diseases. The following month, a 2-day review in depth of relevant knowledge of animal and human experimental models in dermatology will be presented by the Department of Dermatology and Extended Programs in Medical Education, University of California Medical Center, San Francisco, on 3 and 4 February. This programme is designed for the toxicologist, internist and dermatologist interested in the determination of hazards from topically applied chemicals and drugs.

Further information on both meetings may be obtained from: Extended Programs in Medical Education, University of California, Room 569 U, 3rd and Parnassus Avenue, San Francisco 94143, USA (Tel. no. (415)666-4251).

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FORTHCOMING PAPERS

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

Carrageenan: The effect of molecular weight and polymer type on its uptake, excretion and degradation in animals. By K. A. Pittman, L. Golberg and F. Coulston.

Methods for limiting the content of dimethylnitrosamine in Chinese marine salt fish. By Y. Y. Fong and W. C. Chan.

Reproductive dysfunction in rhesus monkeys exposed to low levels of polychlorinated biphenyls (Aroclor 1248). By D. A. Barsotti, R. J. Marlar and J. R. Allen.

Tolerance and acceptable daily intake of chlorinated fumigants in the rat diet. By E. Alumot (Olomucki), E. Nachtomi, E. Mandel, P. Holstein, A. Bondi and M. Herzberg.

Tolerance and acceptable daily intake of ethylene dichloride in the chicken diet. By E. Alumot, M. Meidler, P. Holstein and M. Herzberg.

Potato starch and caecal hypertrophy in the rat. By E. A. El-Harith, J. W. T. Dickerson and R. Walker.

Effets de l'acide tannique et du métabisulfite de potassium, en association avec l'éthanol, sur quelques activités de détoxication enzymatique des microsomes hépatiques du rat. Par D. Gaillard, G. Chamoiseau et M. Suschetet.

Acute and short-term toxicity of ochratoxin A in 10-day-old chicks. By P. Galtier, J. More and M. Alvinerie.

Null carcinogenic effect of large doses of nitrosoproline and nitrosohydroxyproline in Wistar rats. By J. E. Nixon, J. H. Wales, R. A. Scanlan, D. D. Bills and R. O. Sinnhuber. (Short Paper)

Toxin production by 50 strains of penicillium used in the cheese industry. By P. Lafont, J. Lafont, J. Payen, E. Chany, G. Bertin and C. Frayssinet. (Short Paper)

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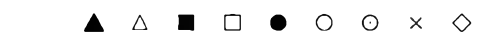

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