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INFORMATION SECTION

ARTICLES OF GENERAL INTEREST*

TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS*

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EFFECT OF AMARANTH, PONCEAU 4R AND/OR VITAMIN A ON ENZYME ACTIVITIES OF THE RAT LIVER

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(*Received 8 September 1977*)

Abstract—Four groups each of five male rats were treated daily by gavage for 9 days with 0·25 M-sucrose (control), amaranth (85 mg/kg body weight/day) in sucrose, vitamin A (115 mg/kg/day) in arachis oil or these doses of both amaranth and vitamin A solutions. The effects of these treatments on the activities of certain liver enzymes were studied. *In vivo* the activity of glucose-6-phosphate dehydrogenase was significantly reduced by amaranth given in combination with vitamin A. Amaranth given alone significantly reduced acid-phosphatase activity in the cytosol. The increase in malate-dehydrogenase activity caused by vitamin A was significantly counteracted by simultaneous administration of amaranth. The activity of α-glycerophosphate dehydrogenase was significantly lower in the amaranth–vitamin A group than in the group given amaranth alone. In *in vitro* studies both amaranth and Ponceau 4R and combinations of either of these substances with vitamin A caused a significant decrease in the activity of rat-liver lysosomal acid phosphatase.

INTRODUCTION

Amaranth (FD & C Red No. 2) was widely used as a food colouring for many years without giving rise to any reports of possible hazard to man.

Extensive toxicity testing of this material, including 2-yr feeding studies in rats, dogs and mice, gave no cause for concern about its safety (Cook, Hewett, Kennaway & Kennaway, 1940; Radomski & Mellinger, 1962; Willheim & Ivy, 1953). However, in 1970 Andrianova reported some experimental data which suggested for the first time that amaranth might have carcinogenic properties, and more recently the teratogenic, carcinogenic and toxic effects of amaranth have been discussed by various authors (Collins & McLaughlin, 1972; Keplinger, Wright, Plank & Calandra, 1974; Khera, Przybylski & McKinley 1974; Larsson, 1975).

In view of the possible toxic effects of amaranth, its isomer, Ponceau 4R, has begun to be widely used in the last few years as a red colouring. The work reported here was an attempt to determine whether amaranth and Ponceau 4R can cause hepatocellular damage. It involved the examination of a number of enzymes, namely α-glycerolphosphate dehydrogenase, malate dehydrogenase and lactate dehydrogenase, all of which can be used to demonstrate cell poisoning (Schmidt & Schmidt, 1963), glucose-6-phosphate dehydrogenase, the first enzyme in the chain leading to the production of D-ribose, a precursor of nucleotide synthesis, which is very important for cellular function, and the lysosomal enzyme, acid phosphatase. Toxic agents can cause tissue injury by inducing the release of lysosomal hydrolases into the cytoplasm (de Duve, 1963). Since vitamin A is known to promote the activity of hepatic lysosomal hydrolases (Fell, Dingle & Webb, 1962), the effect of a combination of amaranth or Ponceau 4R with vitamin A on liver enzymes was also investigated.

EXPERIMENTAL

Test materials. The red colourings, amaranth (FD & C Red No. 2) and Ponceau 4R (new coccine) used in this study were obtained from ICI Ltd., Manchester, England. The amaranth had a dye content of 80%, impurities being 4% NaCl and Na_2SO_4, 3% subsidiary dyes, 4% volatile matter and 0·1% water-insoluble matter. Ponceau 4R had a dye content of 77·6%, impurities present being 8% NaCl and Na_2SO_4, 10% volatile matter, 0·1% water-insoluble matter, 1% subsidiary dyes and 0·2% ether-extractable material. Vitamin A, in the form of transretinol crystalline synthetic type X, was obtained from Sigma Chemical Co., London.

Animals and treatment. Adult male albino rats of the Sprague–Dawley strain weighing 160–180 g were obtained from Anticimex, Sweden, and were divided into four groups of five. These groups were fed a standard diet (R3; Astra, Sweden) and were given by gavage, daily for 9 days, 0·4 ml 0·25 M-sucrose (control group), 0·4 ml 0·25 M-sucrose containing 14·3 mg amaranth (amaranth group), 20 mg vitamin A in 0·4 ml of arachis oil (vitamin A group), or both amaranth and vitamin A solutions in the same doses as were given individually (amaranth/vitamin A group). The animals were weighed daily throughout treatment.

Fractionation of rat liver. The animals were starved for 20 hr (between days 9 and 10) before being killed by a blow on the head. The livers were removed, minced in chilled 0·25 M-sucrose and homogenized in a glass homogenizer at 10% w/v in cold 0·25 M-sucrose–0·025 M-tris-acetate, pH 7·4. The homogenates were then centrifuged for 20 min at 600 **g** to remove the nuclei, debris and unbroken cells. The supernatants were removed and centrifuged for 20 min at 27.000 **g** and then for 60 min at 100.000 **g**. All the procedures were performed at 4°C. The pellets from the 27.000-**g** centrifugation, the lysosome-rich frac-

tions, were suspended in 0·25 M-sucrose–0·025 tris-acetate, pH 7·4, in a concentration of about 15 mg protein/ml, and the suspension was subsequently incubated at 37°C for 40 min. Immediately after the incubation each sample was centrifuged for 20 min at 27,000 **g** and the supernatant was decanted into small glass tubes and maintained at 4°C until assayed for lysosomal-enzyme activity. The 100,000-**g** supernatants were assayed directly for phosphatase activity without incubation.

Estimations of enzyme activity in vivo. The activities of free and total acid phosphatase were measured according to Wattiaux & de Duve (1956) with certain modifications. For total enzyme activity, incubation at 37°C for 40 min was used. Treatment of lysosomes after the incubation was carried out at a final concentration of 0·1% in Triton X-100 in a total volume of 2 ml at room temperature for 30 min. After the treatment, the lysosomal suspensions were centrifuged for 20 min at 27,000 **g** and the supernatants were assayed for lysosomal-enzyme activity. β-Glycerophosphate from Sigma Chemical Company served as a substrate for acid phosphatase. The enzyme was studied in 0·25 M-sucrose buffered with 0·05 M-acetate buffer (pH 5·0). In order to account for the small and variable amounts of products of enzyme activity present in the lysosomal suspensions prior to incubation, aliquots of the suspensions were tested without substrate. These 'blank' values were subtracted from the values obtained for enzyme activity with substrate to yield a more accurate measure of lysosomal enzyme activity. The supernatants obtained after the 600-**g** centrifugation were assayed for lactate dehydrogenase, malate dehydrogenase, glucose-6-phosphate-dehydrogenase and α-glycerophosphate dehydrogenase according to Hess (1963). Proteins were measured by the method of Hartree (1972). All data were analysed statistically by Student's *t* test, the level of significance selected being $P < 0·05$.

In vitro *study*. Homogenates of livers from five animals were used in this experiment. Up to the 600-**g** centrifugation, the procedure for liver fractionation was the same as that used in the *in vivo* experiment. Afterwards, the 600-**g** supernatant was centrifuged stepwise at 3500 **g**, 8000 **g** and 27,000 **g**, rather than immediately at 27,000 **g** as in the *in vivo* experiment. The sediment from each centrifugation was suspended in 0·25 M-sucrose–0·025 M-tris-acetate (pH 7·4) to provide a lysosome-rich fraction, and 1-ml aliquots of these suspensions, containing lysosome-rich fraction prepared from 1 g liver, were diluted in 4 ml buffered sucrose. These suspensions were incubated at 37°C for 40 min with amaranth (1·25, 2·5, 5 or 7·5 mg/5 ml of incubation medium), with Ponceau 4R (3·75, 7·5, 15 or 22·5 mg/5 ml), with vitamin A (1, 2, 3 or 4 mg/5 ml) or with amaranth or Ponceau 4R combined with vitamin A in these dose ranges. After incubation the suspensions were centrifuged at 4°C for 20 min at 27,000 **g**. Free acid-phosphatase activity was determined in the supernatant by the method of de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955), but because the red colour of amaranth and Ponceau 4R disturbed the spectrophotometric analysis based on the blue colour of phosphomolybdic acid, the supernatant was shaken with an isobutanol–benzene mixture (1 : 1) three times to separate the analytically reduced phosphomolybdic acid from amaranth or Ponceau 4R.

RESULTS

In vivo *experiment*

The effect of treatment with amaranth and/or vitamin A on the body weight of the rats over the 9-day treatment period is shown in Table 1. The body-weight increase was lower in the amaranth–vitamin A and vitamin A groups than in the control and the amaranth-treated groups. This effect of vitamin A on the body weight is in agreement with the observations of Misra (1968).

The relation of free enzyme activity to total activity in the lysosome-rich fractions clearly reflects the effect of treatment on the animals (Table 2). The acid-phosphatase activity was somewhat lower in the lysosome-rich fractions from rats treated with amaranth than in those from the control animals, and a more marked decrease in acid phosphatase was evident in the 100,000-**g** supernatant from amaranth-treated rats. However, acid-phosphatase activity was much higher in the lysosome-rich fractions prepared from the livers of animals treated with vitamin A than in those of the control group. This increase in acid-phosphatase

Table 1. *Mean changes in the body weights of rats given by gavage 14·3 mg amaranth and/or 20 mg vitamin A daily for 1–9 days*

Day	Control group	Change in body weight (g/kg body weight/day) in groups given: Amaranth	Amaranth and vitamin A	Vitamin A
2	34 ± 10	31 ± 3	13 ± 12	3 ± 10
3	34 ± 7	38 ± 7	5 ± 19	−6 ± 23
4	42 ± 5	45 ± 10	3 ± 21	−9 ± 19
5	28 ± 6	42 ± 14	−3 ± 25	−5 ± 29
6	29 ± 3	38 ± 5	2 ± 19	−1 ± 18
7	34 ± 4	34 ± 4	1 ± 27	−2 ± 22
8	38 ± 5	37 ± 8	−1 ± 24	−1 ± 24
9	36 ± 9	31 ± 11	3 ± 26	1 ± 21

Values are mean changes ± SD for groups of five rats.

Table 2. *The effect on lysosomal acid phosphatase of daily administration of 14·3 mg amaranth and/or 20 mg vitamin A to rats by gavage for 9 days*

Treatment group	Acid-phosphatase activity (μmol PO_4^- released × 10^{-2}/mg protein/min)			
	In 27,000-g pellet			In 100,000-g supernatant
	Free	Total	Free/total (%)	
Control	1·73 ± 0·21	32·12 ± 3·93	5·44 ± 0·75	1·76 ± 0·15
Amaranth	1·53 ± 0·27	33·16 ± 4·39	4·61 ± 0·39	0·97 ± 0·24[a]
Amaranth and vitamin A	6·64 ± 1·52[a]	20·29 ± 3·68	32·94 ± 5·69[a]	1·80 ± 1·11
Vitamin A	7·89 ± 0·51[a]	22·54 ± 4·01	36·00 ± 8·35[a]	2·37 ± 0·83

Values are mean ± SD for groups of five rats and those carrying a superscript (a) differ significantly ($P < 0·05$ by Student's t test) from the control value.

Table 3. *Enzyme activities of the liver homogenate from rats given daily 14·3 mg amaranth and/or 20 mg vitamin A by gavage for 9 days*

Treatment group	Enzyme activity (μmol NADPH or NADH × 10^{-2}/mg protein/min)			
	G-6-PD	α-GPD	LDH	MDH
Control	1·35 ± 0·06	8·39 ± 0·43	99·02 ± 7·71	29·38 ± 3·39
Amaranth	1·36 ± 0·08	10·19 ± 0·58	90·63 ± 7·57	31·14 ± 2·42
Amaranth and vitamin A	1·01 ± 0·03[abc]	7·83 ± 0·19[b]	97·03 ± 4·87	30·01 ± 0·91[c]
Vitamin A	1·42 ± 0·01	9·52 ± 0·87	108·81 ± 5·97	37·40 ± 2·18

G-6-PD = Glucose-6-phosphate dehydrogenase α-GPD = Glycerolphosphate dehydrogenase
LDH = Lactate dehydrogenase MDH = Malate dehydrogenase

Values are means ± SD for groups of five rats and those carrying superscripts differ significantly ($P < 0·05$ by Student's t test) from the control value ([a]) or from the value for the amaranth group ([b]) or the vitamin A group ([c]).

Table 4. *The* in vitro *effect of various concentrations of amaranth, Ponceau 4R and/or vitamin A on the acid phosphatase of rat-liver fractions*

Added compound(s)	Concn (mg/5 ml incubation medium)	Acid phosphatase activity (% of control value) in lysosome-rich liver fraction after centrifugation at		
		3500 g	8000 g	27,000 g
Vitamin A*	1·0	110	158	110
	2·0	115	385	133
	3·0	123	410	136
	4·0	148	440	247
Amaranth	1·25	39	62	50
	2·5	29	53	40
	5·0	14	42	25
	7·5	12	42	27
Vitamin A and amaranth	3/5	50	310	55
	3/2·5	44	350	50
	2/5	51	290	53
Ponceau 4R	3·75	60	63	30
	7·5	67	60	33
	15·0	57	56	18
	22·5	51	52	15
Vitamin A and Ponceau 4R	3/15	54	77	43
	3/7·5	44	71	27
	2/15	55	71	27

*Used in the form of retinol dissolved in alcohol (100 mg/ml).
Each value is the mean of the results of three incubations carried out in each case in a total volume of 5 ml at 37°C for 40 min. All differences exceeding 15% (the margin of experimental error) were regarded as significant.

activity caused by vitamin A was also described by Weissmann & Thomas (1963) and was slightly, but not significantly, inhibited by amaranth.

Table 3 shows the enzyme activities of the liver homogenates after the 600-**g** centrifugation. Amaranth increased the α-glycerolphosphate-dehydrogenase and malate-dehydrogenase activities and decreased lactate-dehydrogenase activity, but the differences from the control values were not significant. Enzyme activities in the group treated with amaranth in combination with vitamin A were markedly lower than those in the vitamin A group; for the glucose-6-phosphate-dehydrogenase and malate-dehydrogenase activities, the difference was significant. There was a significantly greater decrease in the activities of glucose-6-phosphate dehydrogenase and α-glycerolphosphate dehydrogenase in animals treated with amaranth in combination with vitamin A, than in those treated with amaranth alone. Only in the case of glucose-6-phosphate dehydrogenase was there a significant decrease in activity in the amaranth–vitamin A group compared with the control group.

In vitro *experiment*

Table 4 presents data relating to the effect of added amaranth, Ponceau 4R and vitamin A and combinations of vitamin A with amaranth or Ponceau 4R on the acid phosphatase of lysosome-rich suspensions. As shown in Table 4, amaranth and Ponceau 4R decreased acid-phosphatase activity. Similar results were obtained in the case of amaranth or Ponceau 4R in combination with vitamin A, the enzyme activity being lower than that with vitamin A treatment alone. Vitamin A increased the acid phosphatase activity up to four times. Table 4 shows that the different lysosomal fractions responded differently to the test substances, a finding that may be due to the different phagosome characterization of the lysosomes or to the fact that the different lysosome fractions were derived from different types of liver cell (Davies, 1973).

DISCUSSION

Drugs may modify the lysosomal state by affecting the permeability of the lysosomal membrane, by accumulating in the lysosome and causing a direct inhibition of acid hydrolases or by changing the ability of the lysosomal membrane to fuse with other cell membranes. Analysis of our study suggests that amaranth may act on lysosomal membranes, because the acid-phosphatase activity decreased significantly in the cytosol *in vivo*.

It is known that the effects of drugs on lysosomes can vary in *in vitro* and *in vivo* systems, not only because of the different effect of the drug metabolites but also because, in whole cells, drugs may affect other subcellular components besides lysosomes. However, the much more pronounced effect of amaranth on lysosomes *in vitro* compared with its effect *in vivo* can be explained by the fact that about 85% of ingested amaranth can be reduced by the gut microflora (Ryan, Roxon & Sivayavirojana, 1968). According to Gales, Preda, Popa, Sendrea & Simu (1972) the vitamin A content of the liver decreases after administration of amaranth to rats. In our experiment, however, the release of acid phosphatase observed in *in vivo* systems was almost the same for animals treated with amaranth–vitamin A and for those given vitamin A alone.

Glucose-6-phosphate dehydrogenase is involved in the pentose shunt of the cell. The fact that the activity of hepatic glucose-6-phosphate dehydrogenase was significantly lower in rats treated with amaranth and vitamin A than in the controls or in the groups treated separately with either amaranth or vitamin A was probably the result of the reduction of the nucleotide synthesis in the amaranth–vitamin A group.

An increase in the activity of α-glycerolphosphate and malate dehydrogenases is the first sign of cell poisoning (Rachterich, 1968). In mild cellular damage, lactate-dehydrogenase activity increases only rarely (Schmidt & Schmidt, 1963). Some increase in α-glycerolphosphate dehydrogenase, lactate dehydrogenase and malate dehydrogenase in the vitamin A-treated group can be explained by the fact that a high concentration of vitamin A is poisonous for liver cells. However, it seems that amaranth can change the effect of vitamin A on the cell enzyme activities.

From our observations *in vitro* it seems that both primary lysosomes and phagosomes are influenced by amaranth. This is the reason why centrifugation up to 27,000 **g** was carried out in both the *in vivo* and *in vitro* experiments in the present work.

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HISTOLOGICAL EXAMINATION OF PERINATAL EYE DEVELOPMENT IN THE RAT AFTER INGESTION OF SODIUM CYCLAMATE AND SODIUM SACCHARIN DURING PREGNANCY

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Abstract—Female Wistar rats received 5% sodium cyclamate or 0·4% sodium saccharin in their food for 20 days after mating. The ingested dose of sweetener amounted on average to 1495 mg sodium cyclamate or 98·8 mg sodium saccharin per animal per day. The foetuses delivered by caesarian section on day 20 of pregnancy and the young killed 3 wk after parturition showed no treatment-related anomalies. In particular, there were no histological changes in the lens, the vitreous body, the insertion of the optic nerve or other parts of the eye.

INTRODUCTION

The major investigations of the embryotoxic potential of sweetening agents (Abbott Laboratories, 1973; Food and Drug Research Laboratories, Inc., 1972; Fritz & Hess, 1968; K. Kaziwara and H. Mizutani, unpublished report 1970, cited by Abbott Laboratories, 1973; Lessel, 1970; B. L. Oser, E. E. Vogin, G. E. Cox, S. Carson and S. S. Sternberg, unpublished report 1969; Tanaka, Kawashima, Nakaura, Nagao, Kuwamura & Omori 1973; Tuchmann-Duplessis & Mercier-Parot, 1970) and the numerous reproduction studies (see Abbott Laboratories, 1973) provide no indication of eye damage caused by sodium cyclamate or sodium saccharin. In contrast to these findings, Lederer, Collin, Pottier-Arnould & Gondry (1971) and Lederer & Pottier-Arnould (1970 & 1973) reported ocular anomalies in offspring after feeding sodium cyclamate or sodium saccharin to pregnant Wistar rats. Recently, Lederer (1977) reported that the anomalies found after saccharin feeding must be due to impurities in the sweetener following its synthesis by the Remsen and Fahlberg method. These conflicting reports led us to undertake the studies described below.

EXPERIMENTAL

Groups of 26–28 female Wistar rats (obtained from Mus Rattus Corp., Brunnthal, Munich, and weighing 182–229 g) were mated with 3- to 5-month-old males. The females were assumed to be pregnant when spermatozoa were found in a vaginal smear. The pregnant rats were kept at an ambient temperature of 20–23°C in an atmosphere of 60% mean relative humidity, with a 12-hr electric light/dark cycle. They were given Altromin-R powdered feed (supplied by Altromin Ltd., Lage/Lippe) and tap-water *ad lib.* From mating until day 20 of pregnancy, they were treated with feed containing 5% sodium cyclamate or 0·4% sodium saccharin, complying in each case with the standards of purity established in Germany for the marketed product. The average amount of test substance ingested daily was 1495 mg sodium cyclamate or 98·8 mg sodium saccharin per rat. The difference in mean daily intakes for the diets containing cyclamate (29·9 g) and saccharin (24·7 g) may have been due to the 5% non-calorific component in the former diet or to differences in palatability. The mean daily intake of a control group of similar size was 24·4 g.

One half of the dams in each of the three groups were delivered by caesarian section on day 20 of pregnancy. The other animals were left to deliver their young spontaneously and rear them for 3 wk.

For all the dams, appearance, behaviour, deaths, body weight and food consumption were recorded. Following surgical delivery, the numbers of implantations, foetuses and resorbed embryos were calculated, foetuses were inspected for external malformations, and litter weights, mean foetal weights/litter, numbers of runts (foetuses < 3 g) and mean placental weights were recorded. Data recorded for the rest of the dams in each group included the duration of pregnancy, course of parturition, behaviour during lactation, numbers of implantation sites demonstrated at the end of the experiment by the staining method of Salewski (1964), and pathological changes. For the offspring of these dams, records were kept of weights and sex ratios at birth and after 1, 2 and 3 wk, and of deaths and behaviour, time of eye-opening, formation of fur, ability to run normally, macroscopic changes in the eye and direct pupillary reflexes in response to a flash-light in a darkened room.

For the histological examination of the eyes, the foetuses and young animals were killed with CO_2, and the heads were fixed in Bouin's fluid. For better penetration of the fixation fluid into the area to be examined, the nose, lower jaw and back of the head were removed, the decalcification of bone necessary in the 3-wk-old animals was achieved using sodium ethylenediaminetetraacetate at 38 C. The subjects for

examination were embedded, via methylbenzoate, in Paraplast, so that the microtomy yielded fronto-parallel sections in a cranio-caudal series (Fig. 1). The ocular region, from foetuses and young from each dam, was cut in series or stages, regularly including both lenses and the insertion of the optic nerves. The sections were about 3–5 μm thick and were alternately stained with haemalum–eosin and Masson's trichrome.

RESULTS

The general studies provided no indication of any primary embryotoxic effect of the treatment and the histological examination showed that treatment with the sweetening agents caused no ocular damage. The development of the eyes had followed a normal course in all the foetuses and young animals studied. No case of anophthalmia or microphthalmia occurred. The retina showed no unusual folding, the ocular lenses were of normal structure and there were no group-specific differences between rats treated with sodium cyclamate or sodium saccharin and the control group. In particular, no lytic, necrotizing or vacuolating processes of the ocular lenses were present. The fibres of the lenses showed no increase in circumference or disorientation. The epithelial cells of the lenses had reached their regular position and showed normal mitotic activity. The vitreous body and the implantation of the optic nerves were normal. Figures 2 and 3 show the histological findings in eyes of each of the three groups of animals.

DISCUSSION

Our findings confirmed the conclusions of the many investigations noted earlier (p. 7) showing that the administration of sodium cyclamate or sodium saccharin to pregnant rats is tolerated without embryotoxic effect. Although each dam had ingested considerable amounts of sweetener (on average 1495 mg sodium cyclamate or 98·8 mg sodium saccharin daily), the offspring showed no anomalies, in particular of the eyes, on day 20 of gestation or 3 wk after birth.

The eye changes described by Lederer *et al.* (1971) and Lederer & Pottier-Arnould (1970 & 1973) could possibly have been spontaneous anomalies, since these are not uncommon in the Wistar strain of rat and the authors reported an incidence of 12·4% in their controls. Misinterpretation of histological artefacts is another possible source of apparent eye alterations. With our methods, no notable artefacts were produced in the foetuses (Figs 1 & 2). In the 3-wk-old young, however, the physiological increase in the consistency of the lenses led to occasional splintering, to an equal degree in the control and test groups. These cutting artefacts did not hamper the studies, however, since extensive series of sections were available and plenty of intact regions could be evaluated (Fig. 3).

Our additional studies of postnatal eye function, which Lederer and his co-workers did not examine, also provided no indication of eye damage resulting from the treatment with sweeteners.

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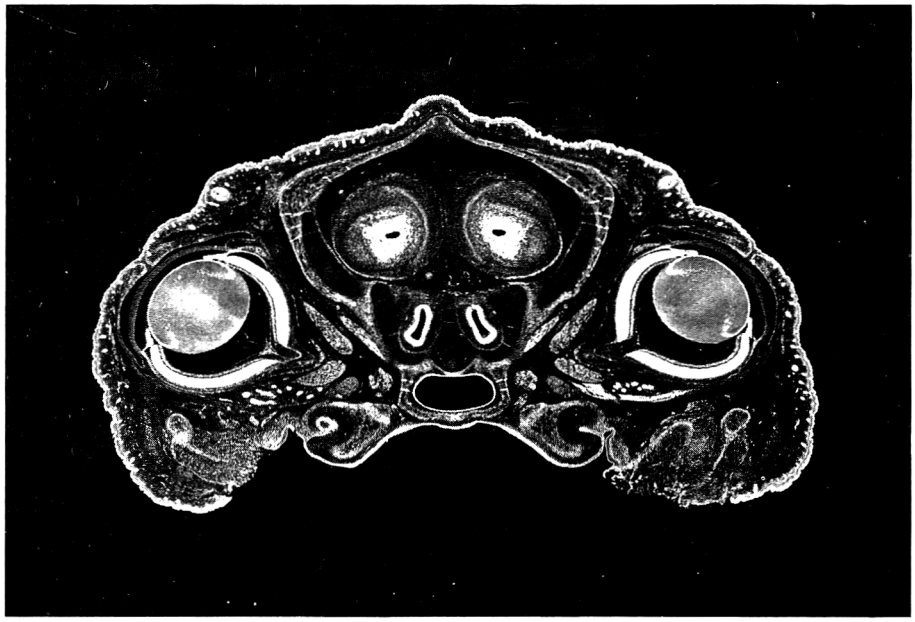

Fig. 1. Transverse section through the head of a 20-day-old rat foetus of the control group, giving a general view of both eyes at the level of insertion of the optic nerves (37th section in a series of 120). Direct projection of a histological section approx. 5 μm thick onto photographic paper. Haemalum-eosin × 12 (approx).

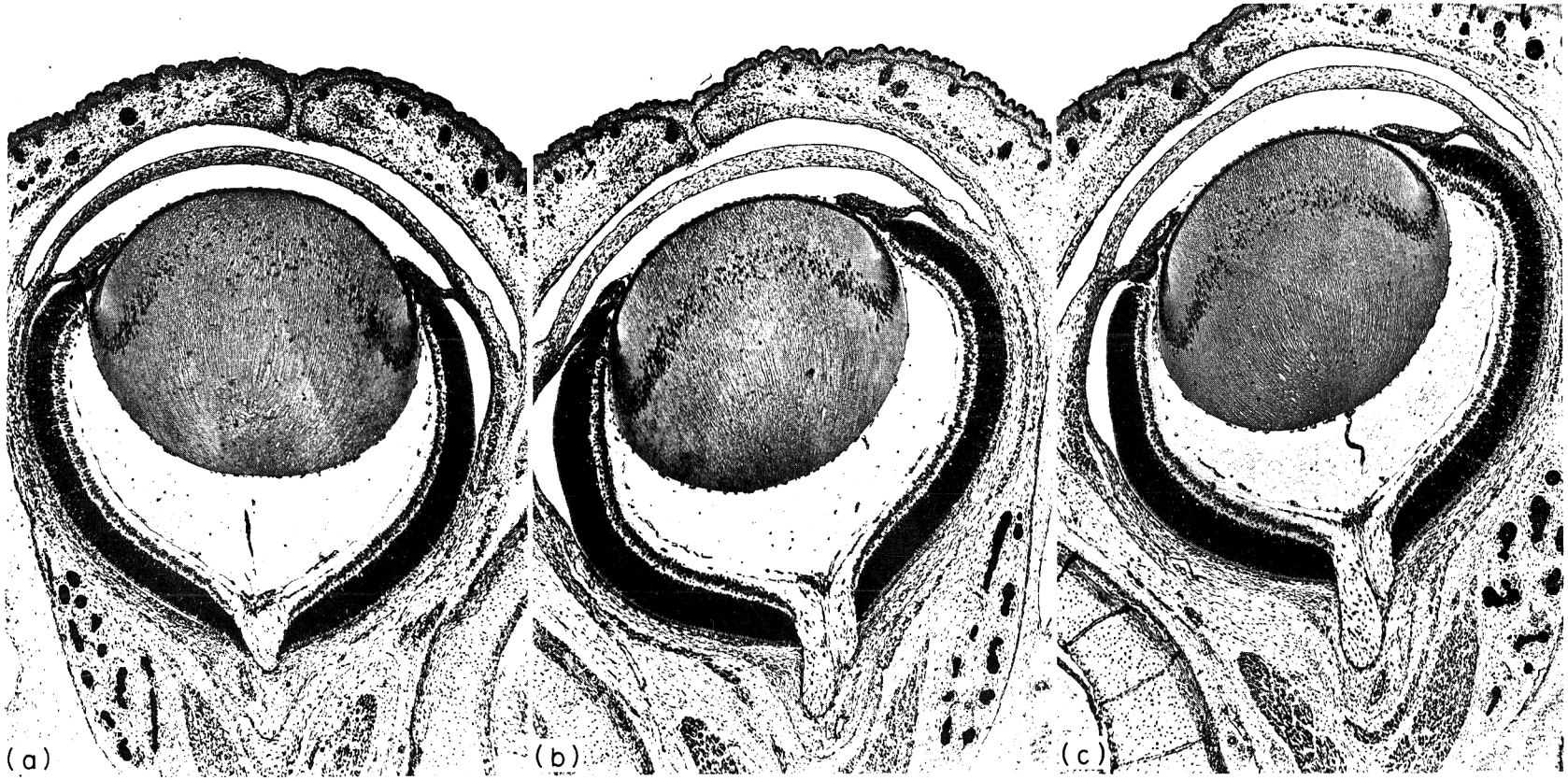

Fig. 2. Normal ocular condition in 20-day-old rat foetuses of (a) the control group, (b) the sodium cyclamate group and (c) the sodium saccharin group. In (a) and (c), remnants of the hyaloid artery are still visible in the vitreous body and at the caudal pole of the lens. The lid sutures are shown at the top and the insertion of the optic fascicle is shown at the bottom of the plates. Haemalum–eosin × 40 approx.

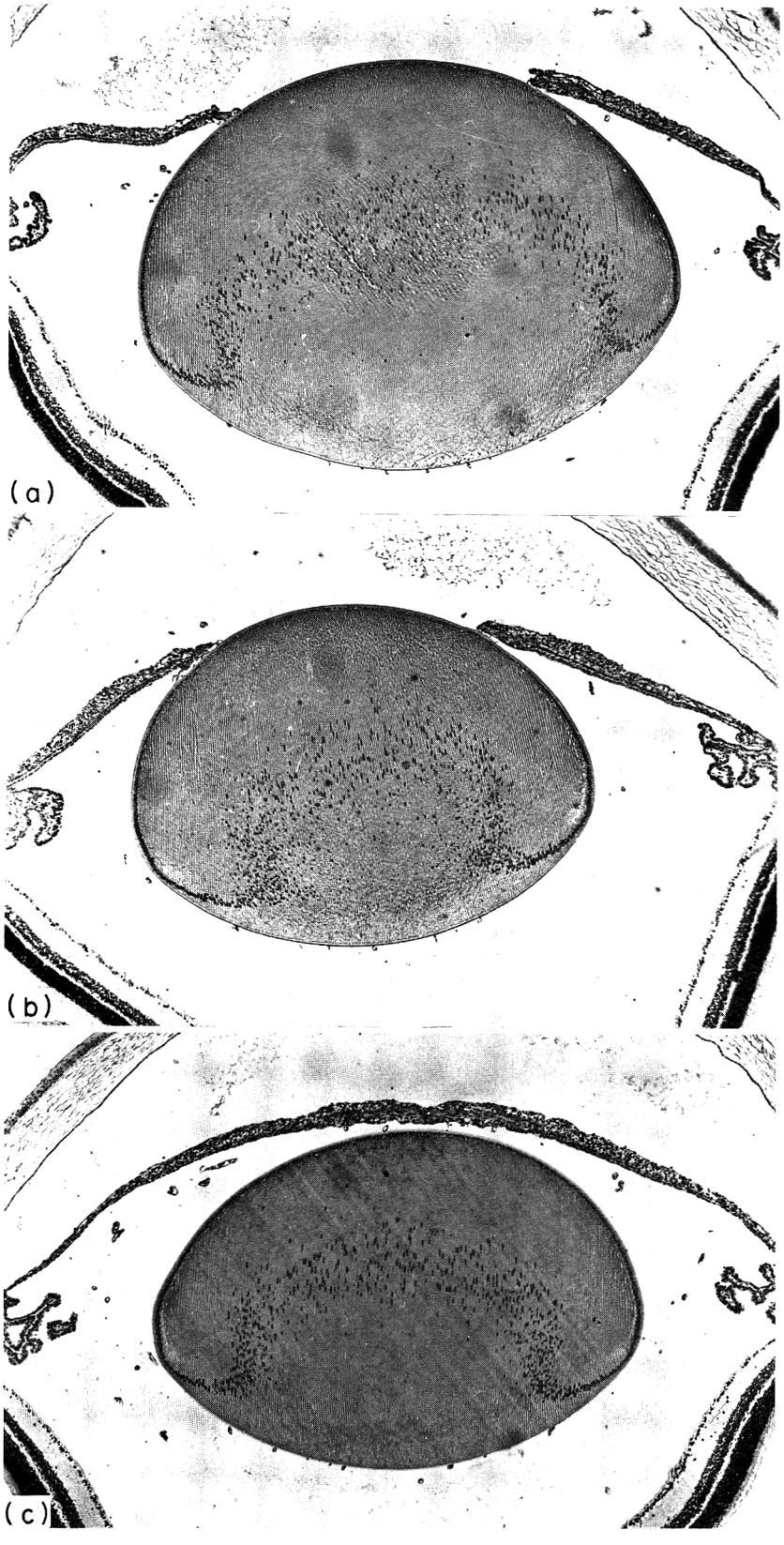

Fig. 3. Normal ocular condition in 3-week-old rats of (a) the control g-oup. (b) the sodium cyclamate group and (c) the sodium saccharin group. Partial view comprising the lens with parts of the cornea, choroid and retina. Haemalum-eosin × 40 approx.

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FORMATION OF METHYLNITROSOCYANAMIDE FROM METHYLGUANIDINE AND SODIUM NITRITE IN ACIDIC SOLUTION

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Abstract—An analytical method developed for determining methylnitrosocyanamide (MNC) and methylnitrosourea (MNU) in aqueous solution and involving extraction with methylene chloride after adjustment of the pH of the solution to 5, separation of the compounds by column chromatography on silica gel, and measurement of their absorbance at 385 nm for MNC and 394 nm for MNU, was used to study the rates of formation of MNC from methylguanidine and nitrite. Initial rates of formation of MNC were proportional to the concentration of methylguanidine and to the square of the concentration of nitrite at pH 1·2 and 2·3. Rate constants of the reaction at these pH values were 1·97 and 0·15 L^2/mol^2 hr, respectively.

INTRODUCTION

Formation of methylnitrosocyanamide (MNC) by nitrosation of methylguanidine (MG) under acidic conditions was first reported by Mirvish (1971). This reaction attracted our attention for several reasons. The first was the structural similarity between MG and *N*-methyl-*N*-nitrosoguanidine, which was shown by McKay & Wright (1947) to be easily convertible by nitrosation under acidic conditions to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), a well-known mutagen (Adelberg, Mandell & Ching Chen, 1965; Mandell & Greenberg, 1960) and gastric carcinogen (Sugimura & Fujimura, 1967; Sugimura, Fujimura & Baba, 1970; Sugimura, Fujimura, Kogure, Baba, Saito, Nagao, Hosoi, Simosato & Yokoshima, 1969). The second was the fact that of the various naturally occurring guanidine derivatives so far tested, MG was the only one showing mutagenicity after nitrosation in gastric juice (Endo & Takahashi, 1973a). The third was that MNC, which was identified as the mutagenic principle of nitrosated MG, is similar to MNNG in its mutagenic quality but its mutagenic potency is about ten times higher (Endo & Takahashi, 1973b). The fourth was the strong carcinogenicity of MNC to the forestomach and oesophagus of rats (Endo, Takahashi, Kinoshita & Baba, 1974). The fifth was the experimental finding that MG was formed from creatine or creatinine by heating with copper ion (Endo, Ishizawa, Endo, Takahashi, Utsunomiya, Kinoshita & Baba, 1977). All these results led us to study the nitrosation of MG in more detail.

In this paper we report on the stability of MNC, the nitrosation product of MG, and on the kinetics of MNC formation from MG. In the nitrosation of MG, methylnitrosourea (MNU) coexists with MNC in the reaction mixture. The stability and formation of MNU and the separation of MNC and MNU are also described.

EXPERIMENTAL

Chemicals. Oxalate, phthalate, phosphate and borate buffer solutions were purchased from Katayama Chemical Industries Co., Osaka. Buffers based on Na_2HPO_4–citric acid and KCl–HCl were prepared according to a manual (Chitani & Kondo, 1958). MG hydrochloride (from Tokyo Chemical Industry Co., Tokyo) was dried to constant weight over silica gel before use. MNC was synthesized according to the method Mirvish, Nagel & Sams (1973) and further purified by fractional distillation. MNU was kindly supplied by Dr. M. Nakadate, National Institute of Hygienic Sciences, Tokyo. Silica gel for column chromatography was Wakogel S-1 (Wako Pure Chemical Co., Osaka), which was activated at 130 C for 3 hr and then deactivated by adding water (5% w/w). The other reagents and solvents used were of special reagent grade.

Apparatus. Ultraviolet absorption spectra were obtained with a UV-200S spectrophotometer (Shimadzu Seisakusho Ltd., Kyoto). Mass spectra of gas-chromatographic peaks (GC–MS analysis) were obtained with a combined JGC-20K gas chromatograph and JMS-D100 mass spectrometer (Japan Electron Optics Laboratory Co., Tokyo). Measurements of pH were made on a F-5 pH meter equipped with a 6028-10T combination electrode (Horiba Seisakusho Ltd., Kyoto). Incubations were carried out in a M-102 incubator (Toyo Kagaku Sangyo Ltd., Osaka).

Stability of MNC and MNU in aqueous solution in relation to pH. Authentic MNC or MNU dissolved in methanol, was added in volumes of 2 ml to 100 ml of buffer solutions ranging in pH from 1·7 to 9·1, and the solutions were incubated at 37°C. At intervals between 2 min and 24 hr, 5-ml samples were withdrawn from each buffer solution for ultraviolet absorption measurements. Absorbance at 385 nm for MNC or at 390 nm for MNU was compared with that of the original solution.

Stability of MNC and MNU in solvents. Authentic MNC or MNU was dissolved in methylene chloride, methanol or ethyl ether in concentrations of approximately 5 mM, and the solutions were kept at 5°C in a refrigerator. After 1, 5 and 24 hr, a sample was withdrawn from each solution and subjected to ultraviolet absorption measurement, the absorbances at 385 or 390 nm being compared with those of the original solutions.

GC–MS analysis of the reaction products from MG and nitrite. A solution containing MG (50 mM) and $NaNO_2$ (80 mM) in 50 ml KCl–HCl buffer at pH 1·2 was kept at 37°C for 1 hr and then extracted with ethyl ether (5 × 10 ml) after adjustment of the pH to 5 by addition of 10% Na_2CO_3. The extracts were concentrated to a small volume and subjected to GC–MS analysis, under the following conditions: glass column (2 mm × 2 m) packed with 10% OV-17 on Chromosorb W AW DMCS (60–80 mesh); helium (30 ml/min) as carrier gas; temperatures of inlet, column and enricher, 90, 60 and 90°C, respectively; ionizing current, 300 μA; ionizing energy, 25 eV.

Formation of MNC and MNU from MG and nitrite. Various concentrations of MG (30–70 mM) and $NaNO_2$ (40–110 mM) were prepared in KCl–HCl buffer solution (50 ml) at pH 1·2 or 2·3 and incubated at 37°C for 15 min–4 hr. After a known reaction time, 10% Na_2CO_3 was used to adjust the pH of the buffer solution to 5 and the solution was then extracted with methylene chloride (5 × 15 ml). The extracts were dehydrated by passing through a bed of anhydrous Na_2SO_4 and concentrated to 5–10 ml in a pear-shaped flask fitted with a Snyder distilling column. During the concentration procedure, the temperature of the water-bath was kept below 60°C. The concentrated methylene chloride was placed on a column of silica gel (3 g), which was eluted with 20 ml methylene chloride, yielding four fractions each of 4 ml, and then with 20 ml methanol. MNC was eluted in two or three of the methylene chloride fractions, while MNU was in the methanol fraction.

Quantitative analysis of MNC and MNU. Ultraviolet absorption spectra of all the fractions were taken in the 350–420 nm range. Concentrations of MNC in the methylene chloride fractions were estimated from a calibration curve showing the relationship between absorbance at 385 nm and the concentration of authentic MNC in methylene chloride. When ultraviolet absorption spectra of the methanol fraction and authentic MNU in methanol were compared, absorbance of the former was relatively higher than that of the latter only in the shorter wave-length region. The base-line method was therefore adopted for the estimation of MNU. For this purpose the calibration curve was depicted in accordance with the relationship between the absorbance obtained from the equation: $A = A_{394} - (A_{385} + A_{403})/2$, where A_λ was absorbance at the wave length of λ nm, and the concentration of authentic MNU in methanol. The calibration curves showed straight lines in the range of concentrations from 10 to 100 μg/ml for MNC and from 20 to 200 μg/ml for MNU.

RESULTS

Stability of MNC and MNU in aqueous and organic solutions

Relative ultraviolet absorbances of MNC or MNU in various buffer solutions are listed in Table 1. Similarly, Table 2 lists relative absorbances of MNC or MNU in organic solvents. Absorbance at 385 nm (MNC) decreased with time when the compound was

Table 1. *Stability of MNC and MNU in different buffer solutions at 37°C*

			Relative absorbance* at							
Compound	pH	Time (min)....	2	5	10	30	60	120	180	24 × 60
MNC	1·7		72	57	40	31	—	—	—	—
	4·0		100	99	97	91	84	70	59	—
	5·0		99	99	98	94	89	78	68	—
	6·0		99	99	99	93	87	75	66	—
	6·8		100	100	99	95	88	76	65	—
	9·1		97	92	80	57	—	—	—	—
MNU	1·7		100	99	100	98	96	93	90	55
	4·0		100	100	100	99	99	99	99	61
	5·0		100	100	99	98	96	94	89	44
	6·0		97	96	92	89	68	47	32	0
	6·8		97	93	92	64	45	31	21	2
	9·1		0	0	0					

MNC = Methylnitrosocyanamide MNU = Methylnitrosourea

When the absorption spectrum of the solution was completely changed from that of the original solution, it is shown as —.

*Absorbance at 385 nm for MNC and at 390 nm for MNU expressed in terms of the initial (0 min) absorbance, designated 100.

Table 2. *Stability of MNC and MNU in organic solvents at 5 C*

Compound	Solvent	Time (hr)....	Relative absorbance* at 1	5	24
MNC	Ethyl ether		100	98	94
	Methylene chloride		100	99	94
	Methanol		99	99	92
MNU	Ethyl ether		90	90	85
	Methylene chloride		93	92	85
	Methanol		100	98	93

MNC = Methylnitrosocyanamide MNU = Methylnitrosourea

*Absorbance at 385 nm for MNC and at 393 nm for MNU in ethyl ether or methanol or at 392 nm in methylene chloride expressed in terms of the initial (0 min) absorbance, designated 100.

dissolved in aqueous solution, especially at pH 1·7 and 9·1. At these two pH values the absorption spectrum of the solution after 1 hr was completely different from that of the original solution. This was also the case with all the MNC solutions at 24 hr, when the spectra were very similar to that of MNU in aqueous solution, suggesting conversion of the MNC in these solutions to MNU. Most of the MNC incubated at pH 4·0–6·8 stayed unchanged for 30 min. MNU was relatively stable when dissolved in aqueous solutions of pH 1·7–5·0, but decomposed very quickly at pH 9·1. Both MNC and MNU in aqueous solution were most stable at pH 5. Therefore, this pH value was used to stop the reaction leading to MNC and MNU formation.

MNC and MNU in ethyl ether, methylene chloride or methanol showed a relatively high degree of stability for up to 24 hr, but they gradually decomposed with time. Quantitative determination of MNC and MNU in these solvents must be completed within a few hours.

GC–MS analysis of the reaction products of MG and nitrite

A total-ion-monitor gas chromatogram of the ethyl ether extract of the MG–$NaNO_2$ reaction product showed only two major peaks, the retention times of which were identical with those of authentic MNC and MNU (3·2 and 28·0 min, respectively). The mass spectra of these gas-chromatographic peaks were also identical with those of MNC and MNU (Figs 1 & 2). These facts indicated that MNC and MNU were the only major compounds produced from the mixture of MG and $NaNO_2$ in acidic solution and extracted with ethyl ether. Separation of the two products as described in the section on analytical procedure, must be effected before they can be estimated quantitatively by ultraviolet spectrophotometry.

Formation of MNC and MNU from MG and nitrite

Recovery rates for 775 μg MNC and 1030 μg MNU dissolved in 50 ml buffer at pH 5·0 were determined by carrying out the analytical procedures, described in the previous section, in triplicate. The recoveries were on average 86% for MNC and 62% for MNU. Values for MNC and MNU reported in this paper were calculated using these recovery factors.

Figure 3 demonstrates the time course of formation of MNC and MNU from a mixture of 50 mM MG and 60 mM $NaNO_2$ incubated at 37 C in KCl–HCl buffer solution at pH 1·2. The concentration of MNC increased in proportion to time during the first 30 min of the incubation period, and subsequently increased less rapidly, reaching a maximum at 1·25–

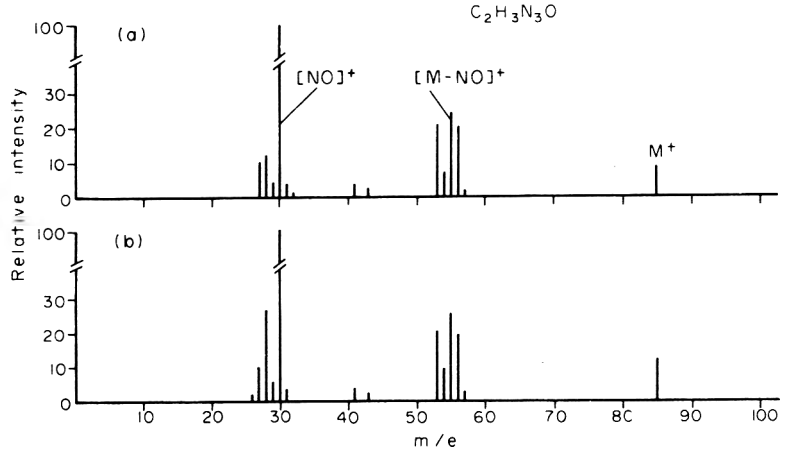

Fig. 1. Mass spectra of gas-chromatographic peak of (a) MNC produced by the reaction of MG and $NaNO_2$ and (b) authentic MNC.

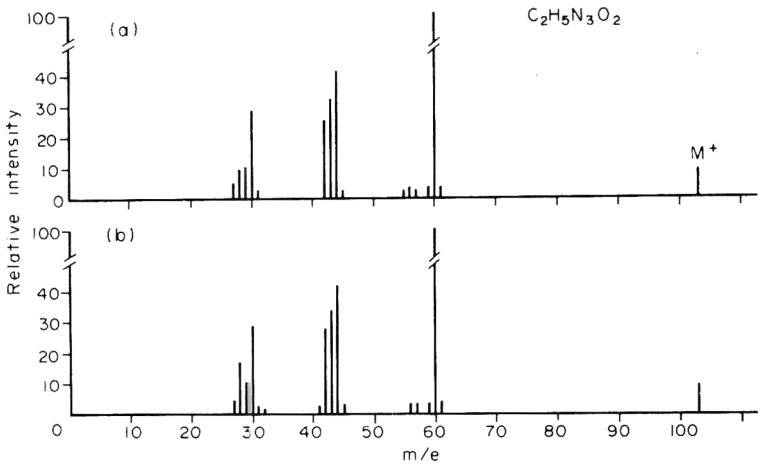

Fig. 2. Mass spectra of gas-chromatographic peak of (a) MNU produced by the reaction of MG and $NaNO_2$ and (b) authentic MNU.

1·5 hr equivalent to a rate of formation from MG of 0·46%. Thereafter it gradually decreased. The formation of MNU increased roughly in proportion to the incubation time, the yield at 3 hr being 2·0%, calculated on the MG.

The time course of formation of MNC and MNU from MG (50 mM) and $NaNO_2$ (100 mM) at pH 2·3 is shown in Fig. 4. The concentration of MNC increased with the time of incubation from 20 min to 2 hr and thereafter remained constant at a level equivalent to a rate of formation of 0·42% calculated on the MG. The formation of MNC was slower at pH 2·3 than at pH 1·2, although in the former experiment the initial concentration of $NaNO_2$ was higher than in the latter. The concentration of MNU increased almost linearly from 20 min to 3 hr, the formation of MNU at 3 hr being 0·98% calculated on the MG concentration.

An incubation time of 30 min was considered most appropriate for the examination of the initial formation of MNC, because formation was proportional to the incubation time for 30 min or more at pH 1·2 and 2·3. The initial rate of MNC formation at pH 1·2 was proportional to the concentration of MG when the concentration of $NaNO_2$ was constant at 60 mM and was proportional to the square of the $NaNO_2$ concentration when the concentration of MG was constant at 50 mM (Table 3). The relationship between the initial rate of MNC formation at pH 1·2 and 2·3 and the initial concentrations of MG and $NaNO_2$ was third order with respect to the MG concentration and the square of the $NaNO_2$ concentration, as shown in Table 3 and Fig. 5. Rate constants of the reaction obtained from Fig. 5 were 1·97 L^2/mol^2 hr at pH 1·2 and 0·15 L^2/mol^2 hr at pH 2·3. Formation of MNC was much faster at pH 1·2 than at pH 2·3, and at both pH 1·2 and 2·3 it was more dependent on the initial concentration of $NaNO_2$ than on that of MG.

The relationship between the initial rate of MNU formation at 30 min and the initial concentrations of MG and $NaNO_2$ did not show any simple relation like that deduced for the formation of MNC.

DISCUSSION

M. Ishizawa, T. Utsunomiya, N. Kinoshita & H. Endo (unpublished data 1977) determined the time of formation of MNC from MG and $NaNO_2$ in simulated gastric juice (pH 1·2) by testing for mutagenic activity in *Salmonella typhimurium*. The mutagenic activity, attributed to the MNC formed, increased with incubation time at the beginning, reached a peak at 40–60 min, indicating approximately 0·5% conversion

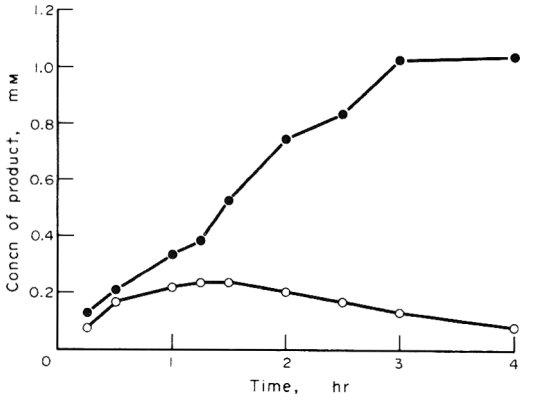

Fig. 3. Time course of formation of MNC (○) and MNU (●) from a mixture of 50 mM-MG and 60 mM-$NaNO_2$ incubated in buffer solution at pH 1·2 and 37° C.

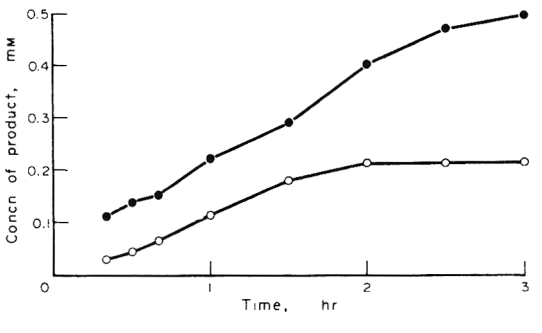

Fig. 4. Time course of formation of MNC (○) and MNU (●) from a mixture of 50 mM-MG and 100 mM-$NaNO_2$ in buffer solution at pH 2·3 and 37°C.

Table 3. *Initial rates of MNC formation on nitrosation of MG with nitrite for 30 min*

pH	Initial concn of MG (mM)	Initial concn of $NaNO_2$ (mM)	MG. $(NaNO_2)^2$ ($mM^3 \times 10^4$)	Initial rate of MNC formation (mM/hr)
1·2	30	60	10·8	0·209
	50	40	8·0	0·174
	50	60	18·0	0·331
	50	80	32·0	0·621
	70	60	25·2	0·501
2·3	50	80	32·0	0·038
	50	100	50·0	0·086
	50	110	60·5	0·092

MNC = Methylnitrosocyanamide MG = Methylguanidine

of MG to MNC, and then decreased. This time course of formation and the yield of MNC was very similar to our results.

The initial rate of MNC formation from MG and nitrite at 30 min was proportional to the MG concentration, to the square of the nitrite concentration, and to the hydrogen-ion concentration. This relationship cannot be explained by the kinetics of the two major nitrosation reactions, namely, nitrosation of secondary amine and nitrosation of amide (Mirvish, 1971). In the former reaction, the initial rate of formation of the nitroso compound is proportional to the amine concentration and to the square of the nitrite concentration, while in the latter case the initial rate is proportional to the concentrations of amide, nitrite and hydrogen ion. As the initial rate of MNC formation was proportional to the hydrogen ion concentration, the kinetics of this nitrosation probably followed the latter with a second molecule of nitrite being used simultaneously in the conversion of methylnitrosoguanidine to MNC. The mechanism of the conversion of MG to MNC could involve the relatively slow nitrosation of MG to methylnitrosoguanidine, formation of which is proportional to the concentrations of MG, nitrite and hydrogen ion, and the rapid reaction of the methylnitrosoguanidine with nitrite to form MNC via diazotization of the guanido group and decomposition of the diazonium salt.

The highest levels reported for MG in foods and nitrite in saliva are approximately 100 and 500 ppm, respectively (Fujinaka, Masuda & Kuratsune, 1976; Tannenbaum, Weisman & Fett, 1976). If both concentrations (about 1 and 10 mM, respectively) persist in the stomach after intake of these materials and the pH of the stomach contents is 1·2, the highest concentration of MNC formed in the stomach after 1·25 hr may be calculated to be less than $1{\cdot}97 \times 10^{-7}$, or about 17 ng/ml. This very low concentration of MNC seems unlikely to cause cancer in the stomach, because the lowest concentration of MNC to induce mutagenic action in *S. typhimurium* was about 85 ng/ml (Endo & Takahashi, 1973b). However, if local concentrations of MG and nitrite happened to be increased more than tenfold, it is possible that MNC might be formed in sufficiently high concentration to cause cancer at those sites in the stomach.

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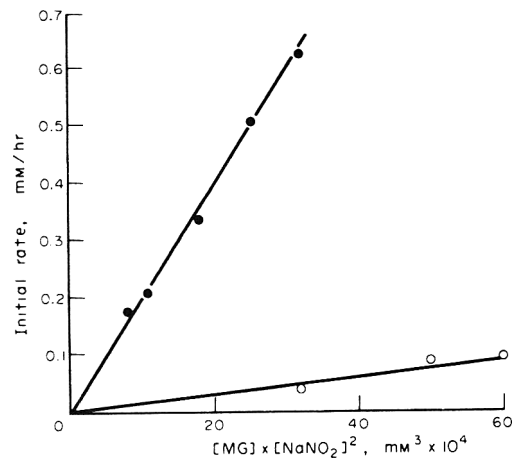

Fig. 5. Relation between initial rate of MNC formation and initial concentrations of MG and $NaNO_2$ incubated at 37°C buffer solution of pH 1·2 (●) or 2·3 (○) for 30 min.

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PESTICIDE-INDUCED MODIFICATION OF HEPATOBILIARY FUNCTION: HEXACHLOROBENZENE, DDT AND TOXAPHENE*

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Abstract—The effect of pre-expose to hexachlorobenzene (HCB), toxaphene (TX) or DDT (100 ppm in the diet for 8 days in each case) on the excretory function of the bile was investigated using isolated perfused rat-liver preparations obtained from treated and control animals. Hepatic uptake, metabolism and biliary excretion of [^{14}C]imipramine (IMP) was examined in these studies. Biliary excretion of endogenously formed metabolites of IMP was enhanced by pre-exposure to HCB, by 18% after 2 hr of perfusion. Pre-exposure to DDT and TX resulted in a decrease in biliary excretion of endogenously formed metabolites of IMP. HCB-induced enhancement of biliary excretion was not associated with either the bile flow or the rate at which IMP was metabolized. However, DDT and TX pretreatments elicited a decrease in the rate of IMP metabolism as well as in bile production, observations commensurate with the decrease in biliary excretion. Biliary excretion of exogenously provided, readily excretable metabolites of IMP was decreased by pre-exposure to HCB as well as to DDT or TX. In the light of these observations an alternative mechanism for HCB-induced modification of biliary excretion has been proposed. This mechanism assumes the existence of a 'synthetic' and a 'post-synthetic' pool of metabolites, the biliary removal of metabolites from each of these pools being subject to modification by HCB. An enhancement of the biliary excretion of IMP metabolites from the 'synthetic' pool and impairment of removal from the 'post-synthetic' pool would be consistent with the above results.

INTRODUCTION

Several recent investigations have suggested interaction of pesticidal chemicals with hepatobiliary function (Mehendale, 1976a & 1977a,b; Reyes, Levi, Gatmaitan & Arias, 1971). Mirex and Kepone, two structurally related chlorinated hydrocarbon pesticides, are known to impair the excretory function of the bile in experimental animals (Mehendale, 1976a & 1977a,b). Pre-exposure to DDT or dieldrin increases hepatic uptake of sulphobromophthalein (BSP) (Reyes *et al.* 1971), and this effect is accompanied by a simultaneous increase in the liver-cytosol binding protein 'Y' now commonly referred to as ligandin. Although the precise mechanism of Kepone- and mirex-induced impairment of biliary excretory function is unknown, it was dissociated from bile flow or an altered rate of metabolism (Mehendale, 1977a,b).

Modification of hepatic drug-metabolizing enzymes by a number of chlorinated hydrocarbon pesticides is well documented (Conney, 1967). Hexachlorobenzene (HCB; Mehendale, Fields & Matthews, 1975), DDT (Bunyan, Townsend & Taylor, 1972) and toxaphene (TX; Fitzhugh & Nelson, 1951) are chlorinated hydrocarbon pesticides of diverse chemical structure (Fig. 1) and are known to induce hepatic drug-metabolizing enzyme activities. There is also evidence that some of these chlorinated hydrocarbon pesticides are excreted via the bile (Paschal, Roan & Morgan, 1974).

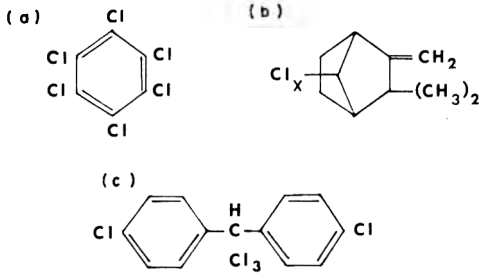


Fig. 1. Structures of (a) hexachlorobenzene, (b) toxaphene and (c) DDT.

These observations suggest some potential for interaction of these toxic chemicals with the hepatobiliary system, affecting biliary excretion of either endogenous or exogenous substances.

The immediate objective of these investigations was to determine whether chlorinated pesticides structurally unrelated to Kepone and mirex were capable of modifying hepatobiliary function. HCB, DDT and TX were chosen for these investigations as models of structurally diverse chlorinated hydrocarbon pesticides, and hepatic uptake, metabolism and biliary excretion of a commonly used antidepressant drug, imipramine (IMP), by isolated perfused rat-liver preparations obtained from treated and control animals was used to test the effect of these toxic chemicals on the hepatobiliary system. Of additional interest was the mechanism(s) by which these chemicals modify hepatobiliary function.

*A preliminary report of these investigations was presented at the Sixteenth Annual Meeting of the Society of Toxicology, Toronto, Canada, 27–30 March 1977.

EXPERIMENTAL

Animals. Male Sprague–Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) were maintained in the central animal facilities away from any known contaminants. The bedding used for these animals was made from untreated corncobs. Animals were provided with water and a standard diet of Purina rat chow blocks *ad lib.* until used for an experiment as a liver or blood donor, except when both treated and control animals received powdered diet. Animals from which blood was collected weighed 250–300 g, whereas the animals used as liver donors weighed 200–250 g. During all surgical procedures, animals were maintained under ether anaesthesia.

Materials. HCB (97% purity, Aldrich Chemical Co., Milwaukee, WI) was recrystallized repeatedly from cold benzene to obtain a purity above 99% as judged by gas-chromatographic analysis. TX (technical chlorinated camphene) was a gift from Hercules, Inc., Wilmington DE., DDT (99% purity, *p,p'*-DDT) was obtained from City Chemical Corp., New York, and [^{14}C]IMP hydrochloride was purchased from Amersham/Searle Corp., Des Plaines, IL. Unlabelled IMP and BSP were obtained from Geigy Pharmaceuticals, Ardsley, NY and Sigma Chemical Co., St. Louis, MO, respectively. [^{3}H]Ouabain was obtained from New England Nuclear Corp., Boston, MA. Emulphor-620 used for introducing any insoluble compound into the perfusate was a gift from GAF Corp., New York. The other chemicals and solvents used in these studies were of reagent or scintillation grade, as required.

Treatment. Rats were pretreated with TX, HCB or DDT at a level of 100 ppm in the daily ration for 8 days. The desired amount of a compound was dissolved in acetone and this solution was mixed with the ground rat chow (50 ml solution/kg food). Control diet was prepared similarly but without any of the above compounds. The final diet was spread in a large pan in a ventilated hood for the acetone to evaporate. Two treated or control animals were maintained in each cage and allowed only to consume either a treated or the control diet and water *ad lib.* On day 9, control and treated animals were used as liver donors for isolated perfused rat-liver preparations.

Perfusions. The liver perfusion apparatus, the 30% rat-blood perfusate, and surgical and perfusion techniques have been described previously (Mehendale, 1976b). The volume of recirculating perfusate (150 or 115 ml) depended on whether or not perfusate samples were required for extraction of metabolites. After the initial equilibration period of 30 min, experiments were commenced by adding a bolus amount of [^{14}C]IMP at an initial concentration of 2×10^{-4} M. The flow rate remained constant throughout the experiment and no differences were observed in the perfusion flow rates between preparations from control and treated animals. Blood perfusate samples (5 or 0·5 ml) were withdrawn at 5, 10, 15, 30, 60 and 120 min. Appropriate corrections were made in the calculations for the amount of radiolabel removed at each time. Bile was collected in 2·5-ml graduated centrifuge tubes at 15, 30, 60 and 120 min. At the end of the 2-hr experiment, the liver was removed from the perfusion system, weighed and homogenized in three parts ice-cold distilled water and the homogenate was either kept ice-cold for immediate extraction or frozen at −20°C for later use.

In some experiments, polar metabolites of IMP were added to the perfusate of isolated perfused liver preparations obtained from treated and control animals. The source of polar metabolites was bile previously collected from analogous isolated perfused-liver experiments with [^{14}C]IMP. This contained less than 2% of the ^{14}C as IMP and desmethylimipramine (Moldowan & Bellward, 1974), the balance consisting of polar metabolites of IMP, principally glucuronides of various hydroxylated metabolites, but also the hydroxylated metabolites themselves and *N*-oxides. The experimental protocol used for these studies was exactly as described above.

In some additional experiments, 55 μmol [^{3}H]ouabain was added to the perfusate (115 ml) of control and treated rat-liver preparations. Blood perfusate (0·5 ml) was collected 5, 10, 15, 30 and 60 min and bile 15, 30, 45 and 60 min after the addition of [^{3}H]ouabain.

Extraction and analyses. Blood perfusate, bile and liver were extracted and analysed for IMP and metabolites by the procedures described by Moldowan & Bellward (1974). Samples of blood perfusate (0·1 ml plasma), bile (0·025 ml) and liver homogenate (0·2 ml 1:3 homogenate) were radioassayed in 10 ml Aquasol (New England Nuclear Corp.). Metabolites were determined by ^{14}C assay after extraction of various samples. In experiments in which biliary excretion of ouabain was studied, samples of perfusate, bile and liver were assayed for ^{3}H as described for ^{14}C. Total ^{14}C and ^{3}H recoveries ranged from 96 to 103% and there were no differences in recovery between control and treated liver preparations. All radioassays were carried out in a Beckman Model LD-250 scintillation spectrometer with an internal standard for quench correction. Statistical analyses were done with Student's *t* test for significance at $P < 0{\cdot}05$.

RESULTS

Biliary excretion of endogenous [^{14}C]IMP metabolites

Results of biliary excretion of endogenously formed metabolites of IMP by control and pretreated liver preparations are illustrated in Figs 2 and 3. Biliary excretion was 18% higher than in the control preparations after pre-exposure to HCB (Fig. 2), as judged by the percentage of the dose excreted in bile after perfusion for 2 hr. During earlier stages there was a slight decrease in biliary excretion which was overcome by about 1 hr. Pre-exposure to TX and DDT had an opposite effect (Fig. 3). TX-pretreated liver preparations had excreted only 30% of the radioactivity after 2 hr of perfusion, representing a 23% decline in biliary excretion compared with control liver preparations. Biliary excretion was also suppressed as a result of pre-exposure to DDT, but this decline (14% at 2 hr) was not statistically significant. The effect of pre-exposure to TX was also more pronounced at the other sampling times.

Rate of bile flow

Biliary excretion of several compounds has been directly associated with the rate of bile flow (Goldstein & Taurog, 1968; Hart, Guarino & Adamson,

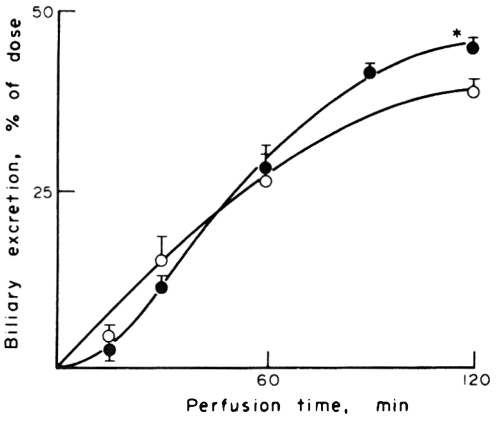


Fig. 2. Cumulative biliary excretion of endogenously formed metabolites of [^{14}C]imipramine by isolated perfused liver preparations from rats fed either a control diet (○) or a diet containing 100 ppm hexachlorobenzene (●) for 8 days. Results, expressed as a percentage of the dose of ^{14}C, are means ± SEM for four experiments and the point marked with an asterisk differs significantly ($P < 0.05$) from the control value.

1969; Klaassen, 1970; Klaassen & Plaa, 1968; Roberts & Plaa, 1967). Hence, the effect of pre-exposure to HCB, TX and DDT on the volume of bile produced was examined (Fig. 4). Generally, bile-flow data have been expressed in the literature in terms of body weight, but expression of the volume of bile produced in terms of liver weight might be more appropriate, since liver mass would be expected to have a more direct influence on bile secretion. Hence, bile-flow data in these experiments have been expressed in terms of unit liver weight (Fig. 4a). For comparison the same data has been expressed also in terms of body weight (Fig. 4b). The overall results are not affected by the method of presentation of the data; rate of bile flow was unaffected by pre-exposure to HCB, but was decreased by pre-exposure to DDT or TX. The decrease in bile flow was greater with TX than with DDT, a result in agreement with the biliary excretion of endogenous metabolites of IMP.

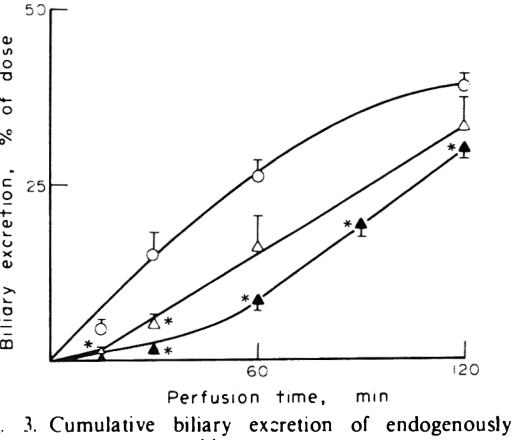


Fig. 3. Cumulative biliary excretion of endogenously formed metabolites of [^{14}C]imipramine by isolated perfused liver preparations from rats fed either a control diet (○) or diet containing 100 ppm DDT (△) or toxaphene (▲) for 8 days. Results, expressed as a percentage of the dose of ^{14}C, are means ± SEM of four experiments and points marked with an asterisk differ significantly ($P < 0.05$) from the control value.

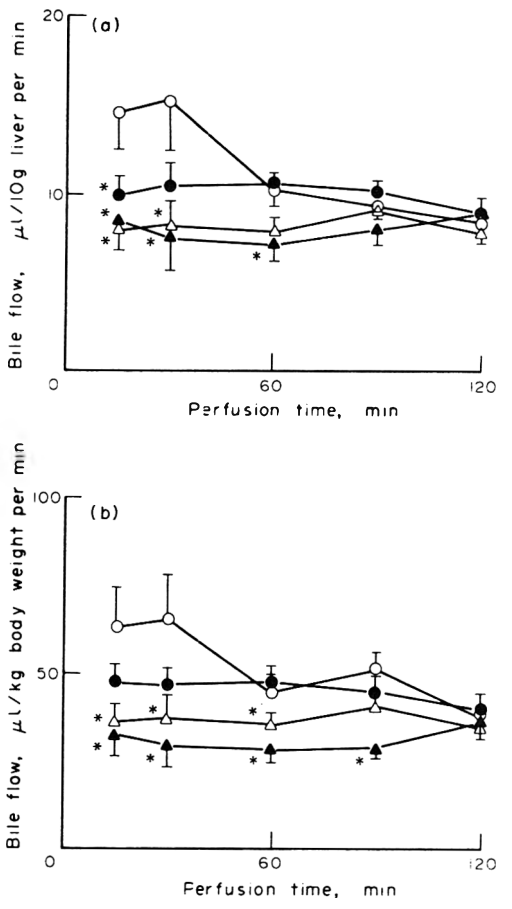
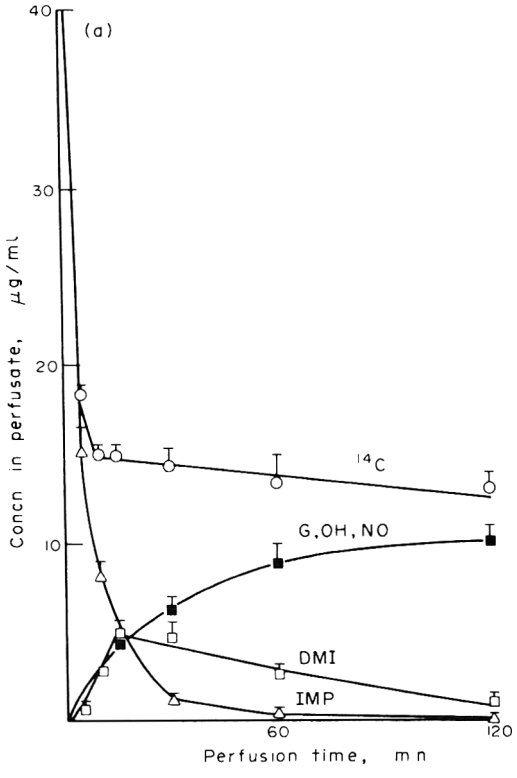


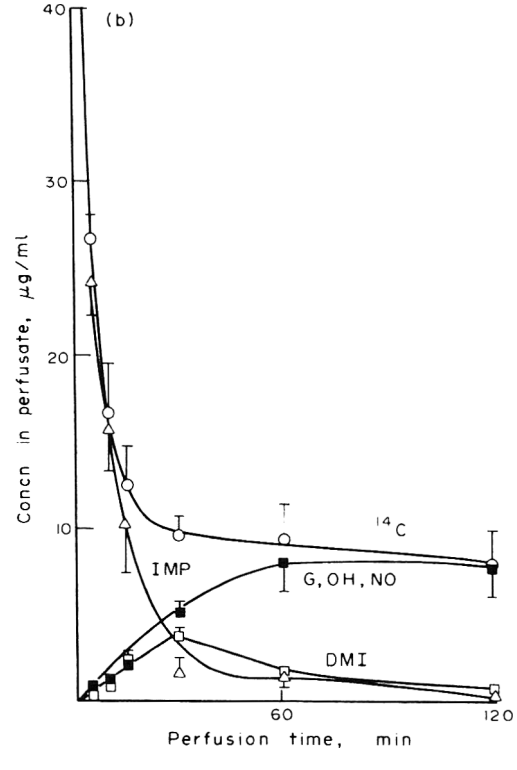
Fig. 4. Effect of 8-day pre-exposure of rats to 100 ppm dietary hexachlorobenzene (●), DDT (△) or toxaphene (▲) on the volume of bile produced by isolated perfused liver preparations and expressed as a function of (a) liver weight and (b) body weight. Results are the means ± SEM of eight experiments and an asterisk indicates those that differ significantly ($P < 0.05$) from the control value.

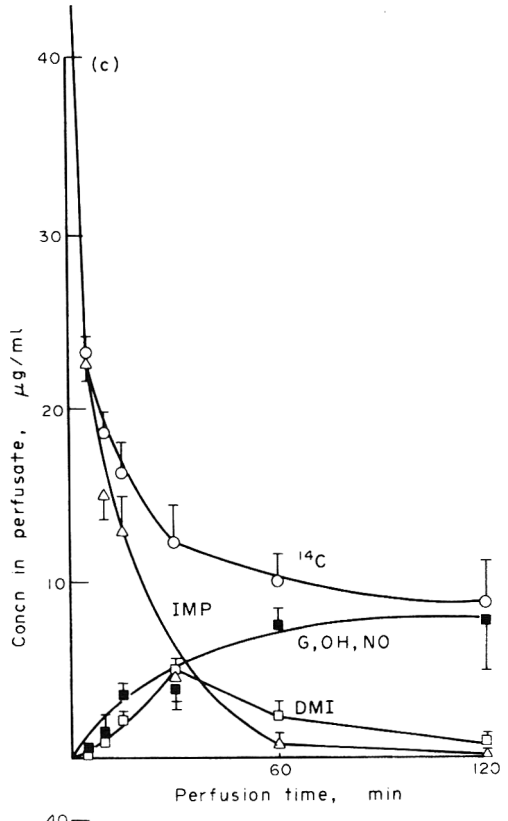
Disposition of [^{14}C]IMP

Data on the disappearance of IMP from the circulating perfusate and the appearance of various metabolites in the perfusate are illustrated in Fig. 5. These parameters were investigated in order to examine whether any alterations in hepatic uptake and metabolism of IMP could account for the modification of the biliary excretion of IMP metabolites. Hepatic uptake and metabolism of IMP were unaffected by pre-exposure to HCB (Fig. 5b). There was a slight decrease in the total ^{14}C and polar metabolite concentrations in the perfusate, observations commensurate with the enhanced biliary excretion (Fig. 2). TX and DDT pretreated liver preparations exhibited a decreased rate of metabolism of IMP as judged by the upward shift in the disappearance curves for IMP (Fig. 5c,d). The decreased rate of hepatic uptake and metabolism persisted for the first hour of the perfusion. This effect was more pronounced with TX preparations, which also showed, in corroboration of this observation, a slightly decreased concentration of polar metabolites up to 1 hr. The tendency for the rate of appearance of polar metabolites of IMP to

be slightly reduced following TX exposure may also be a reflection of a slight decrease in the rate of uptake by the liver. Reduced rate of uptake by the liver and/or subsequent metabolism after pre-exposure to DDT and TX appear to account for the decreased biliary elimination of IMP metabolites.







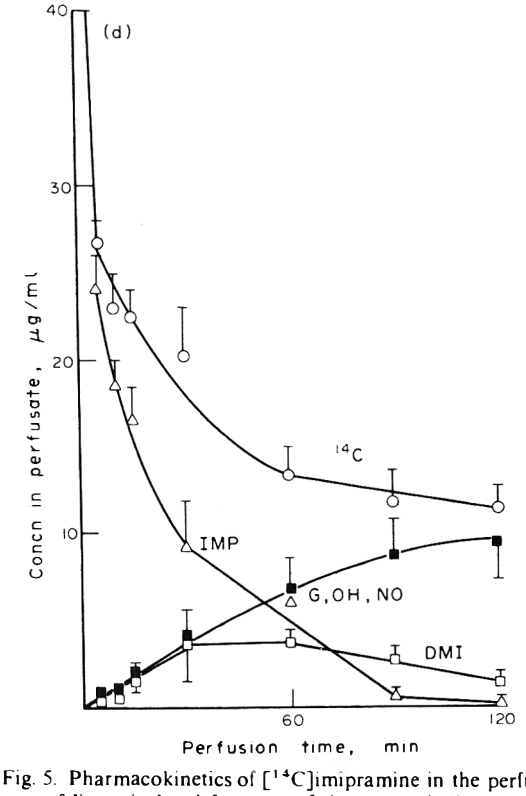


Fig. 5. Pharmacokinetics of [^{14}C]imipramine in the perfusate of livers isolated from rats fed a control diet (a) or diet containing 100 ppm hexachlorobenzene (b), DDT (c) or toxaphene (d). Total radioactivity (^{14}C) was separated into imipramine (IMP), desmethylimipramine (DMI) and polar metabolites, namely glucuronides, free hydroxylated metabolites and *N*-oxides (G, OH, NO). Results are means ± SEM for four experiments.

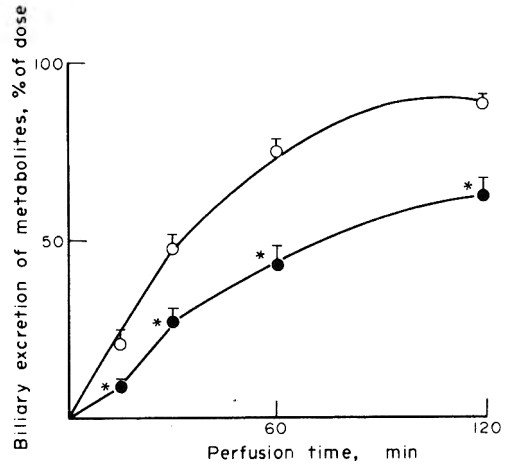


Fig. 6. Effect of hexachlorobenzene pretreatment (●) on the cumulative biliary excretion of exogenously provided polar metabolites of [^{14}C]imipramine by isolated perfused rat-liver preparations. Results are means ± SEM for four experiments and an asterisk marks those that differ significantly ($P < 0.05$) from the control value (○).

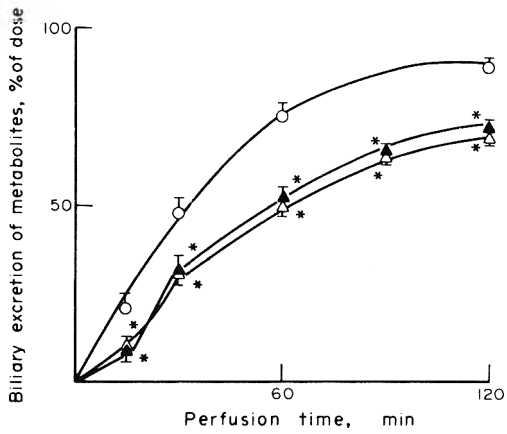


Fig. 7. Effect of DDT (△) or toxaphene (▲) pretreatment on the cumulative biliary excretion of exogenously provided polar metabolites of [^{14}C]imipramine by isolated perfused rat-liver preparations. Results are means ± SEM for four experiments and an asterisk marks those that differ significantly ($P < 0.05$) from the control value.

Biliary excretion of exogenous [^{14}C]imiprmine metabolites

Since it appeared from the above experiments that both bile flow and the rate at which IMP is metabolized might account for the decrease in biliary excretion induced by pre-exposure to DDT and TX, it was of interest to see whether one of those factors was more influential than the other. Moreover, HCB-induced enhancement of biliary excretion could not be accounted for by either of these mechanisms. It was postulated that if the effect of HCB were unrelated to the rate at which IMP was metabolized, then the HCB effect should also manifest itself on the biliary excretion of exogenously provided, readily excretable, polar metabolites of IMP. Furthermore, if the effects of DDT and TX were in fact related to the rate of metabolism of IMP, biliary excretion of exogenous polar metabolites of IMP would not be expected to be affected. Readily excretable polar metabolites of IMP were added, therefore, to the perfusate of liver preparations obtained from control and HCB-, TX- and DDT-pretreated animals. In contrast to the results when IMP was added, HCB pretreatment elicited a 28% suppression of biliary excretion of otherwise readily excretable metabolites of IMP (Fig. 6). Pre-exposure to DDT and TX also suppressed biliary excretion of IMP metabolites, by 21 and 19%, respectively after a 2-hr perfusion (Fig. 7). These results with DDT- and TX-pretreated liver preparations are in agreement with the decrease in biliary excretion demonstrated when the livers were allowed to synthesize the polar metabolites of IMP.

Hepatic content of ^{14}C-labelled material

At the end of the 2-hr perfusion experiments the hepatic content of total radiolabel was determined as an additional index of hepatic uptake. In the experiments in which [^{14}C]IMP was added to the liver preparations, no significant differences between control and treated liver preparations were found in the quantities of the parent compound and its metabolites. Hence data on the parent-compound and metabolite content of the liver are not given. The total hepatic contents of ^{14}C-labelled material at the end of these experiments, as well as in experiments in which polar metabolites of [^{14}]IMP were added, are given in Table 1 for control and treated livers. Although there was a tendency toward increased retention of radioactivity in the treated livers in both experiments, only TX-treated livers retained a significantly higher quantity. In no case was the uptake of the labelled material by treated liver lower than

Table 1. *Content of radioactivity in isolated livers perfused for 2 hr with fluid containing [^{14}C]IMP or its polar metabolites following removal from rats fed for 8 days on diet containing 100 ppm HCB, TX or DDT*

Pretreatment	Liver-content of ^{14}C† following perfusion with	
	[^{14}C]IMP	[^{14}C]IMP metabolites
Control	41·6 ± 1·4	3·5 ± 0·9
HCB	41·7 ± 3·7	5·9 ± 1·5
TX	51·7 ± 2·8*	6·5 ± 0·5*
DDT	47·5 ± 3·4	5·9 ± 0·8

IMP = Imipramine HCB = Hexachlorobenzene TX = Toxaphene

†Expressed as a percentage of the dose of radioactivity to the liver perfusate. Each value is the mean ± SEM of four individual determinations and those marked with an asterisk differ significantly ($P < 0.05$) from the control values.

Table 2. *Biliary excretion of ouabain determined in isolated perfused livers from rats pretreated for 8 days with 100 ppm HCB, TX or DDT in the diet*

Perfusion time (min)	Biliary excretion of ^3H* in livers from rats fed			
	Control diet	Diet containing		
		HCB	TX	DDT
15	8·5 ± 2·1	12·3 ± 2·4	13·8 ± 2·3	14·7 ± 4·9
30	46·1 ± 3·8	56·5 ± 4·5	46·3 ± 7·4	58·3 ± 6·9
60	80·8 ± 2·3	84·6 ± 3·5	82·8 ± 2·0	86·7 ± 1·7
90	89·1 ± 0·8	91·9 ± 1·5	91·6 ± 0·9	93·1 ± 0·5

HCB = Hexachlorobenzene TX = Toxaphene

*Expressed as a percentage of the dose added to the liver perfusate. Each value is the mean ± SEM of four individual determinations.

that of the control. These results suggest that hindered hepatic uptake may not play a big role in the pesticide-induced modification of biliary excretion of IMP metabolites.

Biliary excretion of [^3H]ouabain

It was of interest to see whether pre-exposure to HCB, DDT or TX would affect biliary excretion of a neutral, non-metabolizable model compound. [^3H]Ouabain was used as an example of a compound that is unchanged and does not require to be metabolized prior to biliary excretion. The results are shown in Table 2. None of the pretreatments affected the biliary excretion of ouabain in a 1·5-hr perfusion.

DISCUSSION

Previous studies demonstrated that mirex and Kepone, two structurally related chlorinated pesticidal compounds, were potent modulators of hepatobiliary function (Mehendale, 1976a & 1977a,b). The primary objective of these experiments was to investigate whether other chlorinated pesticides were also effective modifiers of hepatobiliary function. Of additional interest was the mechanism by which these agents may interfere with the underlying processes governing normal biliary secretory and excretory functions. The results show that HCB, DDT and TX are all potential modifiers of biliary excretion, but that interference by these toxic chemicals may not necessarily result in a predictable response. These toxic agents may either enhance or impair biliary excretory function. In addition to introducing an element of unpredictability, these observations suggest that the quest for the mechanisms by which inducing agents modulate hepatobiliary function may not yield simplistic solutions.

Two documented mechanisms have been proposed to explain xenobiotic-induced modification of hepatobiliary function. An association between biliary excretory function and bile flow (Goldstein & Taurog, 1968; Hart *et al.* 1969; Klaassen, 1970; Klaassen & Plaa, 1968; Roberts & Plaa, 1967) was demonstrated for a variety of compounds. On the other hand, Levine (1972, 1973 & 1974) correlated an increase or decrease in biliary excretion with an appropriate modification of metabolism. In the present investigations, pre-exposure to DDT or TX resulted in a reduction in bile flow as well as a reduced rate of IMP metabolism, results which appear to be consistent with the decreased biliary excretion of endogenously formed metabolites of IMP. However, the role of the rate of IMP metabolism in controlling the biliary excretion of IMP metabolites may be questioned in the light of the suppression of biliary excretion of otherwise readily excretable polar metabolites of IMP irrespective of which compound was used for pretreatment. Moreover, the results of HCB-pretreatment studies indicate no relationship between the modification of biliary excretion and either the rate of bile flow or the metabolism of IMP. It is possible that the rate of metabolism as a factor may play itself out of the picture until a critical level of interference with metabolism is achieved.

In the light of these observations, an alternative mechanism consistent with the results of the present investigations may be proposed. There is experimental evidence for the existence of two pools of metabolites in the hepatic circulation (Gessner & Hamada, 1974; Mehendale, 1976b). One, representing the synthetic phase of metabolism, may be termed the 'synthetic pool', i.e. the pool of metabolites that accumulates during the peak of biotransformation when polar metabolites are synthesized on or in close proximity to the smooth endoplasmic reticulum. This pool of metabolites may lend itself readily to biliary removal. During this phase, a proportion of the metabolites may escape into the cytoplasm of the hepatocytes and gain entry into the vascular system, forming the 'post-synthetic pool' of metabolites. The rates at which these two pools of metabolites lend themselves to biliary excretion vary (Gessner & Hamada, 1974; Mehendale, 1976b). The data on modification of biliary excretory function by pre-exposure to HCB may be explained by assuming the existence of more than one pool of metabolites; HCB has an enhancing effect on the kinetics of biliary removal from the 'synthetic pool' and an adverse effect on the biliary removal from the 'post-synthetic pool' of metabolites. When the liver was given an opportunity to synthesize IMP metabolites, pre-exposure to HCB enhanced the removal of the endogenously formed 'synthetic pool' of IMP metabolites and hence increased biliary excretion. When preformed polar metabolites were provided, thus mimicking the 'post-synthetic pool', pre-exposure to HCB resulted in impaired removal of these metabolites.

The results of these experiments may also be

explained by pesticide-induced modification of hepatic-uptake mechanisms. For instance, hindered uptake of [^{14}C]IMP by TX or DDT-treated liver preparations may explain the reduction in biliary elimination. Although this possibility cannot be completely ruled out at the present time, there are some indications that altered hepatic uptake may have played a relatively minor role in these experiments. For example, the hepatic content of exogenously provided polar metabolites of [^{14}C]IMP in TX-treated liver preparations was in fact increased, an observation suggesting adequate uptake but impaired biliary elimination.

These investigations demonstrate that pre-exposure to pesticidal chemicals may result in a modification of hepatobiliary function. Biliary excretion may be either enhanced as in the case of HCB or compromised as in the case of DDT and TX. These observations imply unpredictability in the actions of a variety of chlorinated hydrocarbons. The mechanism(s) by which these toxic chemicals modulate biliary secretory and excretory functions may involve a variety of interactions, combining to culminate in either enhanced or compromised hepatobiliary function. A systematic dissection of these mechanisms would require investigations with prudently chosen models for modulation of hepatobiliary function.

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INDUCTION PAR LE LINDANE DES MONOXYGENASES MICROSOMALES DU FOIE CHEZ LE RAT: EFFETS D'UN REGIME HYPOCALCIQUE

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Résumé—Nous constituons deux groupes de jeunes rats mâles: le premier reçoit une alimentation normale en calcium (1%), le second un régime hypocalcique (0,06%) pendant 6 semaines. Après 2 semaines, chaque groupe est divisé en deux lots: témoin et traité au lindane. Le traitement par le pesticide, qui consiste en l'addition de 60 ppm dans la nourriture, dure 4 semaines. Le foie et les reins sont prélevés et pesés. Nous mesurons dans les microsomes hépatiques les protéines, la phosphatidylcholine et des activités enzymatiques. Le lindane augmente le poids des reins. Il accélère le métabolisme hépatique de l'aminopyrine ou de l'aniline et il induit le système microsomal de transport d'électrons dépendant du NADPH (*P*-450, cytochrome *c* réductase, phosphatidylcholine). Dans nos conditions expérimentales, le régime hypocalcique ne paraît pas diminuer le métabolisme des substances exogènes bien qu'il abaisse la teneur en *P*-450 des microsomes du foie. Le maintien du niveau métabolique peut s'expliquer par un accroissement du transfert des électrons vers le cytochrome à partir du NADPH; la NADPH-cytochrome *c* réductase et la NADPH-oxydase sont induites. Nous observons une interaction entre les effets du lindane et du régime hypocalcique sur l'activité moléculaire spécifique de la NADPH-cytochrome *c* réductase [réduction du *P*-450 (Fe^{3+})]: l'insecticide empêche l'effet propre (induction) de la déficience en calcium.

Abstract—Two groups of young male rats were fed either a diet with a normal calcium content (1%) or a low-calcium (0·06%) diet for 6 wk. After the first 2 wk, each group was subdivided equally into a control group and a group treated for the remaining 4 wk with 60 ppm lindane in the diet. The rats were then killed, livers and kidneys were removed and weighed and hepatic microsomal fractions were prepared for protein, phosphatidylcholine and enzyme measurements. Lindane increased the kidney weights and the rates of aminopyrine and aniline metabolism in the liver, and induced the NADPH-dependent microsomal electron-transport system (cytochrome *P*-450, cytochrome *c* reductase, phosphatidylcholine). Under the experimental conditions used, the low-calcium diet did not appear to affect the metabolism of exogenous substances, although it lowered the *P*-450 content of the liver microsomes. The maintenance of the normal level of metabolism may be explained by an increase in electron transfer towards the cytochrome from NADPH with induction of NADPH-cytochrome *c* reductase and NADPH-oxidase. Lindane treatment prevented the characteristic inducing effect of calcium deficiency on the specific molecular activity of NADPH-cytochrome *c* reductase [reduction of *P*-450 (Fe^{3+})].

INTRODUCTION

Il est bien établi que l'ingestion de lindane (isomère gamma de l'hexachlorocyclohexane) accélère le métabolisme des substances exogènes. Cet insecticide organochloré induit les enzymes liées au cytochrome *P*-450 (oxygénases à fonction mixte) dans les microsomes du foie. L'induction enzymatique apparaît, à moyen terme, pour une quantité de pesticide ingéré relativement faible. Ainsi, nous avons observé que l'addition de 20 ppm de lindane au régime alimentaire du rat mâle, pendant 4 semaines, augmente la *N*-déméthylation de l'aminopyrine (aminophénazone) et l'hydroxylation de l'aniline (Pélissier et Albrecht, 1976). Une analyse mathématique de nos résultats nous a même permis de proposer 4 ppm comme dose vraisemblablement efficace (Lowy, Albrecht, Pélissier et Manchon, 1977).

Le métabolisme des substances exogènes dépend des conditions nutritionnelles. Dans une récente revue générale, Campbell et Hayes (1974) signalaient qu'un apport alimentaire insuffisant en calcium ralentit le métabolisme hépatique des médicaments. Cette affirmation—importante pour la pratique médicale, particulièrement en pédiatrie—se retrouve fréquemment dans la litterature: elle est donc considérée comme 'classique'. Or, à notre connaissance, elle ne repose que sur une seule expérience, déjà ancienne, de Dingell, Joiner et Hurwitz (1966). Ces chercheurs constataient un ralentissement des métabolismes de l'hexobarbital, de l'aminopyrine et de l'acide *p*-nitrobenzoïque chez des rats nourris pendant 40 jours avec un régime déficient en calcium; ils n'observaient pas d'effet après seulement 33 jours. Dingell *et al.* (1966) ne signalaient pas la quantité de calcium du régime carencé et ne mesuraient pas les composants du système microsomal de transport d'électrons (*P*-450, NADPH-cytochrome *c* réductase, etc.). Il nous a donc paru intéressant de reprendre cette expérience, d'essayer de la compléter, et surtout

d'étudier l'éventuelle interaction des effets entre le lindane et la quantité de calcium alimentaire.

METHODES EXPERIMENTALES

Animaux et traitement. Les animaux sont des rats mâles de souche Wistar CF provenant d'un élevage à flore contrôlée SPF; nous les recevons au sevrage à 18–20 jours. Pendant 1 semaine, nous les acclimatons à notre animalerie; nous leur donnons alors une provende commerciale (Biscuits "Extralabo"). Nous répartissons ensuite les rats en deux groupes. Le premier reçoit une alimentation normale en calcium (1%), le second un régime hypocalcique (0,06%). Les régimes sont fournis par les Etablissements U.A.R. (91—Villemoisson sur Orge); ils comportent en particulier 23% de protides (caséine), 6000 UI de vitamine D et respectivement 0,78 et 1,04% de phosphore. Après 2 semaines, chaque groupe est divisé en deux lots: témoin et traité au lindane. Le traitement par le pesticide, qui consiste en l'addition de 60 ppm dans la nourriture, dure 4 semaines.

Après un jeûne d'une nuit, nous pesons puis sacrifions les rats par décapitation. Le foie et les reins sont prélevés, lavés et pesés. Nous préparons des microsomes hépatiques (250 mg foie/ml) selon le procédé que nous avons décrit (Albrecht, Pélissier, Manchon et Rospars, 1973), et nous en déterminons la teneur en protéines (Lowry, Rosebrough, Farr et Randall, 1951). Nous mesurons la calcémie par photométrie de flamme air-acétylène à 554 nm (Herrmann et Alkemade, 1963); la courbe d'étalonnage est établie à l'aide de solutions de Cl_2Ca 'compensées' par 143,5 mequiv/litre de Na^+ et 3,84 mequiv/litre de K^+.

Phosphatidylcholine des microsomes. Nous nous inspirons de Ackerman et Chou (1960) pour l'hydrolyse de la phosphatidylcholine et de Hayes, MgBodile et Campbell (1973) pour le dosage de la choline. A 2 ml de microsomes (500 mg foie), nous ajoutons 2 ml de solution saturée de $Ba(OH)_2$ et 0,5 ml d'éthanol; nous chauffons le mélange au bain-marie bouillant pendant 20 h. Nous amenons à pH 10 avec CH_3COOH puis nous filtrons et complétons avec de l'eau q.s.p. 25 ml. Nous ajoutons 3 ml de reineckate d'ammonium (à 5% dans du méthanol) et laissons en chambre froide pendant 3 à 4 h. Nous filtrons sur Célite. Le complexe de reineckate de choline, qui est retenu sur la Célite, est lavé trois fois avec 2 ml de 1-propanol puis dissous dans 10 ml d'acétone. Nous mesurons l'absorption optique de l'éluat acétonique à 526 nm. Dans ces conditions, 5 µmoles de choline donnent une densité optique égale à 0,053 (cuve de 1 cm d'épaisseur).

Activité enzymatiques des microsomes. Nous indiquons dans un article antérieur (Pélissier, Manchon, Atteba et Albrecht, 1975) les conditions de mesure de la *N*-déméthylation de l'aminopyrine et de l'hydroxylation de l'aniline. Nous déterminons les teneurs en cytochrome *P*-450 et b_5 des microsomes selon les méthodes de Omura et Sato (1964); le milieu comporte de l'HEPES 0,1 M (acide *N*-2-hydroxyéthylpipérazine-*N*-2-éthanesulfonique) à pH 7,4 et des microsomes correspondant à 40 mg foie/ml. Pour mesurer l'activité de la NADPH-cytochrome *c* réductase, nous modifions légèrement la technique que décrivent Hernandez, Gillette et Mazel (1967): le milieu est tamponné à pH 7,4 avec de l'HEPES 0,1 M et les microsomes sont en quantité correspondant à 1 mg foie/ml. Nous nous inspirons du travail de Gigon, Gram et Gillette (1969) pour déterminer la NADPH-oxydase; le milieu contient de l'HEPES 0,1 M, du NADPH 0,2 mM et des microsomes correspondant à 40 mg foie/ml. Nous mesurons l'activité de la NADH-b_5 réductase (NADH ferricyanure réductase) selon la méthode, légèrement modifiée, de Hrycay et Prough (1974); le milieu comporte HEPES 0,1 M, acide éthylène-diaminotétracétique 1 mM, ferricyanure de potassium 3 mM, NADH 1 mM, microsomes équivalent à 1,7 mg foie/ml.

Analyse statistique. Notre expérience comporte quatre groupes; on dit qu'elle est polyfactorielle 2 × 2 puisqu'elle permet l'étude de deux facteurs (lindane, régime), chacun étant à deux niveaux. Nous testons 1) l'absence d'interaction entre les facteurs; 2) l'effet du lindane; 3) l'effet du régime. Les résultats sont établis par l'analyse de variance (Schwartz, 1966), après vérification de sa légitimité par le test de Bartlett (cf. Snedecor et Cochran, 1957). L'organisation de l'expérience permet d'appliquer la méthode des blocs complets (Lellouch et Lazar, 1974).

Nous indiquons sur les tableaux, pour chaque groupe, la moyenne et la valeur estimée de son écart-type; nous signalons aussi, pour chaque paramètre, la valeur de la variance résiduelle s^2. Nous effectuons une analyse de covariance (Lellouch et Lazar, 1974) pour calculer le poids relatif moyen du foie ou des reins et son écart-type (celui-ci est estimé à partir de la 'variance liée' commune). Nous admettons qu'une différence est significative lorsque la probabilité P qu'elle soit nulle est $\leq 0{,}05$ et très significative pour $P \leq 0{,}01$.

RESULTATS

Caractéristiques des animaux

L'ingestion de lindane, 60 ppm dans l'alimentation pendant 4 semaines, ne modifie pas le poids corporel et le poids relatif du foie (Tableau 1). Le poids relatif des reins est significativement augmenté (+12%).

Le régime hypocalcique, 0,06% de calcium pendant 6 semaines, ralentit significativement la croissance pondérale des rats (−9%) et accroît très significativement (+29%) le poids relatif des reins. La calcémie est significativement abaissée de 28% (Tableau 2).

Nous n'observons pas d'interaction entre les effets du lindane et du régime sur le poids corporel, le poids des organes ou la calcémie.

Activité des microsomes hépatiques

Le lindane ne modifie pas la concentration des protéines microsomales, mais il augmente significativement (+16%) la teneur en phosphatidylcholine (Tableau 2). L'insecticide accélère très significativement de 76% la *N*-déméthylation de l'aminopyrine rapportée aux protéines (Tableau 3) et de 45% l'activité/nmol *P*-450 (activité moléculaire spécifique ou *turnover number*). L'hydroxylation de l'aniline est très significativement accrue de 25% par rapport aux protéines, l'activité moleculaire spécifique n'étant cependant pas modifiée. Le lindane augmente très significativement

Tableau 1. *Poids corporel et poids relatif du foie et des reins des rats nourris avec des régimes contenant du lindane*

Régime	Poids corporel* (g)	Poids relatif du foie* (g/100 g rat)	Poids relatif des reins* (g/100 g rat)
Equilibré			
Témoins	235.0 ± 8.95	3.60 ± 0.180	0.858 ± 0.0507
Lindane	250.0 ± 10.4	3.77 ± 0.180	0.917 ± 0.0507
Hypocalcique			
Témoins	212.0 ± 15.1	3.77 ± 0.180	1.072 ± 0.0507
Lindane	228.0 ± 8.55	4.02 ± 0.180	1.224 ± 0.0507
s^2....	429.0	1.041	0.06100

* Moyenne (six animaux/groupe), écart-type sur la moyenne et la valeur de la variance résiduelle (s^2).

de 19% la teneur en *P*-450 (Tableau 3), mais n'a pas d'effet sur la biosynthèse nette du cytochrome b_5. L'activité de la NADPH-cytochrome *c* réductase, exprimée pour 100 mg de protéines, est très significativement plus élevée de 26% chez les animaux traités; le pesticide ne paraît induire l'activité moléculaire spécifique qu'avec le régime équilibré. L'ingestion de lindane ne semble pas modifier sensiblement les activités des NADPH-oxydase et NADH-b_5 réductase (Tableau 3).

Le régime hypocalcique n'a pas d'effet sur la concentration des protéines. Il augmente significativment (+23%) l'activité moléculaire spécifique de la *N*-déméthylation de l'aminopyrine. L'hydroxylation de l'aniline ne paraît pas dépendre, dans nos conditions expérimentales, de la teneur en calcium alimentaire. Le régime hypocalcique diminue significativement (−12%) le *P*-450; il ne modifie pas la biosynthèse du cytochrome b_5. Chez le rat témoin, l'activité moléculaire spécifique de la NADPH-cytochrome *c* réductase rapportée au *P*-450 est très significativement induite (+55%) par la déficience en calcium; l'activité, exprimée pour 100 mg de protéines, n'étant pas modifiée. Le régime hypocalcique accroît très significativement l'activité de la NADPH-oxydase: respectivement de 55 et 66% par rapport aux protéines et au *P*-450. L'activité de la NADH-b_5 réductase, exprimée pour 100 mg de protéines, est significativement abaissée (−19%), mais l'activité moléculaire spécifique, rapportée au b_5, n'est pas modifiée.

Nous observons une interaction significative entre les effets du lindane et du régime hypocalcique sur l'activité de la NADPH-cytochrome *c* réductase: (1) le lindane augmenterait l'activité rapportée au *P*-450 avec le régime équilibré alors qu'il la diminuerait avec le régime hypocalcique; (2) la carence en calcium induit très significativement (+55%) l'enzyme chez le témoin alors qu'elle n'a pas d'effet chez le rat traité au pesticide.

DISCUSSION

Effets du lindane

Nous confirmons certains effets du lindane que nous avons déjà signalés (Lowy *et al.* 1977; Pélissier et Albrecht, 1976; Pélissier *et al.* 1975): 60 ppm dans la nourriture du rat, pendant 4 semaines, accélèrent le métabolisme hépatique des substances exogènes de type I (aminopyrine) et de type II (aniline); l'induction des monoxygénases microsomales est liée à une élévation de la teneur en *P*-450 et à une augmentation de l'activité de la NADPH-cytochrome *c* réductase. Par cette expérience, nous précisons que l'induction enzymatique paraît spécifique des réactions dépendantes du NADPH: ni la NADH-b_5 réductase ni le cytochrome b_5 microsomal ne sont influencés par le traitement au pesticide. D'autre part, le lindane augmente la quantité de phosphatidylcholine des microsomes; ce qui est en accord avec la prolifération du

Tableau 2. *Calcémie, protéines et phosphatidylcholine des microsomes des rats nourris avec un régime contenant du lindane*

Régime	Nombre d'animaux....	Calcémie* (mg/litre) 5	Concentration des protéines microsomales* (g/100 g foie) 6	Phosphatidylcholine* (µmole/100 mg protéines) 5
Equilibré				
Témoins		86.0 ± 14.0	3.51 ± 0.193	28.9 ± 3.44
Lindane		86.0 ± 11.9	3.70 ± 0.328	32.5 ± 4.43
Hypocalcique				
Témoins		51.6 ± 8.57	3.92 ± 0.208	24.8 ± 2.51
Lindane		72.3 ± 4.56	3.78 ± 0.187	29.8 ± 3.58
s^2....		425.7	0.1963	19.85

* Moyenne, écart-type sur la moyenne et la valeur de variance résiduelle (s^2).

Tableau 3. *Activités enzymatiques des microsomes des rats nourris avec des régimes contenant du lindane*

Paramètre	Nombre d'animaux	Activité enzymatique*				s^2
		Régime équilibré		Régime hypocalcique		
		Témoins	Avec lindane	Témoins	Avec lindane	
N-Déméthylation de l'aminopyrine						
nmol aminoantipyrine/min/100 mg protéines	6	11,4 ± 1,56	21,4 ± 2,99	12,9 ± 1,73	21,3 ± 2,59	17,97
/nmol *P*-450	6	0,154 ± 0,0219	0,254 ± 0,0240	0,216 ± 0,0265	0,284 ± 0,0338	0,002583
Hydroxylation de l'aniline						
nmol *p*-aminophénol/min/100 mg protéines	6	23,2 ± 2,62	30,7 ± 3,13	22,5 ± 1,25	26,4 ± 2,50	20,54
/nmol *P*-450	6	0,302 ± 0,0297	0,367 ± 0,0191	0,381 ± 0,0246	0,357 ± 0,0432	0,004039
Cytochrome *P*-450						
nmol/100 mg protéines	6	73,7 ± 3,52	83,3 ± 6,23	60,7 ± 5,86	77,2 ± 4,11	67,98
Cytochrome b_5						
nmol/100 mg protéines	6	87,0 ± 10,6	81,9 ± 6,94	80,5 ± 6,68	74,4 ± 4,91	344,5
NADPH-cytochrome *c* réductase						
µmol/min/100 mg protéines	6	5,53 ± 0,916	7,7 ± 1,31	6,44 ± 0,952	7,38 ± 0,790	1,367
nmol/min/nmol *P*-450	6	75 ± 12,0	93 ± 13,8	117 ± 16,1	99 ± 10,9	277,0
NADPH-oxydase						
nmol/min/100 mg protéines	5	267 ± 51,0	310 ± 113	415 ± 89,1	480 ± 76,3	8420
/nmol *P*-450	5	3,71 ± 0,831	4,7 ± 1,71	7,3 ± 1,58	6,6 ± 1,31	2,350
NADH-b_5 réductase						
µmol/min/100 mg protéines	6	371 ± 55,5	374 ± 26,1	284 ± 34,6	323 ± 17,6	6027
/nmol b_5	6	4,47 ± 0,760	4,69 ± 0,432	3,65 ± 0,502	4,45 ± 0,394	0,8713

* Moyenne, écart-type sur la moyenne et la valeur de variance résiduelle (s^2).

réticulum endoplasmique que signalent les morphologistes (Herbst et Bodenstein, 1972).

Effets du régime hypocalcique

Dans nos conditions expérimentales, 6 semaines de régime ne contenant que 0,06% de calcium, le métabolisme des substances exogènes n'est pas réprimé dans les microsomes hépatiques du rat. Nous ne confirmons donc pas les résultats de Dingell *et al.* (1966). L'étude du système microsomal de transport d'électrons fait apparaître que, bien que le *P*-450 soit en moindre quantité, son "efficacité" enzymatique—pour le métabolisme des substances exogènes—est accrue. En effet, la réduction du *P*-450 (Fe^{3+}) en (Fe^{2+}), qui est une étape essentielle pour le métabolisme, est favorisée par l'induction de la NADPH-cytochrome *c* réductase et de la NADPH-oxydase. Le maintien du niveau métabolique des médicaments peut donc s'expliquer par un équilibre entre effets opposés: d'une part la diminution du *P*-450, d'autre part l'augmentation du transfert des électrons à partir du NADPH. Cet équilibre serait rompu chez des animaux plus sensibles à la carence en calcium. En était-il ainsi dans l'expérience de Dingell *et al.* (1966)? Nous ignorons pourquoi le régime hypocalcique modifie les monoxygénases microsomales. Y-a-t-il une relation avec le métabolisme de la vitamine D_3? On sait en effet que ce métabolisme (hydroxylations) dépend de la calcémie et s'effectue en partie dans les microsomes du foie (Deluca, 1976).

Interaction lindane–apport de calcium

La réduction enzymatique du *P*-450, estimée par la mesure de l'activité moléculaire spécifique de la NADPH-cytochrome *c* réductase, est accélérée à la fois par le lindane et le régime hypocalcique, l'effet du régime étant le plus prononcé. Si l'on associe les deux traitements, les inductions ne sont pas additives et le niveau enzymatique se situe au plus bas; c'est-à-dire comparable à celui de l'animal nourri avec le régime équilibré comportant du lindane. L'insecticide a donc empêché l'effet propre du régime hypocalcique.

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TRANSFER OF POLYCHLORINATED BIPHENYLS TO THE FOETUSES AND OFFSPRING OF MICE

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Abstract— Mice were fed Kanechlor-500 (KC-500) at a dietary level of 0·01 (control), 0·94, 8·4 or 86 ppm from the day of insemination until day 18 of pregnancy and were then killed. The amounts of polychlorinated biphenyls (PCBs) present in the whole body, liver and foetuses of the mice were, respectively, 6–16, 0·8–3 and 0·1–0·2% of the total PCB intake, regardless of the dietary level of PCB, showing that transplacental transfer of PCBs to foetuses occurs to a relatively small degree. Other pregnant mice were fed a diet containing 0·01 or 0·94 ppm KC-500 and maintained on this until their offspring were 5 wk old. The young (one or two from each litter) were killed at weekly intervals from birth and analysed for PCBs. These offspring contained 100 or more times as much PCB during lactation as was present in the foetuses, indicating a considerable transfer of PCBs via the milk.

INTRODUCTION

Polychlorinated biphenyls (PCBs) are known to have been the cause of a mass outbreak of food poisoning called Yusho, which occurred in western Japan in 1968 (Katsuki, 1969; Kuratsune, Yoshimura, Matsuzaka & Yamaguchi, 1972). Foetuses from mothers with Yusho showed some of the characteristics of the disease (Kikuchi, Hashimoto, Hozumi, Koga, Oyoshi & Nagakawa, 1969; Taki, Hisanaga & Amagase, 1969). Several babies who had acquired PCBs from their mothers either transplacentally and/or by breast feeding were also diagnosed as suffering from Yusho (Yoshimura, 1974). Reproductive defects have been reported in animals given PCBs (Dahlgren, Linder & Carlson, 1972; Linder, Gaines & Kimbrough, 1974), and it is evident that PCBs present in mothers can affect their babies. The study described here was undertaken to obtain further qualitative and quantitative data on the transfer of PCBs through the placenta and milk of pregnant and lactating mice.

EXPERIMENTAL

Materials and instrumentation. Ethanol, *n*-hexane, water, sodium hydroxide, anhydrous sodium sulphate and silica gel were purified for PCB analysis as described previously (Masuda, Kagawa & Kuratsune, 1974). Kanechlor-500 and Kanechlor-600 were provided by Kanegafuchi Chemical Industry Co., Osaka. PCB isomers were purchased from Analabs, Inc., North Haven, USA, or synthesized in our laboratory. The gas chromatograph used was a Shimadzu GC-4BM with an electron-capture detector and a glass column (3 mm × 2 m) containing Chromosorb W AW-DMCS (60–80 mesh) coated with 5% SE-30. The column temperature was 210 C and pure nitrogen (99·999%) was used as the carrier gas at a flow rate of 30 ml/min.

Animals and diet. Male and female ddN mice were supplied by the animal centre of Kyushu University. Test diets were prepared by thoroughly mixing 1-kg batches of powdered feed (Oriental M) with ethyl ether solutions containing 0, 1·1, 11 or 110 mg Kanechlor-500 and leaving the mixture exposed to the air overnight to evaporate the solvent. The mixture was then kneaded well with water and with 100 g soluble starch (reagent grade) dissolved in hot water to make a dough, and pieces of the dough weighing 2–3 g were pelleted and dried at 60 C for 15 hr. The dried pellets, calculated to contain 0, 1, 10 or 100 ppm Kanechlor-500, were analysed for PCBs by gas chromatography (see below), the mean levels derived from triplicate determinations being 0·01, 0·94, 8·4 and 86 ppm, respectively.

Experimental procedure. Female mice were divided when 10 wk old into four groups of 7–12 and mated, insemination being confirmed by the presence of a vaginal plug. The pregnant mice were caged individually and fed one of the Kanechlor-500 diets until day 18 of pregnancy, when they were killed. The pellets in each cage were weighed every day and their consumption was calculated. For each dam, the foetuses, liver and rest of the body apart from the digestive tract were analysed for PCBs. Two other groups, one of nine and one of 12 mice, were mated and caged similarly and fed the pellets containing 0·01 or 0·94 ppm PCBs from the day of insemination until the end of the experiment. Young born on day 20 or 21 of pregnancy were raised by their dams for 5 wk, one or two offspring from each litter being killed at weekly intervals from 1 wk after birth for whole-body PCB analysis. The dams were killed at wk 5 and in each case the liver and the rest of the body apart from the digestive tract were analysed separately for PCBs.

Table 1. *Mean concentrations of PCBs in the whole-body homogenate, liver and foetuses of dams fed Kanechlor-500 throughout pregnancy to day 18*

Levels of PCBs in feed (ppm)	No. of dams/group	Basis of calculation*	Concn of PCBs (ppm) in		
			Whole body	Liver	Foetuses
0·01 (control)	8	Total tissue	0·016 ± 0·012	0·056 ± 0·025	0·008 ± 0·011
		Fat	0·27 ± 0·19		0·81 ± 1·16
0·94	12	Total tissue	0·33 ± 0·14	1·4 ± 0·27	0·017 ± 0·007
		Fat	13 ± 6·0		5·4 ± 2·7
8·4	8	Total tissue	5·3 ± 1·1	3·1 ± 0·59	0·20 ± 0·10
		Fat	100 ± 33		26 ± 23
86	7	Total tissue	43 ± 8·9	23 ± 9·1	1·7 ± 0·7
		Fat	990 ± 310		200 ± 88

*Values are means ± SD for concentrations expressed either as a proportion of the whole tissue or of the fat content.

Analytical methods. The official standard analytical method for PCBs established by the Ministry of Health and Welfare (Kawashiro, Ueda, Ueta, Kashimoto, Kitamura, Kuratsune, Tatsukawa, Tanabe, Terashima, Fujiwara, Yoshimura, Masuda & Mizutani, 1972) was used for the extraction, separation and quantitative determination of PCBs. Samples were first homogenized with 100–200 ml *n*-hexane and 20 g sodium sulphate in a Waring blender. The separated *n*-hexane solutions were combined and evaporated to dryness, yielding fatty residues which were saponified with 1 N-NaOH in 50–100 ml ethanol. For liver samples, the saponification was made without extraction. The *n*-hexane extracts of the NaOH solution were combined, concentrated and then chromatographed on a column of silica gel (Wako gel S-1, 2 g) eluted with 150 ml *n*-hexane. The eluate was concentrated and subjected to gas chromatography, the PCBs being determined by comparing the total peak heights of their gas chromatograms with those of a 1:1 mixture of Kanechlor-500 and Kanechlor-600.

RESULTS

All the pregnant mice fed diet containing 0·01, 0·94, 8·4 or 86 ppm PCBs grew well throughout pregnancy, the mean body weights of these four groups on day 18 of pregnancy being 42·3, 42·6, 45·9 and 43·1 g, respectively. There was no significant difference in body-weight gain between these groups. The numbers of foetuses or offspring produced by each dam were normal (7–8) in each group, and there were no abnormalities in appearance.

Tables 1 and 2 summarize the results of the PCB analyses of the tissue samples from the four groups killed on day 18 of pregnancy. The levels of PCBs accumulated in the tissues of dams and foetuses and in the maternal livers increased with increasing PCB content of the diet (Table 1). The liver levels only showed a relatively small increase (×2·2), however, when the dietary concentration was raised from 0·94 to 8·4 ppm. Table 2 shows the total quantities of PCBs retained in the tissues of dams and foetuses. These also, increased when the PCB level of the diets was raised, and the amounts present in the whole body, liver and foetuses accounted, respectively, for 6–16, 0·8–3 and 0·1–0·2% of the total PCB intake. Thus, a much smaller proportion was found in the foetuses than in the other tissues. However, the proportions did not vary widely in the different groups in spite of the marked differences in the PCB levels of the different diets.

The PCB content of 1–5-wk-old offspring of dams given PCBs at a dietary level of 0·01 or 0·94 ppm is shown in Table 3, which also lists the total amounts of PCBs per litter estimated from the number of offspring in the litter and their PCB concentration. While both the concentration and the total amount of PCBs present were always very small in the foetuses of the dams fed PCBs, they were much larger in the young killed during the lactation period (about 2 wk after birth). In offspring weaned 2 or 3 wk after birth, the PCB concentration remained roughly constant, even when they consumed the diet containing 0·94 ppm PCBs. Consequently, the total amounts of PCBs in the bodies of offspring aged 3–5 wk increased with time. A dam given pellets containing 0·94 ppm

Table 2. *Mean weights of PCBs in whole-body homogenates, livers and foetuses of dams fed Kanechlor-500 throughout pregnancy to day 18*

Levels of PCBs in feed (ppm)	No. of dams/group	Total intake of PCBs* (μg)	Total weight (μg) of PCBs* in		
			Whole body	Liver	Foetuses
0·01 (control)	8	1·0 ± 0·1	0·42 ± 0·36 (42·0)	0·14 ± 0·06 (14·0)	0·05 ± 0·07 (5·0)
0·94	12	96 ± 7·0	5·7 ± 1·9 (5·9)	3·1 ± 0·7 (3·2)	0·10 ± 0·05 (0·1)
8·4	8	890 ± 100	140 ± 40 (15·6)	9·2 ± 2·0 (1·0)	1·9 ± 1·1 (0·2)
86	7	9300 ± 300	1100 ± 270 (11·6)	73 ± 16 (0·8)	13 ± 6·9 (0·1)

*Values are means ± SD, the figures in parenthesis being the weight of PCBs in the given tissue expressed as a percentage of the total intake.

Table 3. *Mean concentrations and total weights of PCBs in the offspring of dams fed Kanechlor-500 during and after pregnancy*

Levels of PCBs in feed (ppm)	No. of dams/group	PCB measurement	Age (wk)...	Mean values* for PCBs in offspring of different ages				
				1	2	3	4	5
0·01 (control)	9	Total tissue concn (ppm)		0·006 ± 0·001	0·010 ± 0·006	0·007 ± 0·005	0·005 ± 0·003	0·004 ± 0·003
		Concn based on fat (ppm)		0·12 ± 0·06	0·18 ± 0·07	0·19 ± 0·06	0·17 ± 0·06	0·20 ± 0·14
		Total weight (μg)		0·21 ± 0·03	0·59 ± 0·29	0·59 ± 0·37	0·68 ± 0·36	0·81 ± 0·46
0·94	12	Total tissue concn (ppm)		0·96 ± 0·23	1·1 ± 0·22	0·84 ± 0·35	0·67 ± 0·21	0·73 ± 0·34
		Concn based on fat (ppm)		20 ± 9·4	15 ± 3·4	25 ± 11	26 ± 3·8	25 ± 4·8
		Total weight (μg)		32 ± 7·8	65 ± 13	72 ± 28	89 ± 27	130 ± 45

*Values are means ± SD.

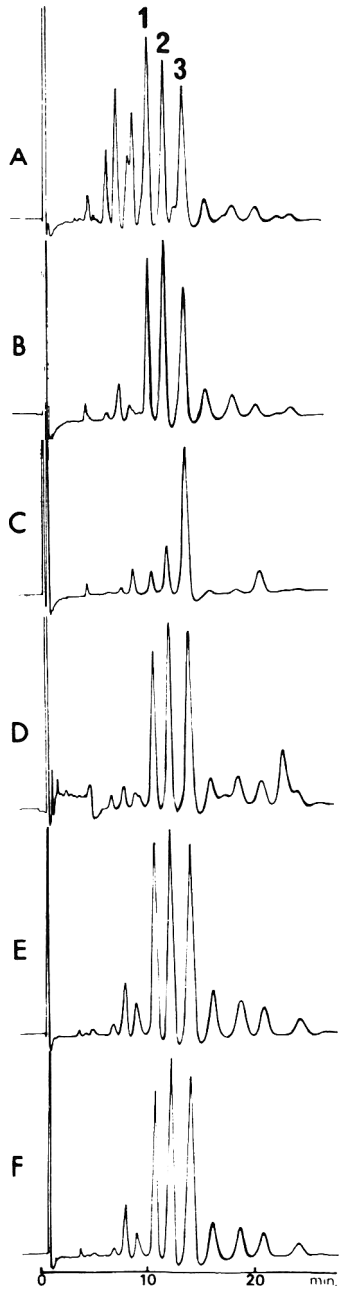

Fig. 1. Gas chromatograms of Kanechlor-500 (A) and of PCBs from the whole-body homogenate (B) the liver (C), a foetus (D), and 2-wk-old (E) and 5-wk-old (F) offspring of mice fed 0·94 ppm PCBs in the diet from the beginning of pregnancy. Peaks 1, 2 and 3 correspond to 2,4,5,3',4'-penta-, 2,4,5,2',4',5'-hexa- and 2,3,4,2',4',5'-hexachlorobiphenyl, respectively.

ingested a total of 147 or 186 μg PCBs on average during 20 days of pregnancy and 1 or 2 wk of lactation. Since the total PCB content of the litter of such a dam averaged 32 and 65 μg at 1 and 2 wk, respectively, after birth (Table 3), the amount of PCBs in the 1- and 2-wk-old offspring accounted for as much as 22 and 35% of the total intake of the dam.

Gas chromatograms of PCBs in the dams, foetuses and offspring of mice fed pellets containing 0·94 ppm PCBs are reproduced in Fig. 1. The peaks recorded show that the 2,4,5,3',4'-penta-, 2,4,5,2',4',5'-hexa and 2,3,4,2',4',5'-hexachlorobiphenyls were the major compounds in the whole-body analyses of all these groups, but the last compound was the main isomer present in the maternal liver.

DISCUSSION

These experiments showed that, in mice, PCBs enter the foetuses through the placenta in relatively small amounts but are transferred to the offspring after birth through the milk in much larger quantities. Similar experimental results were observed by others in studies on rats (Curley, Burse & Grim, 1973; Mizunoya, Taniguchi, Kusumoto, Morita, Yamada, Baba & Ogaki, 1974; Takagi, Otake, Kataoka, Murata, Aburada, Akasaka, Hashimoto, Uda & Kitaura, 1976), although precise comparison of these data is not feasible because of differences in the strain of animal used, in the degree of chlorination of the PCBs and in the feeding conditions. According to our calculations, these studies indicated PCB-transfer ratios from dam to foetuses and to offspring of 0·03–0·2 and 10–20%, respectively, of the amount ingested. The corresponding figures from our experiments were 0·1–0·2 and 20–35%, respectively. Thus, the figures show some level of agreement in spite of the discrepancies in experimental conditions. The experimental conditions used by Mizunoya *et al.* (1974) were very similar to ours, in that PCBs were given to the animals in the diet from the day of insemination, but they used rats and Kanechlor-400 instead of mice and Kanechlor-500 and found PCB concentrations in the foetuses and 2-wk-old offspring to be 0·04 and 2·36 ppm, respectively, when a diet containing 10 ppm Kanechlor-400 was given, while the corresponding figures estimated from our experiment were 0·20 and 10 ppm, respectively, for a dietary level of 8·4 ppm PCBs. (The value of 10 ppm was obtained by a rough extrapolation of the figure obtained when 0·94 ppm PCBs was given.) Thus, the concentrations in mice were about five times as great as those in rats, but the difference may have been due at least partially to the dissimilarity of the two PCB formulations.

Barsotti, Marlar & Allen (1976) obtained some evidence of transplacental and mammary movement of PCBs in monkeys. The concentrations of PCBs in various tissues of the stillborn infant of a monkey that had been fed a diet containing PCBs at a level as low as 5 ppm were much higher than those in the rodents, suggesting a fairly large species variation in the transplacental movement of PCBs. Thus, our present findings may not be applicable to man, and there is an urgent need for qualitative and quantitative data on the transfer and excretion of PCBs in man.

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MYCOTOXICOLOGICAL INVESTIGATIONS ON ZAMBIAN MAIZE

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Abstract—In Zambia there is a statutory requirement that visually diseased kernels should not constitute more than 2% of maize intended for human consumption. Four samples of Zambian maize containing 1·8, 5·4, 13·0 and 20·3% visually diseased kernels were subjected to mycological and chemical analyses and to toxicity trials in ducklings and rats. For all the samples the dominant fungi isolated from surface-sterilized kernels were *Fusarium moniliforme* Sheldon and *Diplodia macrospora* Earle, and the most prevalent fungus isolated from prepared maize meals was *F. moniliforme*. The level of fungal invasion of both kernels and meal was significantly lower for the 1·8%-diseased sample than for the other three. Protein and essential amino acid content tended to increase as the level of diseased kernels increased. None of the samples contained chemically detectable amounts of aflatoxins, ochratoxins, sterigmatocystin, cyclopiazonic acid or zearalenone. Although no samples were lethal to either ducklings or rats, body-weight gains were higher in male and female rats fed the 1·8%-diseased sample for 6 months than in those given the other test diets, the difference being statistically significant in the males. Of seven pure cultures of fungi isolated from the maize samples, five, including *F. moniliforme* and *D. macrospora*, were acutely toxic to ducklings and rats. In view of the indications that unidentified Fusarium and Diplodia toxins present in Zambian maize containing more than 1·8% visually diseased kernels reduce the growth rate of rats fed such samples for 6 months, it is recommended that the 2% maximum level of visually diseased kernels acceptable in Zambian maize for human consumption should be maintained.

INTRODUCTION

Mouldy maize has been reported to cause outbreaks of mycotoxicoses in animals (Marasas & Smalley, 1972; Mirocha & Christensen, 1974; Smalley, Marasas, Strong, Bamburg, Nichols & Kosuri, 1970; Smalley & Strong, 1974) and man (Krishnamachari, Bhat, Nagarajan & Tilak, 1975a,b). Several toxigenic fungi have been isolated from maize, in which the following mycotoxins have been found to occur naturally: aflatoxins, ochratoxin, penicillic acid, kojic acid, zearalenone, T-2 toxin and deoxynivalenol (Hesseltine, 1974; Mirocha, Pathre, Schauerhamer & Christensen, 1976). There are as yet no sensitive methods for the analysis of moniliformin produced by *Fusarium moniliforme* Sheldon, the most prevalent fungus in maize kernels (Cole, Kirksey, Cutler, Doupnik & Peckham, 1973), and other mycotoxins produced by this species have not been chemically characterized (Kellerman, Marasas, Pienaar & Naudé 1972; Marasas, Kellerman, Pienaar & Naudé, 1976). The same situation applies to *Diplodia maydis* (Berk.) Sacc. (Mitchell, 1919; Steyn, Wessels, Holzapfel, Potgieter & Louw, 1972; Theiler, 1927) and consequently it has not been possible to establish the natural occurrence of these Fusarium and Diplodia mycotoxins in maize.

According to current standards relating to the classification, quality and moisture content of maize in Zambia, the maximum percentage of 'diseased grain' acceptable in maize intended for human consumption is 2% (Republic of Zambia, 1974). During some seasons the incidence of cob-rot of maize caused mainly by species of Diplodia and Fusarium is very high (Logan, 1974; MacDonald & Raemakers, 1974), resulting in the rejection of large quantities of maize. The maize-grading regulations of other countries such as the United States of America (United States Department of Agriculture, 1970) and the Republic of South Africa (Republic of South Africa, 1972) make no provision for a separate category of diseased kernels. Mouldy or diseased kernels are included in the maximum percentage allowed for generally damaged kernels, a situation that leads to a wide variation in the level of fungal invasion present in different lots of maize with the same numerical grade.

The maximum percentage of fungal invasion acceptable in maize for human consumption must be low enough to ensure safety from mycotoxin contamination but should also be high enough to be realistic in practice and prevent the unnecessary rejection of a staple foodstuff. The mycotoxicological investigations reported in this paper were conducted in an attempt to determine whether the limit of 2% 'diseased grain' specified by the Zambian maize-grading regulations meets these requirements.

EXPERIMENTAL

Zambian maize samples. Four 20-kg samples of white dent maize grown in Zambia during 1974–1975 were obtained from Mount Makulu Research Station, Chilanga, Zambia, and graded by the Zambian Food Conservation and Storage Unit. The levels of 'diseased grain' as determined by visual inspection (mass by mass) were 1·8% in a hand-harvested sample, 5·4 and 13·0% respectively in two combine-harvested samples, and 20·3% in a sample fortified with hand-

sorted mouldy grains. Moisture content, determined by the Marconi electrical resistance method (Republic of South Africa, 1972), varied between 12·5 and 12·7%. The hand-harvested, 1·8% diseased, sample served as a control for mycological and toxicological comparison with the other samples containing more than 2% diseased kernels. This provided, for the toxicological studies, a control diet which was comparable with the test diets not only in the level of maize meal but also in the type of maize used, all the diets being based on maize of the same cultivar grown under the same environmental conditions. In addition, the 1·8% diseased sample had a lower level of fungal invasion than commercially available maize and the percentage of visually diseased kernels was within the statutory limit.

Mycological analyses of maize samples. One hundred kernels from each sample were surface-sterilized in a commercial 5% aqueous solution of sodium hypochlorite for 1 min, rinsed twice with sterile water and plated out (five kernels/plate) on 1·5% malt-extract agar containing 100 mg novobiocin/litre (Tuite, Shaner, Rambo, Foster & Caldwell, 1974). The plates were incubated at 25°C in the dark and the numbers and kinds of fungi that developed from the kernels, as well as the percentage germination, were recorded after 5–7 days. In addition, samples of the different batches of maize were ground in a laboratory mill and the mycoflora of the resulting meal samples was determined quantitatively by a dilution plate method essentially similar to that of Christensen (1946), except that the isolation medium used was 1·5% malt-extract agar containing novobiocin. Moisture content of the meal samples was determined by drying the samples to constant mass in a forced-draft oven at 103°C and mould counts were calculated on a dry mass basis.

Chemical analysis of maize samples for mycotoxins. The maize samples were analysed for aflatoxins, ochratoxins and sterigmatocystin according to the methods described in the Official Methods of Analysis of the American Association of Analytical Chemists (Horwitz, 1975).

The analytical method for cyclopiazonic acid involved drying a chloroform–methanol (1:1, v/v) extract of a 50-g sample, partitioning the residue between chloroform and 0·1 M-sodium bicarbonate solution, removing the alkaline phase and acidifying it to pH 3 with conc. HCl and subjecting its chloroform extract to paper chromatography using benzene as the developing solvent. Cyclopiazonic acid was then visualized as a mauve spot at R_F 0·4 by subsequent spraying with conc. HCl and 1,4-dimethylaminobenzaldehyde.

Zearalenone was assayed in the maize samples and in culture material of *F. graminearum* Schwabe according to the method of Thomas, Eppley & Trucksess (1975). The identity of zearalenone in positive extracts was confirmed by comparison with an authentic zearalenone standard kindly supplied by Dr. C. J. Mirocha, University of Minnesota, and by UV spectrophotometry of purified extracts (Mirocha, Schauerhamer & Pathre, 1974).

Amino acid and protein analyses. Approximately 25 mg of the finely ground samples were hydrolysed with 2 ml 1:1 HCl in sealed tubes under vacuum for 22 hr. The hydrolysates were dried under vacuum over solid P_2O_5 and NaOH and were then taken up in 1·0 ml pH 2·2 buffer and cleared by centrifuging. Appropriate aliquots of the clear solutions were analysed on a Beckman 120 C analyser and the results were calculated on-line by a Hewlett-Packard 3352 B computer after calibration of the system with Pierce amino acid standard H (batch 07105.17). Tryptophan could not be determined owing to destruction during the acid hydrolysis. Protein values (Kjeldahl nitrogen × 6·25) and moisture content were also determined and all results were expressed on a dry-mass basis.

Toxicity trial in ducklings. Each of the maize samples was ground in a laboratory mill and rations were prepared by mixing 90% maize meal with 10% PVM supplement (Dreyer & Du Bruyn, 1968). The diets containing the different maize meals were fed *ad lib.* to groups of four day-old Pekin ducklings for 14 days, and the ducklings were weighed at the beginning and at the end of the feeding period.

Toxicity trial in rats. Diets containing the different samples of maize meal were fed *ad lib.* to groups of six male and six female weanling inbred BD IX black rats for 6 months. Each diet contained 90% maize meal and 10% PVM supplement (Dreyer & Du Bruyn, 1968) for the first 14 weeks of the experiment, but the 10% PVM supplementation, regarded as the minimal requirement for adequate maintenance, resulted in this case in poor weight gains, and the level of PVM was therefore increased to 20% for the rest of the experiment. Feed consumption was determined over a 7-day period after the rats had been fed the maize diets for 17 wk. The rats were weighed individually at the beginning of the experiment and at weekly intervals thereafter, except during the period of the feed-consumption determination, when they were weighed daily.

At the end of the feeding period all the rats were killed by decapitation and subjected to autopsy. Organs and tissues, including the liver, kidney, lung, heart, brain, oesophagus, stomach, small and large intestine, adrenal and reproductive organs, were preserved in 10% buffered formalin. Paraffin sections stained with haematoxylin and eosin were examined by light microscopy.

Toxicity trials with pure cultures of fungi. The acute oral toxicity of seven species of fungi isolated from the maize samples was determined in the duckling and rat. Yellow-maize kernels (400 g kernels in 400 ml water) were autoclaved in 2-litre glass jars at 121°C for 1 hr on each of two consecutive days and were then inoculated with conidial suspensions of one of the following fungi: *D. macrospora* Earle, *D. maydis*, *F. graminearum* Schwabe, *F. moniliforme*, *F. moniliforme* Sheldon var. *subglutinans* Wr. & Reink., *F. equiseti* (Corda) Sacc. and *Nigrospora sphaerica* (Sacc.) Mason. The maize inoculated with the two Diplodia species was incubated at 25°C for 8 wk (Mitchell, 1919; Theiler, 1927). The *F. graminearum* cultures were incubated at 25°C for 2 wk followed by 6 wk at 12°C (Mirocha & Christensen, 1974). Cultures of all the other fungi were incubated at 25°C for 3 wk. After the required incubation period, the contents of the jars were harvested, dried at 50°C for 24 hr, ground in a laboratory mill and stored in a cold room

until used. Diets containing 50% commercial chicken mash and 50% mouldy meal prepared from cultures of the respective fungi on autoclaved maize were fed *ad lib.* to groups of four day-old Pekin ducklings for 14 days. Similar diets containing 50% commercial rat mash were fed to groups of four weanling male Wistar rats for 14 days. Control diets consisted of 50% maize meal prepared from uninoculated, autoclaved yellow maize and 50% chicken mash or rat mash.

RESULTS

Mycological analyses of maize samples

The dominant fungi isolated from surface-sterilized kernels of the four Zambian maize samples were *F. moniliforme* and *D. macrospora* (Table 1). Other fungi present to a lesser extent were *N. sphaerica*, *F. moniliforme* var. *subglutinans*, *F. graminearum*, *F. equiseti*, *D. maydis* and *Drechslera maydis* (Nisikado) Subram. & Jain (Table 1). The percentage of kernels from which fungi were isolated was significantly less ($P < 0.05$) in the 1·8%-diseased sample than in the other three samples. This was due to a lower value for the combined prevalence of Fusarium and Diplodia infection in this sample (Table 1).

The predominant fungus in the maize-meal samples was *F. moniliforme* and the only other fungi isolated from the meal were *Cephalosporium acremonium* Corda and Aspergillus and Penicillium species (Table 2). Both the Fusarium counts and total fungal counts of the 1·8%-diseased sample were significantly lower ($P < 0.05$) than those of the other three meal samples (Table 2).

Chemical analysis for mycotoxins

None of the four maize samples contained chemically detectable levels of aflatoxins, ochratoxin, sterigmatocystin, cyclopiazonic acid or zearalenone.

Protein and amino acid analyses

The 1·8%-diseased samples had a lower protein content and lower levels of all essential amino acids than the 5·4% diseased sample (Table 3). The highest concentrations of protein and of all amino acids assayed were found in the sample that contained the highest level (20·3%) of diseased kernels.

Toxicity trial in ducklings

Diets containing the different maize samples caused no deaths or significant differences in mean body-weight gain when fed to day-old ducklings for 14 days.

Toxicity trial in rats

No deaths occurred during the entire 6-month feeding period, but marked reductions in body-weight

Table 1. *Mycological analysis of maize kernels*

Fungus	Percentage of kernels infected* in samples with visually diseased kernels constituting			
	1·8%	5·4%	13·0%	20·3%
Fusarium moniliforme	17	30	17	23
F. moniliforme var. *subglutinans*	0	1	0	1
F. graminearum	1	2	2	2
F. equiseti	2	0	2	0
Total Fusarium spp...	20	33	21	26
Diplodia spp.†	8^{a}	$19^{a,b}$	31^{b}	29^{b}
Nigrospora sphaerica	5	6	15	12
Drechslera maydis	0	1	1	1
Total fungi...	33^{c}	59^{d}	68^{d}	68^{d}
Germination (%)...	78	72	63	62

*Based on 100 surface-sterilized kernels/sample. Values appearing in one row and carrying different superscripts are significantly different ($P < 0.05$) according to the Newman-Keuls D-test (Snedecor & Cochran, 1967). Means in rows with no letters are not significantly different.

†Mainly *D. macrospora* and to a lesser extent *D. maydis*.

Table 2. *Mycological analysis of maize meal samples*

Fungus	Propagules/g dry mass ($\times 10^{4}$)* in maize samples with diseased kernels constituting			
	1·8%	5·4%	13·0%	20·3%
Fusarium spp.†	1.0^{a}	16.0^{b}	$19.2^{b,c}$	24.8^{c}
Cephalosporium acremonium	0·6	2·4	3·0	4·4
Aspergillus spp.	1·0	0·6	0·2	0·6
Penicillium spp.	2·6	2·2	1·0	2·2
Total...	5.2^{d}	21.2^{c}	23.4^{c}	32.0^{f}

*Each value represents the mean of five dilution plates. Means appearing in one row and carrying different superscripts differ significantly ($P < 0.05$) according to the Newman-Keuls D-test (Snedecor & Cochran, 1967). Means in rows with no letters are not significantly different.

†Mainly *F. moniliforme* and to a lesser extent *F. moniliforme* var. *subglutinans*.

Table 3. *Amino acid and protein content of maize samples*

Component	Content (g/100 g dry mass) in maize samples with diseased kernel constituting			
	1·8%	5·4%	13·0%	20·3%
Essential amino acids				
Histidine	0·25	0·27	0·26	0·31
Lysine	0·26	0·27	0·27	0·32
Phenylalanine	0·33	0·36	0·33	0·40
Methionine	0·12	0·16	0·14	0·17
Threonine	0·27	0·29	0·28	0·31
Leucine	0·83	0·93	0·84	1·02
Isoleucine	0·24	0·25	0·23	0·28
Valine	0·34	0·37	0·36	0·40
Semi-essential amino acids				
Arginine	0·43	0·44	0·44	0·54
Tyrosine	0·28	0·31	0·29	0·33
Cystine	0·17	0·19	0·14	0·18
Glycine	0·29	0·32	0·32	0·34
Non-essential amino acids				
Serine	0·36	0·38	0·37	0·42
Glutamic acid	1·34	1·45	1·33	1·58
Aspartic acid	0·49	0·52	0·51	0·59
Alanine	0·48	0·52	0·49	0·57
Proline	0·80	0·74	0·78	0·88
Protein	7·94	8·24	8·07	8·76

Analyses were performed by the Division of Food Chemistry, National Food Research Institute, C.S.I.R., Pretoria.

gain were evident in male and female rats fed either the 13·0%- or 20·3%-diseased maize (Table 4). In addition, the mean weight gain of males fed the 1·8%-diseased (control) sample was significantly higher ($P < 0·05$) than that of the group receiving the 5·4%-diseased or more severely affected maize. Although the growth rates of females receiving one of the three more severely diseased types of maize were also reduced compared with the rate in the 1·8%-diseased group, these differences were not significant. No significant differences in feed consumption and feed efficiency were evident in either male or female rats fed the different maize samples (Table 5). No histopathological differences were noted between the group receiving 1·8%-diseased maize and any of the test groups.

Toxicity trials with pure cultures of fungi

The results of toxicity tests in groups of four ducklings or rats fed pure cultures of one of seven species of fungi isolated from the Zambian maize samples are given in Table 6. One of the predominant fungi in all the Zambian maize samples, *D. macrospora*, proved to be extremely toxic and caused the deaths of all four ducklings and all four rats within 8 days. *D. maydis* and *F. graminearum* were also acutely toxic to both ducklings and rats. The dominant Fusarium species in the Zambian maize, *F. moniliforme*, together with its variety *subglutinans*, appeared to be more toxic to ducklings than to rats, but both fungi caused marked weight losses in rats fed the pure culture material for 14 days. Two of the fungi tested, *F. equiseti* and *N. sphaerica*, showed no toxicity to either ducklings or rats.

The toxic culture material of *F. graminearum* (MRC 120) contained approximately 1 g zearalenone/kg. The chemical nature of the mycotoxins produced by the toxic strains of *F. graminearum*, *F. moniliforme*, *F. moniliforme* var. *subglutinans* and *D. macrospora* is undergoing further investigation.

DISCUSSION

In all four samples of Zambian maize, the percentage of surface-sterilized kernels from which fungi were isolated (33–68%) was much higher than the percentage of visually diseased kernels (1·8–20·3%). Several investigators have previously reported that visually sound maize kernels may be infected internally by seed-borne Fusarium species (Edwards, 1941; Futrell, 1972; Lillehoj, Fennell & Hara, 1975; Tuite *et al.* 1974). In the Zambian samples a linear relationship was found, however, between the percentage of visually diseased kernels and the percentage of kernels from which fungi could be isolated ($r = 0·82$; $P < 0·05$). The percentage of visually diseased kernels

Table 4. *Body-weight gains of rats fed diets containing different maize samples for 6 months*

Diet* (% of diseased kernels in maize sample)	Mean body-weight gain (g)†	
	Males	Females
1·8 (control)	243·2^a	138·6^c
5·4	213·8^b	134·5^c
13·0	198·8^b	120·2^c
20·3	199·8^b	121·0^c

*Diets contained 90% maize meal and 10% PVM supplement for the first 14 wk of the experiment and subsequently 80% maize meal and 20% PVM.

†Values are means for groups of six rats. Means in a single column carrying different superscripts differ significantly ($P < 0·05$) according to the Newman-Keuls D-test (Snedecor & Cochran, 1967).

Table 5. *Feed consumption, body-weight gain and feed efficiency of rats fed diets containing different maize samples for 17 wk*

Diet* (% of diseased kernels in maize sample)	Mean feed consumption (g/rat/day)		Mean body-weight gain (g/rat/day)		Feed efficiency†	
	Males	Females	Males	Females	Males	Females
1·8 (control)	14·3	9·0	2·1	0·7	0·149	0·075
5·4	13·1	9·6	1·4	0·6	0·107	0·070
13·0	12·5	10·0	2·3	1·1	0·187	0·110
20·3	12·9	9·7	1·8	1·1	0·144	0·117

*Diets contained 80% maize meal and 20% PVM supplement when these data were recorded over a 7-day period after the rats had been on maize diets for 17 wk.
†Body-weight gain/food consumption.
Values which are means for groups of six rats, do not differ significantly ($P < 0·05$) according to the Newman-Keuls D-test (Snedecor & Cochran, 1967).

Table 6. *Toxicity trials in groups of four ducklings and of four rats fed pure cultures of fungi isolated from Zambian maize*

Fungal component of diet*	Isolate no.	Mean body-weight gain† (g)		No. of deaths‡		Mean time of death (days)	
		Ducklings	Rats	Ducklings	Rats	Ducklings	Rats
Diplodia macrospora	MRC 143	—	—	4	4	5·0	7·0
D. maydis	MRC 141	—	—	4	4	6·0	12·5
Fusarium graminearum	MRC 120	—	−11·0	4	2	6·75	13·5
F. moniliforme	MRC 137	30	−37·8	2	1	6·0	14·0
F. moniliforme var. *subglutinans*	MRC 134	—	−11·3	4	0	6·25	—
F. equiseti	MRC 126	124	44·0	0	0	—	—
Nigrospora sphaerica	MRC 147	174	41·3	0	0	—	—
— (control)	—	157	33·8	0	0	—	—

*Diets contained 50% mouldy meal prepared from pure cultures of the named fungi on autoclaved maize kernels, or 50% autoclaved, uninoculated maize meal in the case of the control group, and 50% commercial chicken feed or rat mash as appropriate.
†Mean body-weight gains from day 1 to 14 for test groups of four day-old ducklings or four weanling male rats.
‡Numbers of animals that died within 14 days out of a group of four ducklings or four rats.

was also inversely related to the percentage germination ($r = −0·94$; $P < 0·05$).

The dominant fungi isolated from the maize kernels were *F. moniliforme* and Diplodia species, while the most prevalent fungus isolated from the meal prepared from these kernels was *F. moniliforme*. It is not clear why the Diplodia species and some other fungi frequently isolated from the kernels were not recovered from the meal. The milling process probably favoured the isolation of highly-sporulating fungi such as *F. moniliforme* and possibly resulted in the release of self-inhibiting factors by the macerated mycelium of other fungi such as Diplodia (Kent, 1940). Both isolation methods, however, indicated a significantly lower level of fungal invasion of the 1·8%-diseased maize compared with the other three samples.

Although none of the samples were lethal to either ducklings or rats, male as well as female rats fed the 1·8%-diseased sample for 6 months showed higher body-weight gains than the three test groups. In the case of male rats, the difference between the weight gains of rats maintained on the 1·8%- and 5·4%-diseased samples was statistically significant. The growth-retarding effect of the samples containing 5·4% and more diseased kernels cannot be attributed to reduced feed intake because of unpalatibility (Table 5) or to differences between the maize samples in terms of amino acid or protein composition (Table 3). The relative increases in the amino acid and protein levels of the samples that contained more than 1·8% diseased kernels were presumably due to the metabolic activity of the kernel-invading fungi, but even if the digestibility and nutritive value of this protein were reduced (Mitchell & Beadles, 1940), these differences would have been masked by the PVM supplement. Since none of the samples contained chemically detectable levels of five known mycotoxins or induced structural changes in the organs of the rats, the differences in growth rate were probably not caused by any of these known mycotoxins. The possible presence of very low levels of known mycotoxins in these samples cannot be excluded altogether, however, because hand-sorted Fusarium-infected Zambian maize kernels have in fact been found to contain zearalenone as well as deoxynivalenol (Marasas, Kriek, van Rensburg, Steyn & van Schalkwyk, 1977) and sensitive analytical methods are not yet available for the detection of moniliformin in natural products (Cole *et al.* 1973).

Several fungi (including the two most prevalent seedborne fungi, *F. moniliforme* and *D. macrospora*) isolated from Zambian maize were acutely toxic to experimental animals (Table 6). The mycotoxins produced by these fungi have not yet been characterized chemically and could not be assayed in the maize

samples. If these unknown mycotoxins were present in the naturally infected maize, their mechanism of action must have been such that it did not induce histopathological changes in rats.

The results of these investigations suggest that unidentified Fusarium and Diplodia mycotoxins present in maize grown in Zambia in the 1974–1975 season and containing more than 1·8% visually diseased kernels reduced the growth rate of rats, particularly males, fed on these maize samples for 6 months. Consequently it cannot be recommended that the statutory maximum acceptable level of visually diseased kernels in Zambian maize should be increased above 2%. MacDonald & Raemakers (1974) similarly concluded from their feeding studies with maize grown in Zambia during 1973–1974 that the 2% level should be maintained. In the United States of America it was found that maize samples containing 5% or more of kernels visually damaged by *Gibberella zeae* (Schw.) Petch (i.e. *F. graminearum*) were almost totally refused by swine, while samples with a 3% level of infected kernels were often associated with a decrease in feed consumption and weight gain (Futrell, Scott & Vaughn, 1976; Tuite *et al.* 1974).

We fully realize that although a 2% level of diseased kernels may be an ideal maximum level to safeguard human health from the potential dangers of mycotoxin contamination in maize, it is probably unrealistically low from the practical point of view. Furthermore, the important consideration in determining the acceptability of maize for human consumption should be the actual concentrations of specific mycotoxins rather than the level of visually diseased kernels. The use of such criteria will obviously have to await the chemical characterization of the relevant Fusarium and Diplodia mycotoxins. Until that time, socio-economic considerations such as the choice between starvation and the possibility of ingesting harmful amounts of mycotoxins, rather than sound scientific considerations, will determine the maximum acceptable level of fungus-infected kernels in maize intended for human consumption. Meanwhile, it can be recommended only that this level should be kept as low as is practically possible, and that all husbandry, harvesting and storage practices that result in a reduction in the amount of fungal development in maize kernels should be encouraged.

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SHORT PAPERS

IS A MIXTURE OF POLYCHLORINATED DIBENZOFURANS AN INDUCER OF HEPATIC PORPHYRIA?

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Summary—Induction of hepatic porphyria by polychlorinated dibenzofurans and chlorinated biphenyls was compared in male SD rats. The polychlorinated-biphenyl mixture (Kanechlor-500) was a potent inducer of hepatic porphyria, while the mixture of polychlorinated dibenzofurans had only a slight effect, if any, at the levels which it proved possible to administer.

Introduction

Polychlorinated dibenzofurans (PCDFs) are contaminants of polychlorinated biphenyls (PCBs) and are highly toxic substances, as well as being structurally related to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), which is one of the most toxic chemicals known. The biological effects of a PCDF mixture in rats have included loss of body weight, atrophy of the thymus and accessory sex organs and development of haemolytic anaemia and lesions resembling chloracne (Oishi, 1977; Oishi, Morita & Fukada, 1977).

Some PCB compounds are strong inducers of hepatic porphyria and TCDD also induces hepatic porphyria in laboratory animals (Goldstein, Hickman, Burse & Bergman, 1975; Grote, Schmoldt & Benthe, 1975; Kimbrough, Linder & Gaines, 1972; Poland & Glover, 1973; Vos & Koeman, 1970; Vos, Strick, Van Holsteyn & Pennings, 1971). On the other hand, the effect of PCDF on the induction of hepatic porphyria has not been reported, and we have examined, therefore, the effect of a mixture of PCDF on biosynthetic systems of hepatic porphyrins and on the excretion of porphyrins in the urine of rats.

Experimental

A mixture of PCDFs synthesized by Dr. M. Morita, consisted of two tetrachloro-, four pentachloro- and four hexachlorodibenzofurans, and the average chlorine number of the aromatic ring substitution was 4·7. The PCB mixture used was Kanechlor-500 (supplied by Kanegafuchi Chemical Industry Co., Osaka). Its main components were the pentachlorobiophenyl isomers and it contained 6·1 ppm PCDF (Morita, Nakagawa, Akiyama, Mimura & Isono, 1977).

From the age of 5 wk, groups of six male SD rats (obtained from Clea Japan, Tokyo) were fed diet containing 1 or 10 ppm PCDF or 100 ppm PCBs mixed in ground Clea Rat Chow for 4 wk. A similar (control) group received untreated diet. At the end of this treatment, blood samples were taken for cell counts and haemoglobin determinations and the rats were killed by decapitation and the livers were removed rapidly and homogenized in ice-cold 0·1 M-phosphate buffer (pH 7·4). The homogenates were used for enzyme assays and determination of porphyrin concentration. The activity of δ-aminolaevulinate synthase (2.3.1.37; ALA-S) was measured by the method of Narisawa & Kikuchi (1966) and that of δ-aminolaevulinate dehydratase (4.2.1.24; ALA-D) by the method of Wada, Yano, Kurashina, Ono, Aoki & Toyokawa (1970). Hepatic and urinary porphyrins were extracted by a modification of the procedure of Dresel & Falk (1956) and determined fluorometrically (Undenfriend, 1962).

Results and Discussion

In PCDF-treated rats there was a marked reduction in haemoglobin concentration, and examination of blood smears revealed haemolytic anaemia.

The activity of hepatic ALA-S was increased significantly in PCB-treated rats and slightly in PCDF-treated rats (Table 1), but in neither case did hepatic ALA-D activities differ from that in the controls. Grote *et al.* (1975) reported that a single oral dose of 2·12 mmol PCB/kg increased ALA-S activity in rats but had no effect on ALA-D activity. On the other hand, the activity of ALA-S was not altered in chicks fed 0·1 μg PCDF/kg/day for 3 wk (Goldstein, McKinney, Lucier, Hickman, Bergman & Moore, 1976).

Concentrations of hepatic porphyrins are shown in Table 2. The copro- plus protoporphyrin concentration showed a significant increase in PCB-treated rats but was unaffected by PCDF treatment. Uroporphyrin concentrations were markedly increased in PCB-treated rats, to a level about seven times that of the controls, and were approximately twice as high as the control level in PCDF-treated rats. PCB-treated rats also showed a slight increase in urinary excretion of porphyrins. Kawanishi, Sano & Mizutani (1977) demonstrated that the porphyrin that accumulated most readily in chick-embryo liver cells cultured

Table 1. *Effect of PCDF and PCB on the activities of ALA-S and ALA-D in male rats*

Test compound	Dietary concn (ppm)	ALA-S (μmol ALA/g protein/hr)	ALA-D (μmol PBG/g protein/hr)
— (control)	0	0·85 ± 0·09	2·48 ± 0·17
PCDF	1	1·21 ± 0·19	2·48 ± 0·18
	10	1·17 ± 0·18	2·57 ± 0·22
PCB	100	1·53 ± 0·09*	2·34 ± 0·14

PCDF = Polychlorinated dibenzofuran PCB = Polychlorinated biphenyl
ALA-S = δ-Aminolaevulinate synthase ALA-D = δ-Aminolaevulinate dehydratase
PBG = Porphobilinogen
Values are the means ± SEM for groups of six rats and that marked with an asterisk differs significantly (Student's *t* test) from the control value: $P < 0.001$

Table 2. *Effect of PCDF and PCB on porphyrin levels in the livers of male rats*

Test compound	Dietary concn (ppm)	Copro- + protoporphyrin (μg/g wet tissue)	Uroporphyrin (μg/g wet tissue)
— (control)	0	1·22 ± 0·12	0·24 ± 0·01
PCDF	1	1·32 ± 0·17	0·49 ± 0·08**
	10	1·31 ± 0·16	0·42 ± 0·04**
PCB	100	1·65 ± 0·15*	1·43 ± 0·20**

PCDF = Polychlorinated dibenzofuran PCB = Polychlorinated biphenyl
Values are the means ± SEM for groups of six rats and those marked with asterisks differ significantly (Student's *t* test) from the control value: $*P < 0.05$, $**P < 0.001$.

in a PCB-containing medium was uroporphyrin, while Bruckner, Khanna & Cornish (1974) reported an increase in urinary coproporphyrin excretion in rats injected ip with PCB.

Although in the present experiment it was necessary to administer the PCDF in much lower doses than the PCB, because of the high toxicity of the former, PCB (Kanechlor-500) appeared to be a potent inducer of hepatic porphyria while the mixture of PCDF had at most a modest effect on porphyrin production in the liver.

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THE ESTIMATION OF AFLATOXIN M_1 IN MILK USING A TWO-DIMENSIONAL THIN-LAYER CHROMATOGRAPHIC METHOD SUITABLE FOR SURVEY WORK

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Summary—An existing method for the analysis of aflatoxin M_1 in milk has been modified by the introduction of two-dimensional thin-layer chromatography and certain confirmatory tests. The improved method is now sufficiently sensitive to demonstrate the presence in cows' milk of aflatoxin M_1 resulting from the ingestion of feed contaminated with aflatoxin B_1 at the very low maximum level permitted by the current UK Fertilizers and Feeding Stuffs Regulations.

Introduction

The method previously reported (Patterson & Roberts, 1975) for the analysis of aflatoxin M_1 in milk described improvements of the earlier procedure of Roberts & Allcroft (1968). The important feature of this modification was the introduction of a membrane clean-up step, which almost completely eliminated the risk of emulsion formation during extraction of the toxin into chloroform, drastically cut analytical working time and greatly increased the sensitivity of the method.

The present note describes further modifications, chiefly the use of two-dimensional thin-layer chromatography (TLC) and the application of confirmatory tests, and demonstrates that this procedure is now sufficiently sensitive for the detection of the trace amounts of aflatoxin M_1 that may appear in milk from cows consuming rations contaminated with aflatoxin at concentrations in the region of those currently allowed by the Fertilisers and Feeding Stuffs (Amendment) Regulations (Statutory Instrument 1976, no. 840, p. 13).

Experimental

The method is fundamentally that described in our earlier report (Patterson & Roberts, 1975) but a residue from the dry chloroform extract (equivalent to 38·5 ml milk) is routinely dissolved in 50 μl chloroform and then analysed, using two-dimensional TLC as described below.

Screening procedure. Aluminium sheets coated with silica gel 60 (0·2 mm thick, Merck Art. 5553, supplied by BDH Chemicals, Poole, Dorset) have recently been introduced because their relatively low cost is an important factor in survey work. Chromatograms are prepared by spotting 5 μl extract and aflatoxin M_1 standard (0·2 μg/ml; obtained from Dr. P. Schuller, Bilthoven, The Netherlands, and more recently from Makor Chemicals, Jerusalem, Israel), as shown in Fig. 1, and developing in two directions, first with chloroform–acetone–propan-2-ol, 80:15:5 by vol. (solvent 1) and then, after drying, with toluene–ethyl acetate–90% formic acid, 60:30:10 by vol. (solvent 2). R_f values for aflatoxin M_1 are 0·28 in solvent 1 and 0·07 in solvent 2, separating the toxin from the majority of chloroform-soluble constituents fluorescing in ultraviolet light. Occasionally a yellow fluorescent area obscures any aflatoxin M_1 present but, if the plate is left overnight in the dark, the interfering spot fades sufficiently to allow aflatoxin M_1 to be identified easily.

A duplicate chromatogram is prepared for co-chromatography in which 5 μl aflatoxin M_1 standard is superimposed on the dried spot of milk extract. The presence on both chromatograms of a single spot with appropriate R_F values in the two solvents and fluorescing blue in ultraviolet light suggests the presence

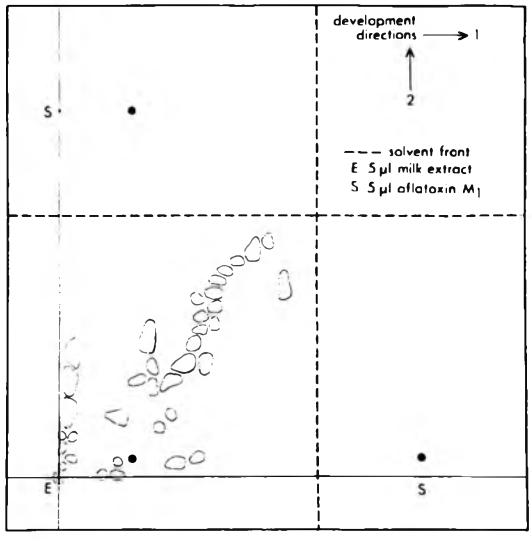

Fig. 1. Separation of aflatoxin M_1 from other constituents of milk fluorescing in ultraviolet light (365 nm). Extract (E) is applied at the origin in an aliquot of 5 μl (equivalent to 3·85 ml milk) and developed with solvents 1 and 2 up to the respective dotted lines (10 cm). For quantitative work, the single standard spot (S) shown only to migrate in solvent 1 is replaced by six standards (0·1 to 1·0 ng aflatoxin M_1) for visual comparison with the unknown.

of aflatoxin M_1 in the milk extract. Confirmation may then be obtained (a) by subsequently spraying the suspected spot with 50% sulphuric acid (cf. Pryzybylski, 1975) and observing conversion to a yellow fluorescent derivative and (b) forming the acetate of aflatoxin M_1 by reacting the dried residue from 25 µl extract in a glass reaction vial with equal volumes (0·2 ml) of dry pyridine and dry acetic anhydride for 30 min at room temperature (Stack, Pohland, Dantzman & Nesheim, 1972). R_F values of aflatoxin M_1 acetate in the two solvents are 0·54 and 0·18, respectively.

A few small variations on the above technique have also been tried. First, preliminary development in the first direction with diethyl ether prior to the use of solvent 1 (same direction) sometimes widens the 'chromatographic window' and hence improves the detection of aflatoxin M_1. Secondly, time and materials may be saved if only half of the aqueous acetone dialysate is taken for subsequent extraction and the analysis is suitably scaled-down. Thirdly, for similar reasons, the 20 × 20-cm sheet may be cut into four 10 × 10-cm squares to provide chromatograms that are usually just as easily interpreted.

Quantitative analyses. These are always carried out batch-wise, using full-size silica-coated aluminium sheets for the analysis of samples found to be 'positive' on initial screening. Two-dimensional chromatograms are prepared from 5 µl aliquots of extract, and any aflatoxin M_1 spots are compared visually under ultraviolet light (365 nm) with standard amounts of aflatoxin M_1 dissolved in the same volume (5 µl) of chloroform and allowed to migrate in solvent 1 only, 0·1, 0·2, 0·4, 0·6, 0·8 and 1·0 ng standard being applied as shown in Fig. 1. A stock solution of aflatoxin M_1 (10 µg/ml) in chloroform is first standardized by ultraviolet spectrometry, aliquots (0·2 ml) are evaporated to dryness and stored at 4°C, and for each quantitative analysis one aliquot is re-dissolved in chloroform (2 ml) and further diluted as necessary.

Use of the procedure in survey work. If milk samples cannot be analysed in the fresh state, they may be preserved with sodium metabisulphite (10 mg/ml milk) for short-term storage and for longer periods they may be held at −20°C. It has been found that in milk stored for a period of 21 days at 4°C, sodium metabisulphite does not diminish the concentration of added aflatoxin M_1 (1·0 µg/litre) nor is there any evidence for reduction of the toxin to M_2 or for the formation of any other derivative.

The smallest quantity of aflatoxin M_1 that is easily visible on two-dimensional TLC is about 0·2 ng when applied in 5 µl chloroform, and quantitation is therefore only feasible when 5 µl milk extract contains approximately this amount. This corresponds to a quantitative analytical limit of 0·052 µg aflatoxin M_1/litre milk, but even smaller amounts of the toxin can be detected if not quantitated, so that the detection limit of the method is about 0·03 µg/litre.

Results and Discussion

The method described above is being successfully used in this laboratory for the routine examination of milk samples, although other workers have experienced difficulty particularly with the dialysis step (see our earlier note, Patterson & Roberts, 1975). Such difficulties are most likely to be due either to the occasional 'pin-hole' in the dialysis tubing or to incomplete soaking of the membrane prior to use. The former can easily be tested for and detected if each prepared dialysis sac is first filled with water, and the latter procedure is best carried out using warm (40 C) water for at least 30 min.

The detection limit of the newly modified method (approximately 0·03 µg/litre) is some 100 times lower than that of the original procedure of Roberts & Allcroft (1968) and shows a threefold improvement on the first modification (Patterson & Roberts, 1975). The latest procedure is almost certainly sufficiently sensitive to detect any aflatoxin M_1 'carried-over' into milk from aflatoxin B_1 present in the diet at the very low maximum level permitted by the current Fertilizers and Feeding Stuffs Regulations. Thus, on the basis of a value of 1/300 for the ratio of the concentrations of aflatoxin M_1 in milk to aflatoxin B_1 in feedstuffs (Rodricks & Stoloff, 1977), it can be calculated that, if such a ration containing 20 µg aflatoxin B_1/kg is consistently fed to dairy cows, their milk would contain around 0·067 µg/litre, which is about twice the detection limit.

Individual estimates of the 'carry-over' of aflatoxin into milk suffer from one or more shortcomings, but that of Rodricks & Stoloff (1977) has been used here because it is an average value derived from data published by several research groups. Even so, the basic data were obtained between 1965 and 1974, when analytical methods were undergoing development, and the various breeds of cattle used were presumably at different stages of lactation and reared on differing husbandry systems. The above calculation is therefore only very approximate, but it is useful in that it determines the working range now required of analytical methods for M_1 and the present procedure is evidently capable of detecting and measuring at realistic concentrations this undesirable naturally-occurring contaminant of milk.

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Review Section

THE BIOLOGICAL EFFECTS OF TALC IN THE EXPERIMENTAL ANIMAL: A LITERATURE REVIEW

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Summary—A critical review of the literature dealing with the biological effects of a variety of talcs shows the mineral to be fibrogenic when administered by various routes to many species of animal. The literature also indicates clearly that the fibrotic response is a function of the dose administered and that there are levels of exposure that are tolerable. The exposures and doses used in the majority of the studies were not chosen on the basis of their relationship to occupational or cosmetic use. In none of the reported studies was there any indication of neoplasia. The literature offers the reader a good descriptive account of the pathogenesis of experimental pneumoconiosis and identifies the critical role played by the alveolar macrophage.

Introduction

The biological effects of talc have been studied extensively by a variety of *in vivo* and *in vitro* procedures but, unfortunately, most of the literature fails to identify the mineral adequately in respect of its source, particle size, particle distribution and content. The responses to varying doses administered by various routes have been described but here, too, the dose and route of administration frequently bore no relation to the usual levels of cosmetic or industrial exposure. It is unclear from the literature what portion of the research was conducted for reasons of occupational or user safety. In fact, most of the work appears to have been done to satisfy scientific curiosity.

One of the most prominent and early workers on the pulmonary effects of dust was Mavrogordato (1918), who established a hypothesis regarding the pathogenesis of various dust diseases. He concluded from his studies that dusts that create problems are dusts that accumulate. Dusts that produce a marked initial reaction are eliminated because they result in shedding of the epithelium. Those that accumulate do not.

Experimental studies

Haynes (1931) published the results of an extensive study in guinea-pigs on a variety of dusts. From these studies, he was able to construct a beautifully detailed description of the pathogenesis of experimental pneumoconiosis, a description which does not differ much from our concept today. One of the dusts studied was talc. He concluded that talc stimulated the elimination of alveolar macrophages and in no way acted as a toxic agent. In animals given a 2-hour/day dust exposure for 2 weeks, bronchial and lymphatic clearance were proceeding moderately briskly 60 weeks later with no sign of pulmonary damage. Haynes (1931) classified talc among the non-harmful groups of minerals that were found to cause no permanent damage in the lungs.

Stüber (1934) studied the cellular response of the popliteal and cervical lymph nodes of dogs to various dusts which had been characterized according to their content of free silica. The dusts were injected directly into the lymphatics at a dose of 50–100 mg, administered in a volume of 10 ml water at a pressure that did not exceed 70 mm Hg. Talc was one such dust, and it was described as containing 60% silica. Within 24 hours, the author observed giant cells crammed with mineral particles; at day 5 most of the particles were present in endothelial macrophages and by day 108 the node appeared normal but, scattered throughout, were groups of mononuclear phagocytes containing mineral particles. The author concluded that in terms of the reaction evoked, talc and other organic and inorganic dusts differed from silica in that they did not stimulate the formation of abnormal phagocytes but instead were retained exclusively in the reticular cells and endothelial phagocytes.

Fossel (1935) postulated that rocks such as kaolin, muscovite, sericite and talc, which have reached their optimal chemical stability in nature, are probably less reactive biologically than those that are less stable. He also appreciated the difficulty of studying mill workers, in whom the definition of dust exposures was impossible at that time. Accordingly, he exposed guinea-pigs to a talc dust for 1 hour/day for as long as 8 weeks. The total exposure was not defined, but the talc was identified as "Naintsch 00/v" containing 62·0% SiO_2, with 55·4% of the particles falling within the 0·06–0·13 mm range; 14·8% were less than 0·007 mm and 5% were greater than 0·06 mm. While the total number of animals exposed was small (ten), the author completed thorough histopathological studies on the lungs of a few animals and found that there was no evidence of nodulation, as in human silicosis, although numerous dust particles were deposited in the lungs. The longer the interval of time

between exposure and autopsy, the fewer inflammatory responses and the less evidence of talc.

Gardner (1938), like other investigators, recognized the difficulty of studying responses to dust in the human population and resorted to an experimental model to satisfy his scientific curiosity. His interests were not to delineate the hazards associated with occupational or user exposure, but mainly to describe the biological response to various mineral dusts. He postulated that connective tissue would respond to particle stimuli regardless of its location within the body, and therefore injected various minerals intravenously into rabbits as 1 and 10% suspensions in saline, while guinea-pigs received 200-mg doses into the abdominal cavity. Gardner (1938) made the first attempt to quantitate the response and offered a logical descriptive response profile which varied from ± to 8+. The response to talc was graded as 2+ which, in this investigation, meant that the cells directly adjacent to the mineral had been irritated and that there was slight evidence of inflammation. The author concluded that "this change has shown no tendency to progress in two years".

Policard (1939–1940) was sufficiently curious about the potential biological effects of inhaled talc to expose rats to an extremely intense dusting with industrial-grade foliated talc (for 3 hours/day for 12 days with a maximum observation period of 32 days). While the particle size was described as less than 5 μm, the concentration of dust in the atmosphere was not known. The author concluded that acute effects of exposure to a high atmosphere of talc could be irritating to the upper respiratory tract and probably accounted for the dyspnoea that he observed in his experimental animals. In contrast to silicate, talc did not appear to damage the nucleus and mummification of the cell was never observed. From histological observations, elimination of talc particles through the lymphatic system did not appear to be very extensive. The particles were trapped in alveolar macrophages and the mineral seemed to be harmless to these cells.

Schulz & Williams (1942) were the first to recognize that differences in the chemical composition of 'talc' may account for the differences in the responses observed. They selected seven commercial 'talcs' with widely different mineralogical compositions and injected guinea-pigs with these sterilized talcs, administering in each case 200 mg in the form of 5% saline suspensions. Animals were killed at intervals ranging from 10 days to 15 months. The major impurities identified were serpentine, carbonate, quartz and dolomite. Grossly, the animals were in good health and all the organs appeared normal, but nodules were observed on the ventral parietal surface of the peritoneum. The lesions became more dense and firm with time. Histological studies confirmed the observations of previous workers that the mineral was mostly phagocytosed by macrophages and giant cells. There was no indication of the proliferation that occurred in silicotic nodules. Interestingly, the carbonate and serpentine components decreased as the experiment progressed and the authors concluded that such talcs were to be preferred from the health aspect inasmuch as smaller amounts of material were permanently stored.

Schepers & Durkan (1955), prompted by the occurrence of a series of deaths from pulmonary disease following exposure to talc mining in upper New York State, initiated a series of animal studies to probe the specific effects of some of the talc contaminants (tremolite, anthophyllite, quartz, serpentine and dolomite). Both the intravenous and intratracheal routes were used in rabbits, rats and guinea-pigs. The size of the dust particles was 3 μm or less for the intravenous studies, while particles and fibres measuring 20–50 μm were used for the intratracheal study. One set of intratracheal experiments was designed to mimic the industrial exposure in New York State. Interestingly, the results of these experiments confirmed that the basic reaction to these dusts was the engorgement of particles by alveolar macrophages and their subsequent immobilization in the lungs, from which they were ultimately extracted or returned to the parenchyma. The degree to which fibrosis occurred depended upon the length of the fibre rather than upon its chemical composition. Particles of 3 μm and less evoked no fibrogenic response, whereas tremolite and anthophyllite fibres of 20–50 μm produced extensive fibrosis. Unfortunately, the investigators did not have talc in a long fibrous form. However, the talc that was used did not lead to collagen deposition. The bronchiolar lesions produced by pure quartz were less marked when talc was added. This protective effect of talc may be explained on the supposition that the koniophores* were effectively immobilized by previous exposure to talc, thus removing the potential impact of quartz.

Wise (1955) compared the reaction of nervous tissue to talc and to starch glove powders. The author did not describe the type or form of talc used but identified the starch as BIOSORB®. His observations, based on applying 75 mg of each powder to the brain, spinal cord and peripheral nerves, revealed that talc produced more scarring than starch. Although the total quantity of powder applied was not in excess of that which could be recovered from a pair of surgical gloves, the method of application did not attempt to mimic the distribution that might occur in actual use; 75 mg would be likely to produce a much greater response when placed at a specific site than if it were dispersed, as might be the case during a surgical procedure.

Lüchtrath & Schmidt (1959), in an excellent and well-documented treatise, emphasized again the importance of defining the mineralogical composition of talc before attempting to develop a causal relationship to lung disease. The authors questioned the value of additional studies in man as a means of advancing our knowledge on talc but were quick to point out that experimental studies have failed to clarify the problem because of the variable composition of dusts used in animal experiments. Their studies were designed, therefore, to compare the pulmonary response in rats to a sample of pure talc containing no quartz and only traces of chlorite, to two samples containing from 1 to 3% quartz plus some chlorite and to a fourth sample containing 8–10% quartz, partly in the form of cristobalite. The latter sample

*It is believed that the authors were referring to macrophages.

was fired in the course of normal manufacturing and was subsequently ground. The test minerals were administered to rats by the intratracheal route at a dose of 50 mg in 1 ml water, but this resulted in extensive dyspnoea and a high death rate as a consequence of bronchial occlusion, so the dose was reduced to offset the acute response. One wonders why investigators who were so meticulous in describing the shortcomings and pitfalls of previous studies erred in the direction of a poor protocol by using a method of administration that did not attempt to simulate occupational or cosmetic exposure. The rats tested by Lüchtrath & Schmidt (1959) developed an acute inflammatory effusion which "assumed pneumonia proportions" but, as the investigators stated, "it is not surprising if one considers the large amount of dust penetrating into the lungs". Following the acute phase, it became possible to discriminate between the reactions to the different forms of talc. It was concluded that the aspiration of pure talc dusts resulted in the development of small nodules consisting of histiocytic storage cells but no major fibrotic reaction was noted. In contrast to this, the dust containing free silica resulted in marked pulmonary fibrosis. On the basis of these animal experiments, Lüchtrath & Schmidt (1959) expressed the opinion that one would not be justified in condemning pure silica-free talc as having a fibrogenic effect on the lung. The authors did recommend, however, that appropriate protective measures be taken to minimize unnecessary industrial exposure.

Kuchling (1961) introduced talc into the peritoneal cavity of five different species of birds, suspending 1·5 g undefined talc in 10 ml saline and injecting volumes of 2·0–0·5 ml, depending upon the size of the bird. While there were minor differences in the responses, it appeared that these were less pronounced than the differences reported in mammalian species. The study added very little to a better understanding of the biological effects of talc.

In 1962, two groups published papers relating to the peritoneal responses of talc. Blümel, Pizá & Zischka-Konorsa (1962) were interested primarily in the response to a starch glove powder and used talc as a reference material. They described neither the strain of rat used, nor the mineral content, source or dose of talc administered. Zullig (1962) also administered talc intraperitoneally and subcutaneously to rats, but he, too, failed to disclose the composition of his talc or its source, when comparing the responses to talc and to starch injected intraperitoneally in a 2-ml dose of a 1% aqueous suspension. Both investigating groups discovered that the tissue response to talc was greater than it was to starch.

A poorly understood and probably nonspecific effect of talc was described by Nishimura, Rosenheim & Klein (1963) following the subcutaneous administration of a large (1-g) dose of talc of USP grade to mice. This dose was equivalent to 33 g/kg body weight or 1·650 kg talc administered to a 70-kg (150-lb) human adult. A transient depression of hepatic catalase was observed, a response that has also been observed in animals with actively growing malignancies. The response was more pronounced in males and probably related to the injury imposed by introducing a large quantity of mineral into the tissue. Although a humoral factor was considered to be a possible cause of the enzyme depression, the investigators suggested inhibition of protein synthesis as an alternative explanation.

In a study designed to demonstrate the irritant action of an aluminosilicate mineral (spodumene), the integument of rabbits was dusted daily with the mineral for 90 days, after which the condition of the skin was compared with that in two other groups of rabbits similarly treated with rock crystal (SiO_2) and talc (Grigor'ev, 1963). The author reported no indication of irritation in any of the animals treated with rock crystal or talc.

Some of the literature on talc contains the results of studies which appeared to be designed to generate basic biological data with seemingly little relevance to occupational hazard or cosmetic use. Three such papers were published by Eger & DaCanalis (1964), Kaltenbach, Radeke, Nishimura & Siddiqui (1966) and Darcy (1966).

Eger & DaCanalis (1964) went to great lengths to introduce talc and asbestos into the portal circulation and later into the splenic vein as suspensions in carboxymethylcellulose. Using the latter technique, they were successful in depositing up to 140 mg/100 g body weight of each mineral into the spleen, from where it was gradually released to the liver. It was possible, using this dose, to block the portal vein and its branches completely, with hepatic necrosis occurring in the survivors. The authors' additional conclusions about differences in degrees of necrosis produced by the three minerals hardly seem warranted when, for example in the case of talc, they had only eight surviving rats to examine at five different times, the longest period being 10 days. They failed to mention the number of surviving animals in the groups treated with the other two minerals, both of which groups were followed for much longer periods of time.

Kaltenbach *et al.* (1966), in an extension of the work previously reported by Nishimura *et al.* (1963), showed that in addition to hepatic catalase depression, there was also a concomitant increase in the incorporation of leucine. Neither observation was of clinical significance and both probably occurred in response to the tissue destruction following the administration of massive doses of talc. Unfortunately, the investigators did not use another mineral at a comparable dose as a control, but it was believed that the catalase depression was nonspecific.

In a somewhat related study, Darcy (1966) reported on the enhancement of glycoprotein synthesis following various forms of tissue damage. Tissue necrosis was produced by the subcutaneous injection of turpentine. Subsequent injections of turpentine or talc at approximately 400 mg/kg resulted in an increase in serum globulin. The author concluded that the effect was not substance-specific but was probably mediated through direct physical damage to the tissues. Further studies of a similar nature were reported by Gordon & Ko (1968).

Rakowski (1964) was of the opinion that except for the work of two Soviet and two American authors, the literature was devoid of studies dealing with the morphological alterations occurring in talc pneumoconiosis. But as described earlier, five papers were published on this subject prior to that of Rakowski

(1964), whose study offered little, if any, new information and, in fact, has been subject to criticism. He failed to describe the talc or give its source, and his method of exposure consisted of scattering the powder by means of a rubber balloon into specifically constructed cages—a somewhat primitive procedure for the early 1960s. For this reason, the author was able to describe only the duration of exposure (2, 4, 6, 8 and 12 months) and had no knowledge of the daily or total dose. The reaction to talc was compared with that to Axotox dust, a product containing 95% talc plus DDT. Both materials produced in the early phases a diffuse inflammation in the parenchyma which later became focal. The response to Axotox was more intense than that to talc. Scarring with collagen deposition did not occur until month 10 or 12.

In a series of experiments directed toward a better understanding of the physiopathology of cobalt-induced epilepsy in the rat, talcum powder was used by Payan (1967) along with other materials. The mineral was packed into a pellet measuring 1 × 2 mm and implanted into the frontal cortex of the animals; 8 days later the convulsive threshold of the animals was checked, and on day 33 they were killed and the brains were fixed in Bouin's solution for histopathological study. The convulsion incidence was not elevated following the talc exposure and the author reported minimal or no reactions to talc, graphite and alumina cream. No mention was made of the source of the talc.

Using an *in vitro* technique to measure the lytic effects of various minerals, MacNab & Harington (1967) added 50 mg of the mineral to a 2% suspension of washed sheep erythrocytes in buffered (pH 7·4) isotonic saline. Significant haemolytic activity was associated with chrysotile, serpentine and all forms of silica tested. The remaining powders, including talc, were either completely inactive or only weakly lytic.

The only reported study dealing with talcum powder in the chinchilla was that of Trautwein & Helmboldt (1967). These authors attempted to prove the hypothesis that chronic interstitial pneumonia in the chinchilla may be a prerequisite for the development of adenomatosis following administration of talc intratracheally as a 2% suspension in saline. The talc, described as pure and obtained from Fisher Scientific Company, Fairlawn, N.J., was administered to each animal in a volume of 2·0 ml. One group of chinchillas received injections at 1, 20, 50, 70 and 90 days and was killed 11 months after the last injection. A second group received a single injection and the animals were killed 24, 48, 72 and 120 hours and 1, 2, 3, 4, 5, 6, 17 and 28 weeks after injection. Animals in a third group were injected weekly for 9 weeks and survivors were killed 1, 2 and 3·5 months after the last injection. Single or multiple administration evoked essentially the same response. The mineral caused chronic irritation of the bronchiolar and alveolar cells and the eventual development of focal adenomatoid changes. The authors were quick to point out that in man and domestic animals, spontaneous pulmonary adenomatosis is often associated with chronic pneumonia caused by viruses, bacteria and other agents. It can be produced in guinea-pigs by Mycobacteria and in rabbits by non-carcinogenic substances such as bacterial toxins, vaccinia virus and HCl. In this study, talc served as a chronic and persistent irritant and as such created a situation that was conducive to the development of adenomatosis.

In a study designed to measure the impact of peritonitis on small-intestine motility, Lill (1967) was unable to find inflammatory changes in the peritoneum of guinea-pigs either macroscopically or microscopically following the injection of 3·0 ml of a 0·5% aqueous talc suspension.

Gross, de Treville, Cralley, Granquist & Pundsack (1970b) studied in rats the pulmonary response to fibrous dusts of diverse composition, including tremolitic talc with a high or low nickel content and an average fibre diameter of 0·3 or 0·1 μm respectively. All the dusts were suspended in water at levels up to 25 mg/ml. Except for four rats that were killed 4 days after the first injection, all animals were allowed to live out their lives. The response to talc was one of proliferative inflammatory foci followed by considerable shrinkage of the lesions and subsequent conversion of the argyrophilic stroma into dense collagen. Gross *et al.* (1970b) made a distinction between "inert dusts" and "reactive dusts" on the basis of lesion reversibility, maintenance of the anatomical integrity of the air space and lack of significant collagenization, all of which are associated with inert dusts. The authors classified talc as a "reactive" material, but in none of the animals was there indication of neoplastic change. In a second paper, Gross, deTreville & Cralley (1970a) reported on studies relating to the carcinogenic effects of asbestos dust. They found that 35% of 72 rats exposed to an average of 86 mg chrysotile/m^3 and surviving for 16 months developed neoplasms in the lungs, but hamsters and guinea-pigs similarly exposed did not. The dust contained significant levels of cobalt and nickel as a consequence of the wearing of the hammer-mill used in its generation. To explore the possible connection between these facts, trace metals were added to synthetic chrysotile and these were injected intratracheally into rats. Other groups of rats were similarly injected with talc having a naturally high and naturally low nickel content. All of the animals were allowed to live out their lives and none developed any tumours.

The first real attempt at mineral definition came from Bethge-Iwańska (1971), who strongly emphasized the need for standardization of the mineralogical nomenclature pertaining to talc. She studied two different grades of talc by exposing rats to an aerosol of dust that varied from 30 to 346 mg/m^3. The dust was blown into the chamber six times a day at 1-hour intervals over a period of 9 months. The exposures were intended to simulate the conditions of exposure encountered by employees in the rubber industry. The two talcs used were described as technical grade and pharmaceutical grade. The technical grade contained a smaller percentage of small particles and a greater percentage of large particles than the pharmaceutical grade. This author discovered, as did others before her, that pulmonary exposure to large doses of talc resulted in a syndrome of nonspecific, chronic inflammatory changes, which initially were of the cellular-reaction type and later became fibrotic in character, resulting in a thickening of the alveolar walls and atrophy of the anatomical components of the respiratory tract and leading to emphysema. However, she

could not distinguish between the two grades of talc. The reason for this may have been the dose used, which must have overwhelmed the respiratory system and made discrimination between the two types impossible.

Hayashi (1971) published an extensive literature review on "Interaction between minerals and the living body, with reference to pneumoconiosis". Talc was among the minerals discussed. The author discovered that the first specific treatise on dust toxicity appeared in *De Re Metallica*, a technical book on minerals and mining written by Agricola in 1556. It is also interesting to note that by the end of the 18th century, it was known that the inhalation of dust was harmful but that the degree of harm depended upon the type of dust inhaled. Hayashi (1971) quickly recognized that the hazard associated with dust inhalation must be considered in terms of particle size, total volume, composition, description of the dust and history of exposure.

Using as an index of cytotoxicity the amount of particles (0·5–2·0 μm in size) required to produce 50% inhibition of the dehydrogenase activity of the macrophage surface, Koshi, Kawai & Sakabe (1959) considered talc, along with sericite and pyrophyllite, to be relatively highly cytotoxic.

Pott & Friedrichs (1972) were intrigued by the reported differences in reactions to dusts of varying physical forms. Accordingly, they designed an experiment to compare the peritoneal response to fibrous dusts of dissimilar chemical composition with that of dusts that were essentially similar chemically but differed in physical form. Each rat received, intraperitoneally, 25 mg of a specific dust suspended in saline up to four times each week. The investigators neglected to mention the total number of injections given to each rat, but they did indicate that the animals were observed for up to 530 days. Chrysotile, normaline and glass, very different chemically but similar physically, produced intra-abdominal tumours to the same degree. The non-fibrous dusts, including talc, did not. They concluded that the carcinogenic action of chrysotile was due to the presence of fibres exceeding 2–3 μm in length.

To understand better the process of mineral fibrogenesis, Davis (1972) introduced 10-mg quantities of various dusts into the pleural cavity of BALB/c mice. Many theories have been advanced over the years as to the mechanism of dust-induced fibrosis and most of these have stemmed from studies involving silica and asbestos. The theories relating to silica have embraced the liberation of silicic acid, the abrasive surface of the dust, the involvement of the autoimmune system, and the destruction of the macrophage cell membrane with liberation of lyosomal enzymes. It appears that fibre length plays a role in asbestos fibrogenesis. The talc samples used by Davis (1972) were not pure; he described them as irregularly shaped plates 1–10 μm in length mixed with small quantities of asbestos fibres 0·05–0·5 μm in diameter and up to 2·0 μm in length. One wonders why a mixed mineral was used in an experiment designed to promote understanding of the process associated with dust fibrosis. Talc, along with five other mineral dusts, was reported to generate an extremely good cellular response. Large granulomas were produced but the particles were widely spaced. A few giant cells were observed. The granulomas became encapsulated but, in the case of talc, were firmly attached to the lung or chest wall. As a rule, the cellular components of the granulomas were replaced by collagen over a period of 6–8 months. Davis (1972) concluded that long fibrous dusts are usually the ones to produce adhesions except in the case of talc, with which adhesions were commonly found. In assessing the significance of this conclusion, it is important to remember that pure talc was not used in these studies but rather a mixture of talc and asbestos.

The effect of talc on the immunoglobulin (Ig) response in mice was studied by Carson & Kaltenbach (1973) following the subcutaneous injection of sterile talc into NZB, NZW and Strong A mice. The talc was not identified as to type or source, but the authors noted that a single injection resulted in an increase in serum IgM at 24 hours and an increase in IgG at 24–48 hours. A double injection did not alter the response nor did a second injection given at a different time. The response was probably mediated through an auto-immune mechanism, wherein the cellular and extracellular proteins were modified into foreign proteins by lysosomal enzymes following the inflammatory response. The modified proteins could have become absorbed on to the talc with subsequent stimulation of the host's immune mechanism. Talc was not thought to act as an antigen.

Schenker & Polishuk (1973) studied the effect of various agents on endometrial regeneration, in experiments prompted by the reported incidence of intrauterine adhesions following physical damage to the mucosa, as might occur during abortion. Using rabbits, they introduced 2 ml of a 10% suspension of talc into one horn of the uterus of one group of animals. Other animals were treated with 10% formaldehyde in saline, 2% formaldehyde in ethanol, copper sulphate pentahydrate pellets, 0·3% sodium lauryl sulphate or quinacrine HCl (50 mg/ml). The uteri were removed after various periods of time and were fixed and subsequently examined for evidence of persistent mucosal damage or regeneration. Talc, lauryl sulphate and quinacrine did not induce changes greater than those produced by curettage alone, while 2% formaldehyde and copper sulphate induced significant uterine pathology but, unlike 10% formaldehyde, did not prevent regeneration.

In May 1973, at a Symposium sponsored by the United States Bureau of Mines, Smith reported on studies, which he had completed but not published, on tremolitic talc. After simple intraplural injection of various doses of chrysotile, amosite, anthophyllite, crocidolite and tremolitic talc into groups of 50 hamsters, the animals were allowed to survive for varying periods of time. Except for the talc, which was administered in a dose of 25 mg, the materials were administered in 25-, 10- and 1-mg quantities. These studies by Smith (1973) proved again what had been known for other carcinogens, namely that the carcinogenic effects of chrysotile are dose-related and that the implementation of appropriate control measures should eliminate any occupational risk. His studies also showed that the fibrogenic activity of tremolitic talc was much less than that of the other four types of

asbestos, although the sample used in these studies contained 50% fibrous talc ranging from 2·5 to 16·5 μm in length and from 1 to 5 μm in diameter. The animals injected with talc survived longer than those in the other groups, and therefore were subject to a longer induction time, yet no mesotheliomas developed. Throughout the test, animals treated with chrysotile were found to have thick fibrous pleural adhesions extending over large areas of the lung, whereas animals treated with talc never showed more than small deposits of mineral particles with relatively little tissue reaction.

Results dealing with the teratological and mutagenic effects of talc were revealed in two HEW-supported studies (Food and Drug Research Laboratories, Inc., 1973; Litton Bionetics, Inc., 1973). The teratology studies, conducted by the Food and Drug Research Laboratories, involved rats, mice and hamsters. The mineral was administered orally at doses of 1600 mg/kg (to mice and rats) and 1200 mg/kg (to hamsters) from day 6 to 15 in the case of mice and rats and from day 6 to 10 in the case of hamsters. The results indicated no effect on the parameters measured.

The mutagenic studies conducted by Litton Bionetics, Inc. tested the same talc as was used for the teratogenicity studies and used four classical test models, namely the host-mediated assay in mice, the *in vivo* cytogenetic assay in rats, the *in vitro* cytogenetic assay, and the dominant lethal assay in rats. For each of the *in vivo* assays, doses of 30, 300 and 3000 mg/kg were used, while doses of 2, 20 and 200 mg/ml were used in the *in vitro* assay. No significant increase in mutant recombinant frequencies were observed in Salmonella and Saccharomyces, no detectable aberrations of bone-marrow metaphase chromosomes occurred, nor were the anaphase chromosomes of human tissue culture cells noted. Lastly, the mineral was considered to be non-mutagenic by the dominant lethal assay.

Pott, Huth & Friedrichs (1974) conducted an experiment in rats to study the tumorigenic effect of fibrous and granular dusts introduced into the peritoneal cavity as a suspension (25 mg/2 ml) in saline. The investigators allowed the animals to live out their normal lives or killed them when they were moribund. All tumours were studied histologically. UICC standard asbestos as well as polygorscite, normalite and glass induced tumours in 30–67% of the rats, while several non-fibrous dusts closely related to chrysotile (such as actinolite, biotite, pectolite and talcum) led to tumours in only a few cases. Histologically nearly all of the tumours were sarcomatous mesotheliomas.

Henderson, Blundell, Richards, Hext, Volcani & Griffiths (1975) studied the behaviour of cultured rabbit-lung fibroblasts when confronted with particles of talc, by adding an undisclosed dose of (Italian 00000) talc to 100 μl of Medium 199, 24 hours after subculturing, and removing samples of the culture at various times for electron microscopy. They were able to show that talc particles up to 10 μm can be phagocytosed by fibroblasts and, like chrysotile, do not penetrate the nuclear membrane.

The most meaningful study relating to the use of cosmetic talc was conducted recently by Wehner, Zwicker, Cannon, Watson & Carlton (1977) Syrian golden hamsters were exposed in a specially designed chamber to a dust cloud of cosmetic talc for varying periods of time based on the calculated median human exposure. The dose was arrived at by simulating the dusting of an infant and collecting the dust cloud at the level of the nares. Mean dusting times of infants also entered into the calculation. Groups of animals were exposed to an atmosphere of talc ($9{\cdot}8 \pm 2{\cdot}4$ mg/m^3), consisting only of particles in the respirable range, for periods of time equivalent to 12–6000 mg hr/m^3. The hamsters were then allowed to live out their lives. Estimates based on infant dusting experiments show that the weekly hamster exposure exceeded the average weekly infant exposure by 30 to 1700 times. Two additional groups of hamsters represented sham control groups. These studies indicated that the exposure of hamsters to an aerosol of cosmetic talc resulted in the deposition of numerous talc particles in the lungs, as determined with the light microscope and confirmed in a subsequent study by the use of neutron-activated talc (Wehner, Wilkerson, Cannon, Buschbom & Tanner, 1977). At these levels of exposure, there was no effect on body weight, morbidity, mortality or type and incidence of histopathological change.

Another recent paper dealing with the biological response to talc describes an experiment conducted to assess the risks of the narcotic addict to parenteral injections of tablets containing talc as a filler. Dogra, Iyer, Shanker & Zaidi (1977) described the particle-size distribution and chemical composition of a talc obtained from Jaipur, India, and used in their studies, in which 25 mg talc in 0·5 ml saline was injected intravenously into guinea-pigs three times at weekly intervals. A significant number of animals died immediately after the second or third dose. The investigators described a mild proliferation of the pulmonary vascular endothelium with a moderate localization of talc in the capillaries of the lung and liver and in the abdominal lymph nodes. The long-term effects (150 days) in guinea-pigs were described as moderate thickening of the inter-alveolar septa, in contrast to the development of granulomas and angiothrombotic lesions seen in the human addict.

Conclusions

Although the talcs used in a variety of studies described in the literature have not been adequately characterized, it is possible to formulate a few general conclusions on the biological response to this mineral. It has been shown that the mineral, in different degrees of purity, is fibrogenic and that the fibrotic response is a function of the dose. It has also been shown that certain levels of exposure are tolerable. Neoplasia has not been an observed response to talc.

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ROLE OF SULTONE CONTAMINANTS IN AN OUTBREAK OF ALLERGIC CONTACT DERMATITIS CAUSED BY ALKYL ETHOXYSULPHATES: A REVIEW

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Summary—An outbreak of severe allergic contact dermatitis occurred in Norway in 1966 in women who had used a new dish-washing product containing an alkyl ethoxysulphate. The dermatitis was sometimes accompanied by adverse systemic effects. Subsequent investigations revealed the presence of unsaturated sultone and chlorosultone contaminants in the alkyl ethoxysulphate component of a particular batch of the detergent. Their presence was attributed to abnormal manufacturing conditions. These sultones were shown to be potent skin sensitizers and the allergic contact dermatitis was attributed to them. The various clinical, toxicological and analytical aspects of this occurrence are reviewed and discussed here. The work represents a model study of this type of problem, in which an occasional contaminant in a commercial product gives rise to untoward skin reactions.

Introduction

Alkyl ethoxysulphates (alkyl ether sulphates; AES) are anionic surfactants that have been in widespread use for over 20 years in a variety of domestic products, including dish-washing liquids, shampoos, bubble-bath preparations, car cleaners, cosmetics and similar products, without any reported evidence of skin-sensitizing ability (Walker, Ashforth, Davies, Newman & Ritz, 1973). In 1966, there was an outbreak of severe allergic contact dermatitis in Norway (Magnusson & Gilje, 1973) in women who had used a new dish-washing product containing AES as a major constituent. Apart from sodium lauryl ether sulphate (21·7%), the product contained nonylphenol ethoxylate (6·0%), tertiary amine oxide (2·0%), ethanol (6·5%), formaldehyde (0·2%) and colour and perfume (0·03%).

Some of these cases of dermatitis were accompanied by systemic symptoms of headache, nausea, fever and depression. The dermatitis was attributed to the AES component of the dish-washing liquid. This was surprising because AES is generally less irritating to human skin than are other surfactants (Smeenk, 1969; Valer, 1969) and pure lauryl ethoxysulphate (LES) exhibits no sensitizing action in the guinea-pig maximization test (Gloxhuber, Potokar, Braig, van Raay & Schwarz, 1974). Furthermore, the systemic symptoms were completely unexpected. Two other unexplained Scandinavian outbreaks of allergic contact dermatitis with products containing LES were also mentioned by Magnusson & Gilje (1973) together with an outbreak in Sweden in 1953–54 associated with a detergent containing sodium lauryl sulphate, which up to that time was believed to be free from such effects; this seemed relevant to the problem since LES apparently contains about 15% sodium lauryl sulphate.

The alkyl ethoxysulphates have the general formula $H{\cdot}[CH_2)_n{\cdot}[OC_2H_4]_m{\cdot}OSO_3^-M^+$ where n usually has values around 12–16, m is usually an average number between 2 and 12, and M^+ is a sodium or ammonium ion. In the alkyl ethoxysulphate implicated in the Norwegian cases of dermatitis, values for n were predominantly 12 and 14 (Connor, Ritz, Ampulski, Kowollik, Lim, Thomas & Parkhurst, 1975). This material was referred to as lauryl ethoxysulphate since lauryl alcohol is the trivial name for $C_{12}H_{25}OH$.

There is now evidence that the Norwegian outbreak was due to chlorinated and/or unsaturated sultone contaminants present in one particular batch of LES (Connor *et al.* 1975). This article reviews the clinical, toxicological and analytical aspects of the problem and some general implications are discussed, because the work represents a model study of the type of problem in which occasionally a toxic contaminant is responsible for untoward toxic reactions elicited by a commercial product.

Clinical observations

Magnusson & Gilje (1973) described the Norwegian outbreak of dermatitis in detail. Generally, a severe dermatitis appeared about 2 weeks after exposure to the dish-washing liquid and the onset was always acute. The dermatitis appeared first on the hands, especially on the fingers and dorsum of the hand, and then often spread to the forearms and face. Severe itching occurred and there was a marked tendency to oedema with swelling of the neck and face and around the eyes. Some patients had systemic symptoms, which included a headache for a day or two, fever, nausea and a peculiar weakness and apathy. It was noted that the picture somewhat resembled the effects produced by poison ivy (Rhus). The dermatitis healed slowly within 3–4 weeks; some cases required systemic treatment with corticosteroids

in hospital. According to Magnusson & Gilje (1973), the total number of cases was estimated to be 500–1000 out of an exposed population of 200,000–500,000.

Sensitization testing

Magnusson & Gilje (1973) performed patch tests on 24 patients who had developed dermatitis after the use of the detergent in question containing LES and on a factory worker who had allergic contact dermatitis and who had been heavily exposed to LES. They also investigated the allergenicity of LES in guinea-pigs. The results of these investigations indicated that the cause of the dermatitis was a particular batch of LES (designated LES 13–2035) which had been manufactured in Sweden. In one of the patients, the relatively small amount of allergen applied in the patch testing was sufficient to produce the systemic symptoms mentioned.

LES 13–2035 and 18 other samples of LES, including one from the same factory that produced LES 13–2035, were subjected to further extensive testing by Walker *et al.* (1973), who used two types of guinea-pig sensitization test. Both a modification of the method of Magnusson & Kligman (1969) and the method of Buehler (1965) showed that the sensitizing ability of LES 13–2035 was not shared by other batches of AES obtained from Sweden, Britain or the USA. Results obtained by the Buehler technique, which involves topical application under closed patches, appeared particularly relevant to the human situation after skin exposure. The sensitizing agent was not LES itself but could be separated from LES 13–2035 by prolonged extraction with petroleum ether (Gloxhuber *et al.* 1974; Magnusson & Gilje, 1973; Walker *et al.* 1973). It appeared to be relatively non-polar and stable, because by the time these experiments were performed LES 13–2035 was several years old (Gloxhuber *et al.* 1974; Walker *et al.* 1973).

Identification of the sensitizers

Connor *et al.* (1975) investigated the nature of the sensitizing agents present in a hexane extract of LES 13–2035 by thin-layer chromatography (TLC), gas-chromatography–mass spectrometry (GC–MS) and nuclear magnetic resonance spectroscopy (NMR). The allergenic activity of the fractions was monitored during separation by bioassay in guinea-pigs sensitized to a hexane extract of LES 13–2035. One of the skin-sensitizing components was identified as 1-dodecene-1,3-sultone ($C_{12}^{=}$) after comparison with authentic material, and 1-tetradecene-1,3-sultone ($C_{14}^{=}$) was considered to be another. Two chlorosultones, 2-chloro-1,3-dodecanesultone ($C_{12}Cl$) and 2-chloro-1,3-tetradecane sultone ($C_{14}Cl$) were also tentatively identified as sensitizing agents (Conner *et al.* 1975). The formulae of these and related compounds are shown in Fig. 1. The concentration of $C_{12}^{=}$ in samples of LES 13–2035 ranged from 1·6 to 4·8 ppm, the variation being attributed to the different storage conditions. Although a precise estimate is not possible, the activity of LES 13–2035 probably decreased by 66–90% during the 3-year period 1972–74 (Connor, Kowollik & Thomas, 1976). It can be calculated, therefore, that LES 13–2035 may originally have contained sultone contaminants ($C_{12}^{=}$ and $C_{14}^{=}$) to a level approaching 100 ppm.

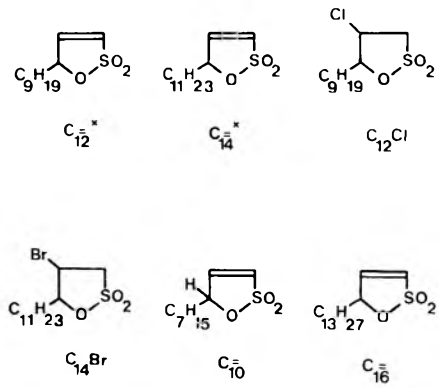

Fig. 1. Structure of unsaturated sultones and chlorosultones investigated by Ritz *et al.* (1975) for sensitizing ability in the guinea-pig. The two compounds marked* were positively identified as present in LES 13–2035; $C_{12}Cl$ and $C_{14}Cl$ were tentatively identified in LES 13–2035 by Connor *et al.* (1975).

The sensitizers $C_{12}^{=}$, $C_{14}^{=}$, $C_{12}Cl$ and related compounds consisting of 2-bromo-1,3-tetradecanesultone ($C_{14}Br$), 1-decene-1,3-sultone ($C_{10}^{=}$) and 1-hexadecene-1,3-sultone ($C_{16}^{=}$) (see Fig. 1) were tested in guinea-pigs for sensitizing potential (Ritz, Connor & Sauter, 1975) using single challenge and re-challenge procedures. All the sultones were found to be potent sensitizers and extensive cross-reactivity occurred, most compounds being more potent than 1-chloro-2,4-dinitrobenzene (DNCB), a well-known sensitizer in guinea-pigs and man. With induction doses of 7·1 nmol, C_{12} and C_{14} compounds appeared to be more potent than C_{10} and C_{16}, whereas at lower doses the order of potency was $C_{16} > C_{14} > C_{12} > C_{10}$.

It was suggested that the above findings might not be relevant to the human situation because sensitization was induced by injection of the unsaturated sultones in Freunds Complete Adjuvant (FCA) rather than by epicutaneous application. However, several workers (Magnusson & Kligman, 1970; Maguire, 1973; Maguire & Chase, 1967) consider that FCA is unlikely to affect the ranking of sensitizing ability. Another factor is that the injection procedure used by Ritz *et al.* (1975) may have maximized the sensitizing potential of the compounds, since it would tend to eliminate variation due to differences in dermal absorption. Nevertheless, the previous work of Walker *et al.* (1973) showed that LES 13–2035 caused sensitization in guinea-pigs after topical application. The evidence therefore seems fairly convincing that unsaturated sultone and chlorosultone contaminants were the cause of the allergic dermatitis produced by LES 13–2035.

Ritz *et al.* (1975) observed that $C_{12}^{=}$ and $C_{12}Br$ had similar potency with respect to primary sensitization in guinea-pigs, whereas $C_{12}Cl$ was less potent. In rechallenge experiments, the animals sensitized to $C_{12}^{=}$ and $C_{12}Br$ reacted more strongly to $C_{12}Cl$ than those sensitized to $C_{12}Cl$ itself. This led to the interesting suggestion that the sensitizing ability of $C_{12}Br$ may be due to $C_{12}^{=}$ and that $C_{12}Br$ is possibly more readily dehydrohalogenated than $C_{12}Cl$ to $C_{12}^{=}$ in the skin. It was proposed that the unsaturated sultone ($C_{12}^{=}$)

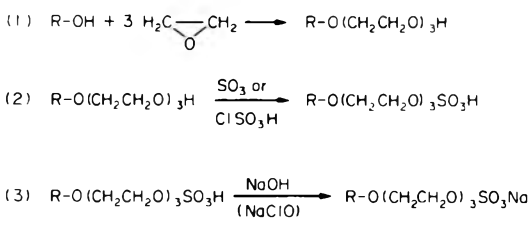

Fig. 2. Stages in the manufacture of lauryl ethoxysulphate.

or some further product then combines with skin proteins to initiate the response, the magnitude of the effect being determined by the speed of dehydrohalogenation and the amounts of the specific conjugate required for maximal elicitation of the response.

Origin of sultone contaminants

The most likely origin of the sultones was in the manufacture of LES 13–2035. The manufacture of LES is basically a three-step process (Fig. 2): (1) ethoxylation by ethylene oxide of fatty alcohol prepared either from vegetable oil ('natural' fatty alcohol) or from petroleum hydrocarbons ('synthetic' fatty alcohol); (2) sulphation of the resulting ethoxylated alcohol by sulphur trioxide or chlorosulphonic acid; (3) neutralization of the product with alkali (Magnusson & Gilje, 1973; Walker *et al.* 1973). Both ethoxylation and sulphation are highly exothermic reactions, and inadequate dissipation of the heat of these reactions may discolour the product, which then has to be bleached with either hypochlorite or peroxide. It should be emphasized that under properly controlled conditions, discoloration does not occur and therefore bleaching is unnecessary. The LES product usually contains a small amount of unsulphated matter, owing to incomplete sulphation, and this may be extracted with petroleum ether.

According to Connor *et al.* (1975), under the unusual conditions encountered during the processing of LES 13–2035, small quantities of dodecene and tetradecene present in the material to be sulphated during stage (2) of the manufacturing process could have become sulphonated to give 2-dodecene-1-sulphonic acid and 2-tetradecene-1-sulphonic acid. These compounds could have been converted to the chlorosultones by hypochlorous acid generated in the bleaching step that may be used in the manufacturing process, if the pH of the bleach reaction dropped below 9·5 (Fig. 3), and could have persisted in the paste or final product if the conditions were not conducive to hydrolysis of the chloro- and/or unsaturated sultones. Partial dehydrochlorination of the chlorosultones would have yielded the unsaturated sultones shown in Fig. 1, which must presumably have persisted through the stages of neutralization, formulation and storage.

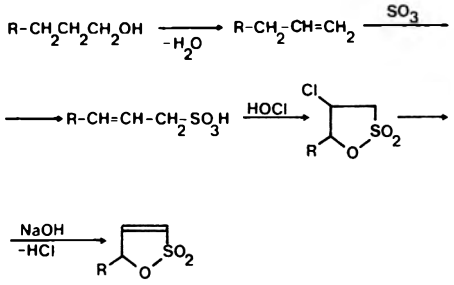

Fig. 3. Proposed mechanism for the formation of sensitizing agents in the manufacture of lauryl ethoxysulphate.

Connor *et al.* (1975) demonstrated that the treatment of sodium 2-dodecene-1-sulphonate with hypochlorite at pH 7 produced a solution that was able to evoke strong skin reactions in guinea-pigs already sensitized to the hexane extract of LES 13–2035. When a hexane extract of this solution was subjected to TLC, and the sultone region was removed and treated with collidine under dehydrohalogenation conditions, the product was identified as 1-dodecene-1,3-sultone (C_{12}) by NMR. This provides good evidence of the manner in which both types of sultone contaminants could have arisen during the manufacture of LES 13–2035.

Quantitative analysis of unsaturated sultones and chlorosultones

Sultones may be determined quantitatively by conventional titration or colorimetry after hydrolysis to the corresponding sulphonic acid (Martinsson & Nilsson, 1974) but this procedure suffers from a lack of sensitivity and specificity. An isotope-dilution method has recently been developed for estimating 1-alkene-1,3-sultone (Connor *et al.* 1976) in which a known weight of 1-[3-^{13}C]dodecene-1,3-sultone is added as an internal standard to the sample of LES being investigated. The mixture is extracted with petroleum ether, and the extract is dehydrohalogenated to convert any chlorosultones present to unsaturated sultone. TLC is used to concentrate the sultones, which are then measured by GC–MS. This method, used to determine sultone levels in samples of LES 13–2035, has a sensitivity of 0·01 ppm and is specific for 1-dodecene-1,3-sultone (C_{12}) and 2-chlorodecane-1,3-sultone ($C_{12}Cl$). However, the general applicability of the method is limited by the non-availability of other ^{13}C-labelled sultones and because it does not permit recovery of the sample for biological testing.

A simplified method involving ion-exchange chromatography, TLC and high-pressure liquid chromatography has been developed (MacMillan & Wright, 1977). This allows qualitative and quantitative determination of sultones at a level of 0·2 ppm and the required fractions can be collected for further chemical analysis or biological testing. It is less sensitive than the GC–MS method but is likely to be very useful in quality control work.

General discussion

The extensive biological and chemical investigation of LES 13–2035 has identified several unsaturated sultones and chlorosultones as potent skin-sensitizing agents. The work provides good evidence that the Scandinavian outbreak of allergic contact dermatitis in patients who had been exposed to detergents containing LES was due to low concentrations of these sultone contaminants produced in one particular batch of LES under abnormal manufacturing conditions.

Although the problem of this particular outbreak

of allergic contact dermatitis appears largely to have been solved, it would be interesting to know whether the sultone contaminants or their metabolites were the actual sensitizing agents, particularly since Ritz *et al.* (1975) have implicated the possible dehydrohalogenation of $C_{12}Br$ and $C_{12}Cl$ to $C_{12}^{=}$ by the skin in the sensitizing process. Unfortunately, there is little published information on the general pharmacological and toxicological properties of long-chain sultones. Further investigation of unsaturated sultones and chlorosultones may throw light on why systemic symptoms were sometimes observed in conjunction with the contact dermatitis, since the lipophilicity of these compounds suggests that they may readily penetrate the blood–brain barrier. Recent reports (Doak, Simpson, Hunt & Stevenson, 1976a,b) suggest that propanesultone is a skin carcinogen in mice. However, there is no evidence that long-chain sultones have this property.

The work described represents a model study of the type of problem in which occasional toxic contaminants give rise to untoward toxic reactions to commercial products. The series of events leading to the formation of unsaturated sultones and chlorosultones in LES 13–2035 is so unusual that no similar occurrence of such untoward happenings has ever been recorded, despite extensive monitoring of AES paste and products containing it. Thus it is possible to prevent a further occurrence of the problem in question now that the origin of the sultone contaminants is reasonably well established and appropriate methodology is available for adequate quality control. The particular unsaturated sultones and chlorosultones present in LES can be added to the list of highly toxic impurities cited by Golberg (1975) as having been found in major commercial products, including *p*-dioxin in 2,4,5-trichlorophenoxyacetic acid, cyclohexylamine and dicyclohexylamine in cyclamates, *trans*- and *cis*-dichlorohexafluorobutene in halothane, and *o*- and *p*-toluenesulphonamides in saccharin. It reinforces Golberg's point that much toxicological effort may be nullified by failure to pay attention to supposedly unimportant trace impurities in commercial products.

Acknowledgements—We should like to thank The Procter & Gamble Company and Environmental Resources Limited for help in the preparation of this review. We are also very grateful to Dr. J. Törnquist, Berol Kemi AB, Stenungsund, Sweden, for the information used in Figs 2 and 3.

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Fd Cosmet. Toxicol. Vol. 16, pp. 63–64. Pergamon Press 1978. Printed in Great Britain

REVIEWS OF RECENT PUBLICATIONS

Wholesomeness of Irradiated Food. Report of a Joint FAO/IAEA/WHO Expert Committee. *Tech. Rep. Ser. Wld Hlth Org.* 1977, **604**, pp. 44. Sw.fr. 6.00 (available in UK through HMSO).

In its 1970 report, the Joint FAO/IAEA/WHO Expert Committee recommended temporary acceptance of wheat and wheat products irradiated up to 75 krad (0·75 kGy) and of white potatoes irradiated up to 15 krad, but not of onions irradiated up to 15 krad. The latter decision was due to the inadequacy of the available toxicological data.

The progress that has since been made in the safety evaluation of irradiated food intended for human consumption can be attributed mainly to the direction of the International Project in the Field of Food Irradiation and is reflected in the 1977 Joint Expert Committee's recommendations for unconditional acceptance of wheat and ground wheat products irradiated up to 100 krad and of potatoes irradiated up to 15 krad and for provisional acceptance of onions irradiated up to 15 krad. Moreover, newly-evaluated irradiated foods received favourable scrutiny, unconditional acceptance being granted for chicken (up to 700 krad), papaya (up to 100 krad) and strawberries (up to 300 krad) and provisional acceptance for cod and redfish (up to 220 krad) and rice (up to 100 krad). No decision was reached on irradiated mushrooms, again because of inadequate data.

For the wholesomeness of irradiated food to be established, standards of microbiological, nutritional and toxicological acceptability must be attained. The toxicologist's brief is to demonstrate the absence or otherwise of any significant amounts of toxic products formed in the food as a result of the irradiation process.

This 1977 report, like its predecessor, recognizes that the principles accepted for testing food additives or chemicals in general are inapplicable to irradiated food. In the latter context, a distorted picture will emerge if there is over-exaggeration of either the radiation dosage or the level of the irradiated food incorporated into the animal's diet.

The advent, therefore, of a new approach to this problem could not be more timely, and the report draws attention to the important contribution of radiation chemistry studies undertaken on major food components. It notes that radiolytic products formed in irradiated food are present only in the ppm range following irradiation with doses up to 6 Mrad and asserts that the concentrations of radiolytic products are even less significant at lower radiation doses. It is also apparent that the radiolytic products identified so far either in whole foods or in food components present no toxic hazard at the concentrations found.

For foods irradiated up to 1 Mrad, the report accepts that toxicity data may be extrapolated from one member of a food class to another in the same class. This decision should minimize requirements for toxicity testing. The report strikes an optimistic note in suggesting that under certain circumstances, and when further experience has been gained from continuing radiation-chemistry studies and toxicological experimentation, a purely chemical approach to the wholesomeness evaluation of irradiated food may be possible. The Committee makes various recommendations for future work, not only in respect of further studies on specific irradiated foods but also in more general areas of study. The latter include the further identification of radiolytic products and, where relevant, a determination of their toxicity, comparison of the toxicity of volatile components of irradiated and non-irradiated foods, and studies of the nature and toxicity of radiolytic products of lipids with special reference to *cis-trans* isomerization.

The Joint FAO/IAEA/WHO Expert Committee has put its weight behind the development of irradiated food, but it remains to be seen whether this new impetus will be translated into a greater degree of acceptability by the regulatory authorities throughout the world over the next few years.

Seventeenth Progress Report of the Standing Technical Committee on Synthetic Detergents. Department of the Environment. HMSO, London, 1977. pp. v + 25. £0.90.

This latest report notes that UK use of anionic and cationic surfactants, particularly the latter in applications such as clothes-washing and post-wash fabric softening, has continued to increase in recent years. Despite this, removal of anionic surfactants at treatment plants continues to be in the range 90–95%, and there has been virtually no change overall in the average concentrations in the final effluent. Removal of nonionic surfactants has now reached a similar level of efficiency. Surfactant levels in rivers used for public supply are often at or below the limits of detection, and are generally too low to produce significant foaming. The long-standing foaming problems in West Yorkshire have largely disappeared, but this is thought to be due, at least in part, to reduced activity in the local textile industry.

Boron levels in an Essex river, monitored since 1970, have remained at 1 mg/litre. Because this concentration could affect certain sensitive crops, particularly those grown by new techniques which use a very small quantity of soil between plastic sheets, it is recommended that water authorities should monitor the boron content of river water intended for irrigation. There has been no evidence of any long-term changes in phosphate concentrations, and replacement of polyphosphate by nitrilotriacetate in detergent powders is regarded as unnecessary. Agreement

was reached on the last point with the German Detergent Committee at two meetings, held in April 1975 and May 1976. It was also agreed that there was no need for a statutory fish test for surfactants, as envisaged by the EEC, and that, although control of use of cationic surfactants was unnecessary at present, their increasing use meant that the situation should be kept under surveillance.

The main activity of the Committee during the year was the negotiation with the Soap and Detergent Industry Association of a voluntary notification scheme for new substances or for increased use of existing components in domestic detergents and related products if this is considered likely to have a significant environmental effect. Guidelines for test methods for assessing effects on sewage treatment and environmental impact have been proposed, and details will be specified later by the Technical Subcommittee. Only domestic detergents have been considered so far, but it is hoped that the scheme may be extended to industrial cleaning compositions.

Fd Cosmet. Toxicol. Vol. 16, pp. 65–68. Pergamon Press 1978. Printed in Great Britain

BOOK REVIEWS

Environmental *N*-Nitroso Compounds—Analysis and Formation. Proceedings of a Working Conference held at the Polytechnical Institute, Tallinn, Estonian SSR, 1–3 October 1975. Edited by E. A. Walker, P. Bogovski and L. Griciute. IARC Scientific Publications no. 14. International Agency for Research on Cancer, Lyon, 1976. pp. xviii + 512. Sw.fr. 110.00.

This volume contains papers presented at a working conference organized by the IARC at Tallinn, Estonia, in October 1975. Since 1965, the IARC has organized these biennial conferences on the analysis and formation of *N*-nitroso compounds, and the growing importance attached to this problem in the context of environmental contamination and human exposure is reflected in the current listing of nearly 80 participants and 52 communications.

The papers are divided into four sections, the first of which deals with the analysis of *N*-nitroso compounds. An introduction to this section, by P. Bogovski, presents interim data on an attempt to correlate environmental levels of certain volatile nitrosamines in parts of Iran with a high incidence of oesophageal cancer. The evidence obtained so far is equivocal and the role of *N*-nitroso compounds in oesophageal cancer is far from clear, but further investigations still in progress may reveal the identity of the causative agent. The analytical papers deal with the mass spectrometry of volatile nitrosamines and suitable derivatives of nitrosoamino acids, and describe the isolation and determination of non-volatile nitrosamines by the new technique of high-performance liquid chromatography with novel detection systems, including the thermal energy analyser. The analysis of volatile nitrosamines in foods and the atmosphere is described in six contributions.

The second section, concerned with the formation of *N*-nitroso compounds, is subdivided into groups dealing with specific aspects of the problem. The first group of six papers discusses the catalytic effects on nitrosamine formation exerted by a number of diverse factors ranging from formaldehyde and other carbonyl compounds to surfactants and metal complexes. The formation of nitrosamines from naturally occurring amines and from drugs occupies a further 13 contributions. The third section deals with the occurrence of *N*-nitroso compounds in foods and tobacco smoke, and the final section, on "miscellaneous studies", reports various biological and biochemical studies, investigations into the degradation of nitrosamines, and some studies on the carcinogenicity testing of nitroso derivatives and on the effect of other compunds, such as ascorbate and vitamin A, on their carcinogenic activity. Finally an excellent report on the state of the analytical methodology and of work on the formation and occurrence of *N*-nitroso compounds is included in this book, together with recommendations for future work prepared by three sub-committees set up at the conference.

The reviewer can only restate the self-evident fact that this collection of papers will be invaluable to chemists and biologists involved in studies relating to the problem of nitrosamines in the human environment.

Radiation Chemistry of Major Food Components. Its Relevance to the Assessment of the Wholesomeness of Irradiated Foods. Edited by P. S. Elias and A. J. Cohen. Elsevier/North-Holland Biomedical Press, Amsterdam, 1977. pp. xii + 220. $25.75; Dfl. 63.00.

The needed increase in the world's food supply could be provided by raising the levels of both food production and food preservation. The potential of irradiation as a means of food preservation has not so far been exploited, partly because of the extreme caution of the regulatory authorities in accepting existing safety-evaluation data and partly because public concern over the hazards of radiation generally may be reflected in a distrust of irradiated foods.

It is now generally accepted that the principles followed in food-additive testing cannot be applied to the testing of irradiated food, since in the latter case, attempts to unmask any toxic potential by exaggerating test conditions (by inflating the radiation dosage or the level of irradiated food in the animal's diet) is likely to yield misleading results. Clearly, an understanding of the chemical changes induced in food by irradiation would assist the assessment of the safety of irradiated foods or animal feeds. The International Project in the Field of Food Irradiation therefore commissioned several experts to provide critical reviews of the radiation stability and chemistry of major food components and these reviews have been brought together in the book named above.

In a general introduction, the Project Director, Dr. P. S. Elias, explains the need for a new approach to replace the conventional, laborious, costly and not altogether satisfactory animal feeding studies on which, until now, the assessment of the wholesomeness of irradiated foods for human consumption has relied. In the individual chapters on the radiation chemistry of lipids, proteins, carbohydrates and vitamins, particular attention is paid to the radiation-induced changes in the content of the food component in question and to the nature and concentration of the radiolytic products formed. As far as possible, each author has compared the response of the particular food component to radiation in the dry

solid state, in aqueous solution, in model systems or complex mixtures and in the whole food product.

It is apparent that the major food components respond to irradiation in uniform ways and that the radiolytic products detected so far in irradiated foods, including many found also in non-irradiated foods, are not present in toxicologically-significant concentrations. Further chemical analyses of radiolytic products, coupled in some instances with toxicity tests on the compounds identified, will facilitate greatly the evaluation of irradiated food.

This book will be valuable to chemists, to biochemists, to food scientists and technologists and to toxicologists working in the field of food irradiation, as well as to scientists engaged in government departments and international agencies concerned with the control of food preservation. Above all, it should stimulate interest in the application of radiation-chemistry studies to the evaluation of the wholesomeness of irradiated foods and promote an appreciation of the potential value of this approach.

Pesticide Manual. Basic Information on the Chemicals used as Active Components of Pesticides. 5th Ed. Edited by H. Martin and C. R. Worthing. Issued by the British Crop Protection Council, 1977. pp. vii + 593. £15.00.

The rate of production of novel pesticidal formulations and information about them is such that useful coverage of this vast subject can only be provided by frequently revised editions. This new fifth edition of the "Pesticide Manual" has made its appearance little more than 2 years after publication of the fourth.

In an attempt to keep up with pesticide developments, the manual includes compounds that had reached only the stage of submission to outside authorities for field studies. In contrast, those compounds that were formerly marketed or widely reported but have now fallen out of favour are listed in the appendix.

The editors have collated details on nomenclature, history, manufacture, physical, chemical and biological properties, formulations and analysis. Although appropriate references are included in almost all other areas, references for the toxicity data are sparse, precluding any attempt to evaluate the original source or check the accuracy of the data. For example, the manual provides an oral LD_{50} for aminotriazole of 1100–2500 mg/kg in rats. In contrast, the FAO/WHO publication "Pesticide Residues in Food" (1974) indicates an LD_{50} for the rat of 25,000 mg/kg by the same route. Without the original source one cannot be certain that the manual's figures are reliable.

Despite these limitations the manual can be very useful. Not only is the Chemical Abstracts Registry Number provided for each compound, but the index includes common, chemical and trade names as well as code numbers. In this respect, the publication is a 'must' for consultation before any literature search on a pesticidal compound is undertaken.

For the majority of the pesticides, the structures and molecular formulae are shown, together with their Wiswesser Line Formula Notations (WLN). These notations, which are becoming increasingly popular in chemical documentation, are strings of symbols constructed by strict rules to provide a compact, unique and unambiguous description of the molecular structure in linear form. Although the principles of encoding structures into WLN require some weeks of training, the manual's two-page 'do-it-yourself WLN' provides an interesting insight into the scheme.

The manual describes its approach as 'basic'. It is indeed a basic necessity when information on pesticides is required.

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

It has become almost axiomatic in science that increasing advancement causes established disciplines to become fragmented into a number of separate specializations, which in turn expand only to be further subdivided. In the case of pharmacology, this process has led to a plethora of new names, such as systematic pharmacology, autopharmacology, pharmacokinetics, pharmacodynamics and pharmaco-therapeutics. Although some of these divisions are justified, others must be suspected of serving only to placate man's ego. This volume on perinatal pharmacology and therapeutics, however, must dispel any criticism that may arise from the appearance of yet another subdivision and helps to establish its subject as a viable discipline in its own right.

The book deals with the effects and interactions of drugs and other chemicals in both the foetus and neonate. The first three chapters deal with prenatal pharmacology and the fifth with postnatal pharmacology, whilst the fourth spans both periods of development. Following the pattern of many successful and authoritative books, each chapter has been written by people pre-eminent in that particular field. The first chapter considers the transfer of pharmacologically active molecules across the placenta, whilst the second discusses the capacity of human and other placental tissues to metabolize both endogenous and exogenous compounds by the classic biotransformation reactions of oxidation, reduction, hydrolysis and conjugation. The distribution and metabolism of drugs in the foetus forms the main topic of chapter 3, thus completing the prenatal section of the book. This is followed by a long and detailed consideration of the ways in which drugs, administered during the pre-, peri- or postnatal periods, can affect neuronal development and subsequent patterns of behaviour in young animals and in the human child. The fifth and final chapter concentrates on the clinical implications of neonatal pharmacology, including the interplay of factors involved in the manifestation of teratogenic effects, foetal pharmacology and the treatment of foetal disease *in utero* and the transfer of drugs and other chemicals from the lactating mother to the neonate in the milk.

Although the prime interest of this book lies with man, particularly in the last chapter, a great deal of information derived from animal studies is mentioned

and discussed. The provision, where appropriate, of anatomical, histological and physiological background to the various systems considered allows the chapters to be read without reference to other texts. Altogether, the book should prove an asset to anyone concerned with the effects of chemicals on the developing embryo and neonate, and it is not, by current standards, unreasonably priced.

Hepatocellular Carcinoma. Edited by K. Okuda and R. L. Peters. John Wiley & Sons, New York, 1976. pp. x + 499. £27.60.

Tumours of the liver may arise either in the absence of any previous hepatic disease or in association with chronic hepatic disorders. Both types present a challenge to the diagnostician and pathologist.

Primary hepatic tumours in man are relatively rare in temperate climates but occur quite frequently in tropical countries. This is particularly the case with tumours that arise from liver cells. Tumours arising from the bile ducts are universally infrequent and cancers of other constituents of the liver, such as the blood vessels or connective tissue, are extremely rare in all climes.

Since liver-cell tumours associated with liver disease are fairly common in man, the pathological lesions associated with liver cancer are carefully considered in this book. Cirrhosis, particularly the macronodular type with wide bands of fibrosis, is more often associated with carcinoma than is any other hepatic lesion, inflammatory or reactive. Other types of cirrhosis are less liable to be associated with cancer, the least liable being the fine micronodular type of cirrhosis. This and other aspects of the physiopathology of liver cancer are well covered and particular attention has been paid to the clinical manifestations of the disease and to the means available for its diagnosis and for monitoring its progress.

Each of the 21 chapters has a separate author and the book is divided into four parts, dealing in turn with pathogenesis, aetiology, pathology and clinical features. Apart from the first chapter, the whole book is concerned with the disease in man. The first chapter is a good and concise account of the mode of induction and progression of experimentally induced carcinoma. Unfortunately it keeps too much to the 'central dogma' of chemical carcinogenesis, implying that all chemically induced cancers are the result of damage to DNA. The validity of this mechanism in many chemically induced tumours is unquestionable but other mechanisms involving chronic repeated injury cannot be disregarded, particularly in the light of the strong evidence that in man cirrhosis predisposes to carcinoma. First chapters are usually reserved for topics considered to be particularly interesting, and the prominence given in this case to the pathogenesis of experimentally induced tumours leads the reader to expect more on the same topic. Unfortunately this expectation is unfulfilled and the vast amount of material on the mechanisms of induction of hepatocellular carcinoma in experimental animals remains virtually unconsidered. Furthermore, in the various chapters on the disease in man, little effort is made to include experimental material which many would regard as highly relevant to the human situation.

These omissions leave important gaps in what would otherwise have been a good and up-to-date review of liver cancer. Perhaps the title should have stressed that the book is heavily biased towards hepatocellular carcinoma in man.

Glutathione: Metabolism and Function. Kroc Foundation Series, Vol. 6 Edited by I. M. Arias and W. B. Jakoby. Raven Press, New York, 1976. pp. xiii + 382. $28.50.

This volume contains 25 papers which were presented at a workshop sponsored by the Kroc Foundation and the Liver Research Center of Albert Einstein College of Medicine and held in June 1975. The aim of the workshop was to discuss and evaluate the recent advances made in elucidating the unique role of this tripeptide in such apparently diverse areas as transport mechanisms and the metabolism, disposition and detoxication of foreign compounds, including carcinogens. The importance of the subject attracted a number of eminent scientists, and dissertations of exemplary standard by the 46 contributors and the high quality of the ensuing discussions apparently made the meeting an outstanding success.

This is clearly reflected in the book, which is divided into four sections, the first of which consists of seven papers dealing with the general aspects of glutathione metabolism. These range from the chemical properties of glutathione and the enzymic processes mediated by it to a review of conjugation reactions and mercapturic acid excretion. The second section examines the regulatory mechanisms governing the oxidation/reduction states of glutathione, and the third deals with the structure, regulation and function of glutathione *S*-transferases and ligandin. The importance attached to this family of catalytic binding proteins, representing an extraordinarily adept system for detoxication, is reflected in the fact that this aspect of the subject occupies almost half of the book. The final section is concerned with the role of glutathione in chemically induced injury and carcinogenesis. In each of the sections, critical discussions of the papers constitute a valuable adjunct to the text.

The diverse and essential functions of glutathione in regulatory mechanisms of the body are being increasingly recognized and this book presents information of considerable importance on the subject.

Biochemical Toxicology of Environmental Agents. By A. De Bruin. Elsevier North-Holland Biomedical Press, Amsterdam, 1976. pp. x + 1544. Dfl. 320.00.

The growing awareness of toxic hazards in man's chemical environment has led to an increasing emphasis on the study of the toxicity of food additives, drugs, pesticides and industrial chemicals. Additionally, particular attention is being paid to the biochemical processes underlying the onset of overt toxic

effects and to the role of defence mechanisms in altering or averting chemically-induced injury to organisms. An inevitable consequence of this widespread research effort has been a literature explosion which is placing a considerable, and continuing, burden on scientists trying to keep up with the subject.

Dr. De Bruin has attempted to ameliorate this situation by collecting and collating relevant information, and the end-product is a massive tome of some 1500 pages. These are divided into 42 chapters on subjects ranging from general features governing the biodynamics of xenobiotic compounds and the metabolism of environmental chemicals, pesticides, carcinogens and nutrients to immunological and acquired-tolerance mechanisms. The amount of information provided is enormous and over 13,000 references are cited.

This book should provide an invaluable work of reference despite the inevitable obsolescence of some of the material it contains. It should be noted, however, that this is not a critical treatise but rather a compendium of information. Nevertheless, the author deserves to be congratulated on accomplishing an extremely arduous task.

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Information Section

ARTICLES OF GENERAL INTEREST

NITROSO COMPOUNDS AND CARCINOGENESIS

Introduction

Many nitrosamines are potent carcinogens, and their possible synthesis by the reaction of amines naturally present in food with nitrite added to foods in curing processes has prompted a great deal of research into the conditions under which nitrosation could possibly take place in the human gut (*Cited in F.C.T.* 1971, **9**, 739; Lane & Bailey, *Fd Cosmet. Toxicol.* 1973, **11**, 851; *Cited in F.C.T.* 1974, **12**, 156 & 1977, **15**, 655).

Mirvish (*Toxic. appl. Pharmac.* 1975, **31**, 325) has reviewed the possibilities for nitrosation not only of amines but of other environmental chemicals that are reactive with nitrite by virtue of an N–C linkage. Nitrosamines are formed from dialkyl, alkaryl, diaryl or cyclic secondary amines; nitrosamides are formed from *N*-alkylureas, *N*-alkylcarbamates and simple *N*-alkylamides. In addition, nitroso derivatives may be derived from cyanamides, guanidines, amidines, hydroxylamines, hydrazines, hydrazones and hydrazides. The scope for *in vivo* nitrosation is therefore wide. The conditions under which nitrite can react with such compounds are also varied. Most carcinogenic nitroso compounds result from acid-catalysed reactions, but nitrosamines may be formed at pH values close to neutral under the influence of heat or of bacterial activity, which reduces nitrate to nitrite in the presence of the amines. This synthesis is known to occur in the large intestine, the infected urinary bladder or the infected vagina as well as in the stomach. Thiocyanate, bromide and chloride ions catalyse the nitrosation of amines, so it is possible that the additional thiocyanate found in the saliva of smokers may affect the degree of intragastric nitrosation.

The formation of nitroso compounds can be minimized. Ascorbic acid reacts rapidly with nitrite in acid substrates to yield nitric oxide and dehydroascorbic acid, and might therefore be used as a competitive inhibitor against nitrosation of amines and amides in the stomach. This inhibition has been demonstrated in guinea-pigs (Akin & Wasserman, *Fd Cosmet. Toxicol.* 1975, **13**, 239). Other practicable blocking agents include erythorbic acid, gallic acid, tannic acid, sulphite and cysteine (Mirvish, *loc. cit.*).

Weisburger & Raineri (*Toxic. appl. Pharmac.* 1975, **31**, 369) have pointed out that there is at present very little evidence that exposure to nitrosamines produces cancer in man as it does in animals. However the biochemical evidence suggests that man is susceptible and it seems likely, therefore, that stomach and perhaps oesophageal and liver cancers can result from the endogenous nitrosation of absorbed amines and amides by nitrite derived from microbiological reduction of nitrate in foods kept at room temperature. If this is so, low temperature storage or the addition of inhibitors such as ascorbic acid may serve to reduce the incidence of such cancers in the future.

Role of drugs

Drugs belonging to the chemical classes discussed above are just as likely to undergo nitrosation when exposed to nitrite under the right conditions as are normal food constituents. Consequently, the incorporation of appropriate amounts of ascorbic acid, or some other suitable blocking agent, has been recommended for formulations containing potentially nitrosatable drugs (Mirvish, *loc. cit.*), and the likelihood of nitrosamine formation may also be reduced by administering such drugs parenterally.

LaBar & Sander (*Z. Krebsforsch.* 1975, **84**, 299) reported that 30–50% of a single dose of 100 mg aminopyrine given in 100 ml water to fasting volunteers remained in the gastric juice after 1 minute, and that its concentration fell to about one quarter after 10 minutes. When sodium nitrite in broth made from 100 g cured ham was given under similar conditions, its concentration in the gastric juice was halved during the first 2 minutes and fell to one quarter after 10 minutes. When boiled ham itself was consumed instead of the derived broth, retention of nitrite in the stomach was prolonged. *In vitro* experiments with broth from cured ham, pork and spinach incubated with aminopyrine showed that significant quantities of dimethylnitrosamine (DMN) were formed at nitrite levels as low as 4 mg, both from acid spinach broth and alkaline ham broth. There is thus a possibility that taking aminopyrine in conjunction with a meal containing nitrite might result in nitrosation and carcinogenesis. LaBar & Sander (*loc. cit.*) suggested that aminopyrine might well be withdrawn from the open market in countries where it was still included in over-the-counter analgesic formulations, and recommended that when used under medical supervision it should be taken with plenty of fluid, not in conjunction with meals, and combined with an adequate inhibitory dose of ascorbic acid. The same investigators (*idem, Arzneimittel-Forsch.* 1976, **26**, 1340) found that rats given 2 mg DMN/kg by mouth excreted 0·003% of the dose unchanged in the urine, but that when the dose was increased to 15 mg/kg excretion in the urine was 0·08%. However, they found a wide variation between individual animals. From the mean quantity of DMN excreted after an oral dose of 10 mg aminopyrine/rat followed immediately by 10 mg sodium nitrite/rat, they estimated that the nitrosation

rate of aminopyrine was about 30%. The method proved too insensitive to be applied to human studies, however.

Lijinsky & Greenblatt (*Nature New Biology* 1972, **236**, 177) administered by gavage 35 mg aminopyrine/rat, 40 mg sodium nitrite/rat or both compounds together, and examined the animals 68 hours later. Livers from those given both compounds showed severe centrilobular necrosis, typical of the observed effect of DMN (at doses of 3–7 mg/rat), while in neither of the other groups did such changes occur. Treatment of rats with the same doses of these compounds on three consecutive days killed all the animals exposed to the combination and their livers showed severe necrosis. When graded doses of nitrite were used, a significant yield of DMN apparently resulted with doses as low as 10 mg. Another study by Lijinsky *et al.* (*Nature, Lond.* 1973, **244**, 176) entailed giving groups of rats drinking-water containing either heptamethyleneimine hydrochloride and sodium nitrite, each in concentrations of 0·2%, or aminopyrine and sodium nitrite, each at 0·1% or at 0·025% on 5 days/week, for 28, 30 and 50 weeks, respectively. The animals were observed for their lifetime. In the high-dose aminopyrine trial, all animals died within 1 year and 29/30 showed haemagioendothelial liver sarcomas with frequent lung metastases. In the low-dose aminopyrine trial, 14/30 died within 1 year, 12 with liver tumours, one with a parotid tumour and one with a large mammary tumour; in six of these rats, lung metastases appeared. In the heptamethyleneimine trial, 23/30 died within a year and 20 developed primary tumours, mostly oesophageal papillomas and squamous-cell carcinomas of the lung.

The doses used in these studies did not greatly exceed those that could be encountered by man, so the interaction of these and similar compounds cannot be ignored as a possible factor in human cancer. On the other hand, in 30 rats given comparable doses (0·1% in the drinking-water) of oxytetracycline and sodium nitrite for 60 weeks (Taylor & Lijinsky, *Int. J. Cancer* 1975, **16**, 211), only four liver tumours (one cholangioma and three of hepatocellular origin) were found. Even in the absence of any tumours in rats given nitrite or oxytetracycline alone, the significance of this finding must be considered doubtful.

The appetite suppressant phenmetrazine would be expected to undergo nitrosative dealkylation to 3-methyl-2-phenyl-*N*-nitrosomorpholine (nitrosophenmetrazine; NPM). Greenblatt *et al.* (*Nature New Biology* 1972, **236**, 25) introduced 1 mmol (177 mg) phenmetrazine suspended in water containing sodium nitrite (in a molar proportion of 1 :4) into the ligated stomachs of two rabbits and after 5 hours found NPM yields equivalent to 13 and 18% of the theoretical (26·5 and 37·5 mg respectively) in the stomach contents. In a parallel experiment with the related phendimetrazine, no NPM was detected. Serial sampling of the stomach contents after phenmetrazine/nitrite administration showed that nitrosation was quite rapid at pH 3·5, with a decline in both phenmetrazine and nitrite concentrations after the first hour. With an increase in nitrite from 1 to 4 mmol/mmol drug, the yield of nitrosamine rose proportionately. Intragastric injection of phenmetrazine and sodium nitrate into rats produced no more than a trace of nitroso compound within 5 hours, and elimination of nitrate from the stomach was much slower than that of nitrite. The status of NPM as a carcinogen is unknown, but these findings suggest that phenmetrazine may pose a hazard if taken together with food containing nitrite.

Alkylurea derivatives

The possible hazard of urea derivatives as nitrosation substrates has been studied by Mirvish *et al.* (*J. natn. Cancer Inst.* 1972, **48**, 1311). Mice given drinking-water containing 0·1% sodium nitrite in conjunction with feed containing 0·54% methylurea or 0·64% ethylurea for 6 months developed more than four lung adenomas per mouse and showed a small increase in malignant lymphomas. The production of multiple lung adenomas by the administration of nitrosoureas is well-documented. When the alkylureas were fed alone there was no increase in tumour incidence over control levels. The medical use of urea derivatives such as tolubutamide, tolazamide, sulphanilylurea, neohydrin-^{203}Hg and nitrofurazone, and the use of phenylureas such as monuron and diuron as herbicides, makes the possible nitrosation of these compounds by dietary nitrite a significant possibility.

Mirvish & Chu (*ibid* 1973, **50**, 745) gave fasting rats 21·5 mg methylurea or 25·5 mg ethylurea by stomach tube, together with 10 mg sodium nitrite. After 45–80 minutes the stomach contents showed a mean yield of 27% of the theoretical for methylnitrosourea (MNU) and 8·7% for ethylnitrosourea (ENU), calculated on the nitrite content. When enough sodium bicarbonate to neutralize the gastric acid was given before the methylurea and nitrite, no nitroso derivative was found in the stomach contents, indicating that an acid-catalysed reaction was involved. When incubated with gastric juice, MNU was stable for at least 1 hour at 37°C. Intubation of 5 mg MNU or ENU into the rat stomach led to recovery of only 27 and 29%, respectively, after 45–60 minutes, so the actual yields of these nitrosoureas from their precursors in the rat stomach were probably considerably higher than the demonstrated 27 and 8·7%. Rustia (*Cancer Res.* 1974, **34**, 3232) found that concurrent daily intragastric administration to hamsters of 100 mg ethylurea/kg and 50 mg sodium nitrite/kg for 40 doses produced multiple types of tumours. More than three different types of tumour per affected animal were seen in 85% of these hamsters, whereas only single tumours appeared in the other groups, affecting 11% of those given ethylurea alone and 16% of untreated controls. Vascular tumours of the spleen and liver, papillomas of the forestomach and vagina, ovarian tumours and tumours of nervous tissue originating in the peripheral nervous system were included among the results of the ethylurea/nitrite treatment, a more extensive range of tumours than that seen in hamsters exposed to these precursors transplacentally.

Rats given drinking-water containing 0·04 M-*n*-propyl-, isopropyl- or *n*-butylurea together with food containing 0·28% sodium nitrite developed malignant abdominal reticuloses within 6 months (Schneider *et al. Arch. Geschwulstforsch.* 1974, **44**, 126). The unifor-

mity of tumour type within these groups of animals indicated that the tumours resulted from endogenous nitrosation of the alkylureas.

Amino acids and heterocyclic compounds

Braunberg & Dailey (*Proc. Soc. exp. Biol. Med.* 1973, **142**, 993) have pointed out that L-proline and hydroxy-L-proline are the secondary amines to which human populations are most exposed through ingestion of food. Proline offers the greater hazard of the two amino acids, since it may be nitrosated to *N*-nitrosoproline (NP), which then undergoes decarboxylation to form nitrosopyrrolidine (NPR). In an experiment with young fasting rats intubated with test doses of ^{14}C-labelled L-proline and nitrite, the amount of NP in the stomach 15 minutes after treatment was found to be directly related to the doses of the reactants used (Braunberg & Dailey *loc. cit.*). Greenblatt & Lijinsky (*J. natn. Cancer Inst.* 1972, **48**, 1389) reported that mice given drinking-water containing 0·1% sodium nitrite and 0·5% proline, hydroxyproline or arginine for 38 weeks failed to show any increase in the incidence of lung adenomas. The addition of NP directly to the drinking-water at a level of 0·05 or 0·1% also failed to induce a significant number of lung tumours, but direct feeding of nitrosopiperidine in a total dose of 65 mg/mouse increased lung adenomas tenfold. NPR in a total dose of 15 mg/mouse also increased the incidence of lung adenomas, and at the same time proved severely hepatotoxic, with the production of nodular hyperplasia and paracentral necrosis.

Another study (Garcia & Lijinsky, *Z. Krebsforsch.* 1973, **79**, 141), involving administration of the nitrosamino acids, nitrosohydroxyproline, NP and nitrosopipecolic acid, to rats at levels of 0·015% in their drinking-water for 75 weeks, indicated that at such a concentration none was carcinogenic. Other rats given 0·05% sodium nitrite with 0·025% pyrrolidine, piperidine, piperazine, morpholine or heptamethyleneimine for 75 weeks also failed to develop tumours. Piperidine given at 0·1% with 0·2% sodium nitrite was not tumorigenic. It appears that strongly basic amines do not readily form nitrosamines in dilute solution in sufficient quantities to induce tumours (Garcia & Lijinsky, *loc. cit.*). A similar conclusion was drawn from a study in hamsters (Sen *et al. Fd Cosmet. Toxicol.* 1975, **13**, 423), the strong base in that case being diethylamine.

The biosynthesis of NPR has been demonstrated by Mysliwy *et al.* (*Br. J. Cancer* 1974, **30**, 279), who gave dogs with a gastric fistula 50 ml of 0·1% sodium nitrite and 0·02% pyrrolidine unbuffered at pH 11·2. The pH of the stomach contents fell over 30 minutes, while the concentration of nitrite fell to 10% of its initial value during the same period. From 1 minute after the introduction of the solution, NPR was detectable, and its concentration rose to 0·96 ppm after 2·5 minutes in one dog and to 0·12 ppm after 7 minutes in another. Thereafter it declined rapidly, probably as a result of absorption. Since the production of NPR was negligible when the reagents were maintained at 37°C and pH 3 *in vitro*, it appears that some additional factor is involved in promoting the synthesis of NPR in the dog's stomach.

The real significance of these demonstrations of tumour induction following administration of nitrosamine or nitrosamide precursors to rats and other experimental animals can still not be assessed adequately in terms of the likely course of events in man. However, these studies do suggest ways in which the formation of nitroso compounds in the gastro-intestinal tract may be discouraged and some of these are clearly relevant to the human situation. Until man's susceptibility or resistance to nitrosamine carcinogenesis can be more clearly established, it seems advisable to take any possible steps to reduce his exposure to this class of compound.

[P. Cooper—BIBRA]

IRRADIATED DIETS EXAMINED

We last took a broad look at the problems of irradiated foodstuffs in 1969 (*Cited in F.C.T.* 1969, **7**, 171). Since then, additional investigations of the effects of feeding irradiated products to animals for long periods have been undertaken, many with the support of the International Project in the Field of Food Irradiation, and limited approval for specific types of irradiated food has been granted by some authorities.

Animal feedstuffs

Chauhan *et al.* (*Fd Cosmet. Toxicol.* 1975, **1**, 433), mated untreated female Swiss mice over a 4-week period with males that had been fed for 8 week on diet irradiated with 2·5 Mrad and who found at mid-pregnancy no differences in implantation rates and dead implantations between these females and a similar group mated with males fed non-irradiated diet. The same investigators (*idem. Int. J. Radiat. Biol.* 1975, **28**, 215) mated 14- and 15-week-old male Wistar rats, of a third generation fed with ration irradiated at 0·2 or 2·5 Mrad, with females aged 9–11 weeks and again found no evidence of dominant lethal mutations, there being no significant differences between these two test groups and the controls in respect of pregnancy rates and pseudopregnancies, total implantations (live foetuses plus dead implantations), dead implantations and overall mean corpora-lutea counts.

A host-mediated assay in Swiss mice fed food irradiated at 0·75, 1·5 or 3·0 Mrad and injected ip with *Salmonella typhimurium* TA1530 was carried out by Johnston-Arthur *et al.* (*Stud. biophys.* 1975, **50**, 137). No difference from controls was seen in Salmonella implanted in animals fed 0·75 Mrad-irradiated ration, a slight difference was associated with the 1·5 Mrad irradiation and a significant increase in mutation frequency occurred with 3 Mrad. The increase in

mutation frequency seen with food given the highest irradiation dose was not accompanied by any obvious lethal effect on the test bacterium. Another host-mediated assay using the same bacterial strain and Swiss mice fed a diet irradiated with 3 Mrad failed to show any increase in mutation frequency (Münzner & Renner, *Zentbl. VetMed.* 1976, **23B**, 117). An *in vitro* test, in which an aqueous extract of the irradiated food was incubated with an agar culture of the Salmonella, also gave no indication of increased mutation.

Milk powder

Following a study in which rats or mice fed for 8 weeks on a diet with a relatively high level of free radicals, derived from its content of 35% whole-milk powder irradiated with 4·5 Mrad, had shown no mutagenic or other adverse effects on reproductive capacity (*Cited in F.C.T.* 1974, **12** 427), Renner & Reichelt (*Zentbl. VetMed.* 1973, **20B**, 648) fed a similar ration to six generations of rats over a 3-year period. Although succeeding generations were killed after 1 year, the parent generation was kept on test for just over 2 years. At the end of the first year, animals taking the irradiated diet weighed significantly less than the controls, probably because of their low vitamin intake, but this difference was eliminated during the second year. There was no evidence in the treated animals of any toxicity in the form of blood abnormalities or changes in serum-enzyme activities, organ weights or histology, no increase in tumour incidence was detected and fertility was unaffected.

Wheat and potatoes

George *et al.* (*Fd Cosmet. Toxicol.* 1976, **14**, 289) reported that when Wistar rats were fed a diet containing 70% irradiated wheat (75 krad) for 1–6 weeks, their bone-marrow picture showed no greater frequency of polyploid cells than was found in others fed 70% non-irradiated wheat. Even when the wheat was fed within 24 hours of irradiation it had no observable effect on the bone-marrow picture. No adverse effect on the sexual physiology of female rhesus monkeys fed a diet containing both similarly irradiated wheat flour (75 krad) and potatoes irradiated with 10 krad, a dose of the order of that used to inhibit sprouting, was observed by Sialy *et al.* (*Int. J. Radiat. Biol.* 1976, **29**, 555). Monkeys fed this diet for 3 months showed no external changes in reproductive organs and no alteration in the menstrual cycle, vaginal cytology or oestrogen and pregnanediol production. The histology of the reproductive organs remained normal.

Levinsky & Wilson (*Fd Cosmet. Toxicol.* 1975, **13**, 243) described a negative dominant lethal study in which an alcoholic extract of potatoes irradiated with about 12 krad was given by gavage twice daily to male Swiss mice, in a daily dose equivalent to 3 g potato, for 7 days, immediately after which sequential mating was started. Females were examined on days 11–17 of gestation. Pregnancy rates, total numbers of implantation sites and numbers of foetal deaths did not differ in the treated mice and controls.

The effect of malnutrition as a factor in the possible toxicity of irradiated foods was investigated by Vijayalaxmi & Rao (*Int. J. Radiat. Biol.* 1976, **29**, 93). Groups of 16 male Wistar rats were fed either an 18%- or a 5%-protein diet for 8 weeks, after which four from each group were mated over a 4-week period while the rest were fed either non-irradiated or irradiated wheat (75 krad) in a diet containing 9% protein for another 12 weeks before mating. The mutagenic index (percentage of dead embryos among total implantations) in females mated with rats taking the 18%-protein diet was consistently lower than that associated with the 9%-protein diet. The rats fed only the 5%-protein diet were probably not sexually mature, since none of the females became pregnant by them. The mutagenic index associated with initially good nourishment did not differ significantly from that of initially malnourished rats subsequently fed non-irradiated wheat, but in both groups ingestion of irradiated wheat increased the mutagenic index. The irradiated feed also reduced the number of germ cells in the testes of malnourished although not of well-nourished animals. In view of the evidence for an increase in dominant lethality and the decrease in germ cells in the testes following feeding of this irradiated diet, particularly to malnourished rats, it should be noted that the wheat was fed to the rats within 20 days of its irradiation.

Of particular interest is a paper by Bhaskaram & Sadasivan (*Am. J. clin. Nutr.* 1975, **28**, 130) describing nutritional studies in small groups of children. Three groups each of five children suffering from severe protein and calorie malnutrition were fed diets containing non-irradiated, freshly irradiated (75 krad) or irradiated and subsequently stored wheat in amounts equivalent to 20 g/kg body weight for 6 weeks. All groups showed similar clinical and biochemical responses to the feeding. In four children who received freshly irradiated wheat, polyploid cells were first detected 4 weeks from the start of the test feeding and their incidence rose from 0·8 to 1·8% at 6 weeks. Other abnormal cells with split centromeres and widely separated chromatids also appeared. Totals of abnormal cells at 4 and 6 weeks accounted for 1·2 and 3·8%, respectively, but their incidence had decreased substantially in two children re-examined after 16 weeks, and none were apparent at 24 weeks. Children given stored irradiated wheat showed an incidence of 0·6% polyploid cells at 6 weeks. No abnormal cells appeared in children fed non-irradiated wheat. The numbers involved and the type of examinations performed limit the value of this study. Evidence from numerous other studies suggests a 0–2% range in the incidence of polyploidy in normal human populations, and a recent extensive study carried out for the International Project for Food Irradiation showed no effect on polyploidy incidence in rats fed freshly irradiated wheat (P. S. Elias, personal communication, 1977).

Irradiation has been shown to be a very effective means of prolonging the storage life of certain foodstuffs, by controlling insect infestation of stored grains, for example, and discouraging the sprouting of potatoes and onions. Priyadarshini & Tulpule (*Fd Cosmet Toxicol.* 1976, **14**, 293), however, reported that irradiation markedly increased aflatoxin production in cereals, onions and potatoes heavily inoculated with *Aspergillus parasiticus* and suggested that irradiation induced in the substrate biochemical changes

that favoured aflatoxin production. On the other hand, the Joint FAO/IAEA/WHO Expert Committee (*Tech. Rep. Ser. Wld Hlth Org.* 1977, 604, 17 & 18) found no evidence that, in practice, contamination of grain with aflatoxin-producing fungi or levels of toxin production were enhanced by irradiation.

[P. Cooper—BIBRA]

SOYA PROTEIN AND CHOLESTEROL

Although the connection is not universally accepted, it is now widely agreed that high serum-cholesterol levels predispose to coronary heart disease. In addition to genetic factors, the cholesterol level in the blood is influenced by diet—particularly the ratio of saturated to polyunsaturated fats (Keys *et al. Metabolism* 1965, **14**, 747). The suggestion has also been made that the amount and source of protein in the diet influence serum-cholesterol levels (Olson & Vester, *Physiol. Rev.* 1960, **40**, 677; Hodges *et al. Am. J. clin. Nutr.* 1967, **20**, 198). This suggestion is supported by the lower serum-cholesterol levels of vegetarians compared with non-vegetarians (West & Hayes, *ibid* 1968, **21**, 853; Sacks *et al. New Engl. J. Med.* 1975, **292**, 1148) and by the hypocholesteraemic effect produced in rabbits by vegetable protein but not by animal protein (Carroll & Hamilton, *J. Fd Sci.* 1975, **40**, 18).

Sirtori *et al.* (*Lancet* 1977, **I**, 275) have carried out a study in which a soya-bean textured protein totally replaced animal proteins in the diets of 22 people with type II hyperlipoproteinaemia. In each of these patients, the effects of a soya-protein diet were compared with those of a standard low-lipid, low-cholesterol diet. All the patients had been on a low-lipid, low-cholesterol diet for at least 3 months before the study. After adaptation to the hospital environment, each patient was given either a low-lipid diet followed by the soya-protein diet or *vice versa*, each dietary period lasting for 3 weeks. Both diets contained a high ratio of polyunsaturated to saturated fats and a low cholesterol level; the fibre content of the two regimes was the same. Plasma-cholesterol and triglyceride levels of the patients were determined three times each week after a 12-hour overnight fast and plasma lipoproteins were determined once a week. The study was completed by ten males and ten females aged from 22 to 68 years. The soya-protein diet, whether given before or after the low-lipid diet, significantly reduced cholesterol levels in the plasma (by an average of 21% in 3 weeks), whereas the low-lipid diet had very little effect. The extent of the reduction in plasma cholesterol was significantly related to the pretreatment plasma-cholesterol level. Plasma triglycerides were slightly decreased by both diets during the first treatment period but tended to stabilize during administration of the second diet.

In a second study by Sirtori *et al.* (*loc. cit*), eight more patients were given a diet in which the animal protein was replaced with textured soya protein for two successive 3-week dietary periods, with half of the subjects receiving 500 mg cholesterol daily for the first 3 weeks while the remainder were given the cholesterol supplement only for the last 3 weeks. The added cholesterol influenced neither the rate of decrease of plasma cholesterol during the first feeding period nor the stability of plasma cholesterol during the second. These authors state that this hypocholesteraemic effect is far in excess of that to be expected from the small difference between the two diets in the ratios of polyunsaturated to saturated lipids and may help to explain the variations in lipid levels found in populations with differing vegetable intakes. Knowledge of this effect may assist the formulation of diets for the treatment of hyperlipidaemia.

The underlying mechanism has not been established but it has been suggested (*Lancet* 1977, **I**, 291) that it might be related to the low-methionine content of soya protein compared with meat protein. However, Gatti & Sirtori (*ibid* 1977, **I**, 805) produced experimental evidence to refute this suggestion. They found that addition of methionine to the soya-protein diet of five patients did not appear to hinder the hypocholesteraemic effect.

Helms (*ibid* 1977, **I**, 805) put forward a number of objections to the conclusions of Sirtori *et al.* (*loc. cit.*). His first point was that this theory contrasted with the fact that Eskimos on very high animal-protein diets had low plasma-lipid concentrations. Then, criticizing the lack of detailed diet specifications in the report of the studies, he estimated that the daily carbohydrate intake from the textured soya-protein source alone was probably 23 g, and that about 15–20 g of this was indigestible, non-cellulose polysaccharide. Since there is considerable evidence that such polysaccharides have some capacity for lowering blood cholesterol. Helms suggested that the effects of the textured soya protein on blood lipids could be ascribed to the carbohydrate rather than the protein fraction.

Responding to this suggestion, Gatti & Sirtori (*loc. cit.*) agreed that the textured soya protein did indeed contain about 8 g more non-digestible, non-cellulose carbohydrate than the control diet, but pointed out that this amount of indigestible fibre was unlikely to produce a significant hypocholesteraemic effect. In support of this claim they cited the findings of Durrington *et al.* (*ibid* 1976, **ii**, 394) and Jenkins *et al.* (*ibid* 1976, **ii**, 1351), who showed that even 15 g of daily pectin or guar gum decreased cholesterol levels by only 7–9%, far less than the decreases produced by soya protein. Whilst the activity of the indigestible fibres cannot be ruled out, it seems that the effect on blood lipids is more likely to be due to the protein fraction of the soya bean.

The lack of effect of the cholesterol supplement in the second study of Sirtori *et al.* (*loc. cit.*) could have been caused by the low dietary lipid content and high

ratio of polyunsaturated to saturated lipid according to Hermus *et al.* (*ibid* 1977, **1**, 905), who pointed out that Sirtori *et al.* (*loc. cit.*) did not state whether a daily cholesterol supplement affected plasma-lipid levels in patients on the control low-lipid diet.

Hermus *et al.* (*loc. cit.*) also viewed the extrapolation of the effect of soya protein to other vegetable proteins as unwarranted. They demonstrated that hypercholesteraemia in casein-fed rabbits could be largely prevented by replacing part of the casein by other animal proteins such as gelatin and fish protein and tentatively attributed this to differences in the amino-acid composition of the proteins.

Although the mechanism involved is far from clear, the demonstration of the hypocholesteraemic effect of textured soya-bean protein has important implications in the fight against coronary heart disease. Possible therapeutic benefits of soya protein in this context would add to its already considerable value as a source of low-cost protein.

[M. A. Thompson—BIBRA]

PBB AND THE LIVER

The aftermath of the accidental contamination of Michigan cattle with a polybrominated biphenyl (PBB) in 1973/4 exposed wide gaps in our knowledge of the hazards of this group of compounds. Following this notorious incident, deaths, stillbirths and high rates of abortion were observed in animals exposed to high levels of a hexabrominated biphenyl (*Food Chemical News*, 1974, **16** (10), 34). In man, neurological, muscular and gastro-intestinal disorders were later linked with PBB ingestion (*Food Chemical News*, 1977, **18** (43), 32).

The effects of PBBs seem to be particularly marked in the liver. Residues accumulated mainly in the fat and liver of a cow and a sheep fed a diet containing 50 ppm PBB for 15 and 30 days, respectively (*Cited in F.C.T.* 1977 **15**, 157) and marked glandular hyperplasia was produced in the intrahepatic bile ducts of the cow and the gall bladder of the sheep. Mice given a diet containing 1000 ppm PBB for 11 days had enlarged livers and increased levels of cytochrome *P*-450, as well as swollen hepatocytes and focal areas of coagulative necrosis (*Cited in F.C.T.* 1977 **15**, 257).

More evidence of the action of PBBs in the liver has recently accumulated. In a number of studies in which rats were given a mixture of PBBs at dietary levels from 1 to 1000 ppm, liver- to body-weight ratios were increased significantly (Dent *et al. Res. Commun. chem. Path. Pharmac.* 1976, **13**, 75; *idem*, *Toxic. appl. Pharmac.* 1976, **38**, 237; Lee *et al. ibid* 1975, **34**, 115; Sleight & Sanger, *J. Am. vet. med. Ass.* 1976, **169**, 1231). The mixture used in all these studies except that of Lee *et al.* (*loc. cit.*) was Firemaster BP6, the contaminant in the Michigan disaster. In guinea-pigs fed PBBs in the diet, Sleight & Sanger (*loc. cit.*) found no significant increase in liver weight at the 1- or 10-ppm levels but at 100 and 500 ppm the liver-to body-weight ratios were increased, although loss of body weight accounted for a considerable part of this.

Lee *et al.* (*loc. cit.*) found that after 2 weeks on a diet containing 100 or 1000 ppm PBBs, rats had accumulated similar and very high levels of bromine in both fat and liver, and much lower levels in muscle. However, after 4 weeks on the 100-ppm diet, the bromine level in fat was twice that in the liver. Two weeks after withdrawal of PBB from these diets, bromine levels in the liver had decreased markedly but those in the fat had not, while 6 weeks after withdrawal, bromine was gradually decreasing in the liver and muscle but was still building up in the fat of the 1000-ppm group. However, even 18 weeks after withdrawal, all three of these tissues in treated animals retained amounts of bromine considerably higher than those in controls.

Microscopic lesions, predominantly extensive swelling and vacuolation of cells, were found by Sleight & Sanger (*loc. cit.*) in the livers of rats fed 10–500 ppm PBB for 30 days. Most of the vacuoles were small except in a few areas; they were shown by staining to contain fat. Areas of inflammation and necrosis were rare, the extent of cell damage being dose-related. Lee *et al.* (*loc. cit.*) gave a more detailed account of the hepatic cell damage produced by feeding rats with 100 or 1000 ppm PBB for 2 weeks. The hepatocytes were hypertrophied, with basophilic cytoplasm clumped around the cell membranes, and the pale, foamy and granular or vesicular perinuclear cytoplasm contained cytoplasmic inclusions, the capsules of which were shown by staining to be phospholipids. It was demonstrated that the inclusions varied in size, shape and structure, were formed within the hepatocytes and tended to cluster to form giant inclusions. The larger inclusions had thicker laminated capsules and the number and size of these structures were related to dosage and duration of treatment and to the degree of hepatocellular change. During the recovery period, the inclusions decreased in number and size in parallel with regressing hepatocellular changes.

Electron microscopy revealed proliferation of the smooth endoplasmic reticulum (SER) in both the studies mentioned above. Lee *et al.* (*loc. cit.*) described the structure of the fully developed inclusions as a thick concentrically laminated capsule of paired arrays of smooth membrane containing several lipid droplets. Hepatocytes with marked proliferation of SER showed considerable depletion of glycogen and moderately increased lipid droplets in the form of cytoplasmic globules which were not always related to the cytoplasmic inclusions. There was a proportional depletion of glycogen as the SER proliferated. Sleight & Sanger (*loc. cit.*) also observed this proliferation of the SER. In addition they reported a significant increase in the size of the mitochondria in rats fed 1 or 10 ppm PPB, but this increase was less distinct at the higher levels and contrasted with the

generally normal mitochondria found by Lee *et al.* (*loc. cit.*).

The latter goup maintain that myelin figures and the pathological changes in the hepatocytes produced by their sample of octabromobiphenyl are similar to those produced by DDT, thiohydantoin compound, phenobarbitone and toxic fat. They suggest that the myelin figures develop to enhance the surface area of the membrane for maximum contact of the toxin with detoxifying enzymes. In the light of the cumulative build-up of bromine in the fat, the toxic materials could well be located in the membrane-bound lipid bodies in which drug-metabolizing enzymes would have easy access to the fat-soluble material, but this is speculation.

An increase in the activity of hepatic microsomal enzymes at all feeding levels (1–500 ppm) was recorded by Sleight & Sanger (*loc. cit.*), and in the two papers cited earlier, Dent *et al.* describe the effects on liver-enzyme activities of administering PBBs either in the diet or as a single ip injection. The liver-enzyme activities of rats given 4·69, 18·75, 75 and 300 ppm PBBs in the diet showed a dose-response relationship between exposure and induction, and several enzymes were highly sensitive to PBB (Dent *et al. Res. Commun. chem. Path. Pharmac.* 1976, **13**, 75). At 300 ppm all the enzymes measured were significantly induced and at 75 ppm cytochromes *P*-450 and b_5, epoxide hydratase, aniline hydroxylase, ethylmorphine-*N*-demethylase, ethoxycoumarin-*O*-deethylase and benzo[a]pyrene hydroxylase were induced by NADPH-cytochrome *c* reductase was not. At 18·75 ppm all the enzymes except cytochrome b_5 and aniline hydroxylase were significantly induced, while at 4·69 ppm only epoxide hydratase was induced. The inducing properties of PBB appeared to be similar in some respects to those of both phenobarbitone and 3-methylcholanthrene, which classically represent two distinct types of inducer. Because of the persistence of PBBs, particularly in adipose tissue, enzyme induction would be expected to be a long-lasting effect.

In their second study, Dent *et al.* (*Toxic. appl. Pharmac.* 1976, **38**, 237) gave rats a single ip injection of 25 or 150 mg PBB/kg. Significant induction of cytochrome *P*-450, epoxide hydratase, ethoxycoumarin-*O*-deethylase, ethylmorphine-*N*-demethylase, aniline hydroxylase and arylhydrocarbon hydroxylase took place in all cases from 48 hours through until 336 hours at the 150-mg/kg level, whereas at the 25-mg/kg level the induction of ethylmorphine-*N*-demethylase was not significant until 336 hours and induction of epoxide hydratase never reached a significant level. When the pattern of induction by the PBBs was again compared with those of phenobarbitone and 3-methylcholanthrene, the PBB mixture was implicated once more as a mixed inducer. Separate components of the Firemaster BP6 mixture could be responsible for the different inducing effects, although a single component may be involved in both actions. It is suggested that the enzyme-inducing properties of the mixture might even be due to a minor contaminant; chlorinated dibenzofurans, which are amongst the most potent enzyme inducers known, have been identified in polychlorinated biphenyl mixtures and similar compounds could be present in Firemaster BP6.

While it is not clear which components of Firemaster BP6 are responsible for the different parts of its mixed enzyme-inducing ability, it is certain that the mixture has significant effects on the liver and there is clearly a possibility that with such enzyme-inducing capacity the mixture could influence the toxicity of other compounds.

[M. A. Thompson—BIBRA]

Fd Cosmet. Toxicol. Vol. 16, pp. 77–88. Pergamon Press 1978. Printed in Great Britain

TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS

COLOURING MATTERS

3237. How pigs deal with Orange RN

Larsen, J. C. & Tarding, F. (1976). Studies on the metabolism of Orange RN in the pig. *Acta pharmac. tox.* **39**, 525.

For the azo dyes widely used for colouring foodstuffs, the main route of metabolism involves reductive cleavage of the azo link by the gut flora with the consequent formation of primary amines. The aniline produced in this way by Orange RN (monosodium 1-phenylazo-2-naphthol-6-sulphonate), for example, has given rise to anaemia and Heinz-body formation in various species of laboratory animal (*Fd Cosmet Toxicol.* 1971, **9**, 619) but in addition, a Danish study carried out a few years ago revealed a lesion in the livers of pigs fed Orange RN (Olsen *et al. Toxicology*, 1973, **1**, 249). This unexpected finding prompted further metabolic studies in this species.

After Orange RN had been given iv or by stomach tube to female pigs, 24-hr urine specimens were examined for metabolites. Following an iv dose of 7·8 mg/kg, the urine contained unchanged dye (31% of the theoretical yield), 1-(4-hydroxyphenylazo)-2-naphthol-6-sulphonic acid (3%), *p*-aminophenol, the product of aniline hydroxylation (34%) and *o*-aminophenol (4%); 1-amino-2-naphthol-6-sulphonic acid was present but was not measured. The remainder of the phenylazo moiety appeared as *p*-aminophenol when the collection of urine was continued up to 72 hr. The excretion pattern suggested that Orange RN was partly excreted from the circulation into the bile, thereafter undergoing azo reduction in the gut. After administration of a dose of 78 mg/kg by mouth, the urine contained Orange RN and 1-(4-hydroxyphenylazo)-2-naphthol-6-sulphonic acid, the two compounds together representing only 0·4% of the theoretical yield, *p*-aminophenol (52%), *o*-aminophenol (6%) and aniline (0·3%), again with a small quantity of 1-amino-2-naphthol-6-sulphonic acid. These results confirm that Orange RN undergoes considerable reduction at the azo link after oral administration to pigs. It appears, however, that compared with earlier findings in the rabbit (Daniel, *Toxic. appl. Pharmac.* 1962, **4**, 572), 1-amino-2-naphthol-6-sulphonic acid is a relatively unimportant urinary metabolite.

FLAVOURINGS

3238. Sensitizing sebacate

Berlin, A. R. & Miller, F. (1976). Allergic contact dermatitis from ethyl sebacate in haloprogin cream. *Archs Derm.* **112**, 1563.

A 13-yr-old girl who developed contact dermatitis affecting the dorsal aspect of her feet and toes was found to be sensitized to a haloprogin cream which she had applied to relieve the irritation associated with the wearing of gym shoes. She had previously used this preparation for a short time a few months before. When the later use of the cream was discontinued the rash faded, and the girl was then patch-tested with the ingredients of the cream. Ethyl sebacate, which was included as a solubilizer, was found to be the culprit.

Ethyl sebacate is widely used as a flavouring agent in certain foods, and is also included in some cosmetic creams as a solubilizer. The authors discuss the theoretical possibility that people already contact-sensitized to this ester might suffer allergic reactions when they subsequently ingest it, and speculate that the lack of reports of this type of response may be due to complete metabolism of the compound in the gastro-intestinal tract. They also suggest that the extreme rarity of cases of sensitization to ethyl sebacate in cosmetic preparations may be an example of inhibition of contact dermal sensitization by prior ingestion of the additive in foods, an effect known as the Sulzberger-Chase phenomenon.

3239. Aspartame and phenylketonuria

Koch, R., Schaeffler, G. & Shaw, K. N. F. (1976). Results of loading doses of aspartame by two phenylketonuric (PKU) children compared with two normal children. *J. Toxicol. envir. Hlth* **2**, 459.

Aspartame (L-aspartyl-L-phenylalanine methyl ester) was cleared by the FDA for use as a sweetening agent in July 1974 (*Federal Register* 1974, **39** (145), 27319). However, because of questions raised as to the validity of the research data submitted by G. D. Searle & Co., the effectiveness of the regulation was stayed before any aspartame had been marketed (*Food Chemical News* 1975, **17**(45), 22). Aspartame is hydrolysed in the gut to methanol, phenylalanine (PA) and aspartate (*Cited in F.C.T.* 1977, **12**, 778), and the authors cited above point out that its use as a sweetener could provide as much as 340 mg PA daily. This could represent a significant amount for phenylketonurics, who lack the enzyme responsible for converting PA to tyrosine and must therefore be given

special diets with a low PA content. In the study cited above, the effect of single loading doses of aspartame and PA in normal and phenylketonuric subjects was investigated.

Two phenylketonuric and two normal adolescents were each given a single dose of 34 mg aspartame/kg, followed 2 wk later by the molecular equivalent amount of L-PA (19 mg/kg). The two phenylketonuric subjects were concurrently receiving diets providing PA at dose levels of 17 and 70 mg/kg/day, respectively, and in the former case the experimental dose of 1072 mg PA exceeded the dietary intake of 965 mg. Analysis of urine, collected for three 8-hr periods before and after each dose and then at 48 and 72 hr afterwards, in no case revealed a significant change in the output either of PA or of its metabolites *o*-hydroxyphenylacetic acid, phenylpyruvic acid or phenylacetylglutamine. The phenylketonuric patient on the more restricted diet did not excrete phenylpyruvic acid after either aspartame or PA, and although he showed slight increases in urinary PA and phenylacetylglutamine these had no clinical meaning. In the serum, sampled before each dose and at intervals up to 72 hr afterwards, PA levels increased by 4–6 mg/100 ml in the two phenylketonurics after both the aspartame and PA doses, but in the normal subjects serum PA was not significantly changed by the treatment. In no cases were serum tyrosine levels markedly altered, and no methanol was found in any serum or urine samples.

This limited study indicated that aspartame, at intake levels higher than those to be anticipated from its use as a sweetener, was well tolerated by the phenylketonuric adolescents. However, in younger phenylketonurics its contribution to the total permissible PA intake would be correspondingly higher, and the authors consider that the amount of aspartame ingested by such subjects may need to be controlled.

3230. No mutations from ingested cyclohexylamine

Machemer, L. & Lorke, D. (1976). Evaluation of the mutagenic potential of cyclohexylamine on spermatogonia of the Chinese hamster. *Mutation Res.* **40**, 243.

Cyclohexylamine (CHA) is used as a boiler-water additive and is also well known as a metabolic product of cyclamates. Mutagenicity studies on CHA have given somewhat conflicting results, but is noteworthy that there has been no evidence of mutagenic effects after its oral administration (*Cited in F.C.T.* 1977, **15**, 69). Further negative findings after administration by this route are now presented.

Male Chinese hamsters were given an aqueous solution of CHA sulphate by oral intubation at a dose level of 150 mg/kg/day for 5 days. Colchicin (4 mg/kg) was injected 18·5 hr after the last dose to inhibit mitosis, and the animals were killed after a further 5·5 hr. One hundred spermatogonial metaphases from each animal were then compared with similar numbers from positive and negative controls given, respectively, 100 mg cyclophosphamide/kg/day for 5 days and water alone. In the CHA group, only 0·87% of the metaphases displayed aberrations (gaps and breaks), or 0·37% when gaps were excluded, and no translocations were found. These results did not differ significantly from the 1·24% incidence of gaps and breaks (0·25% breaks) in the negative controls. In contrast, in the cyclophosphamide group the incidence of aberrations was 3·41%, or 1·99% when gaps were excluded, and 0·71% also displayed translocations. Although the incidence in this positive group was relatively low, suggesting that some damaged cells might have been eliminated during the treatment period, the differences from the negative control group were statistically significant. The study thus provided further evidence of the safety of CHA in this connexion.

AGRICULTURAL CHEMICALS

3231. Zinc depletion and dithiocarbamate teratogenesis

Larsson, K. S., Arnander, C., Cekanova, E. & Kjellberg, M. (1976). Studies of teratogenic effects of the dithiocarbamates maneb, mancozeb, and propineb. *Teratology* **14**, 171.

The teratogenic powers of the dithiocarbamate fungicides maneb, mancozeb and propineb are compared in the work cited above. Preliminary work by these authors had indicated that these compounds act by depleting body zinc. The three formulations (manganese ethylenebis(dithiocarbamate), a zinc-maneb complex containing 20% manganese and 2·5% zinc, and a polymer of zinc propylenebis(dithiocarbamate), respectively) all of technical grade, were given by gavage to mice on days 9 or 13 and to rats on day 11 of gestation. Mice received 400, 770 or 1420 mg maneb/kg or 380, 730 or 1320 mg mancozeb/kg, while rats received similar doses of these two compounds or 400, 760 or 2300 mg propineb/kg. No adverse effects appeared in maternal or foetal mice. In rats the frequency of resorptions rose after treatment with 770 or 1420 mg maneb/kg to 56 and 40% respectively, compared with a control figure of 3%. All surviving foetuses showed malformations (severe limb and craniofacial defects) after exposure to the two highest doses of maneb, and malformations were seen also in 25% of surviving foetuses exposed to mancozeb at the 1420 mg/kg level. The frequency of resorptions increased to 34% in rats given 2300 mg propineb/kg, and at this dose level foetal malformations were almost universal; at lower propineb levels there were no more malformations than in controls. Adverse reactions, including paralysis of the rear extremities and loss of weight, were observed in all three groups of pregnant rats 3–4 days after treatment with propineb; recovery generally occurred within 4 days. The addition of zinc to maneb at 15 mg zinc acetate/kg did not alter the 100% frequency of foetal malformations associated with the 750 mg/kg dose, but addition of 30 or 60 mg/kg reduced the malformation rate for this dose to 64 and 11%, respectively.

However with 1380 mg maneb/kg, even 108 mg zinc acetate/kg did not reduce the frequency of malformations.

It seems likely that the teratogenic effect of maneb is closely related to the degree of zinc depletion, suggesting that the zinc-chelating potency of the dithiocarbamates may determine their teratogenicity. According to Hurley & Mutch (*J. Nutr.* 1973, **103**, 649), the zinc plasma concentration is higher in rats than in any other species and accumulation of zinc begins *in utero*; this could explain, at least partially, the species difference in the effect of the dithiocarbamates. Alternatively there may be differences in the gastro-intestinal absorption of these compounds by the two species. Although the doses used in these studies were far greater than the amounts likely to be involved in the ingestion of food-spray residues by pregnant women, the authors consider it prudent to favour the zinc-containing dithiocarbamates as pesticides.

3232. The metabolism of ethylene thiourea

Kato, Y., Odanaka, Y., Teramoto, S. & Matano, O. (1976). Metabolic fate of ethylenethiourea in pregnant rats. *Bull. env. contam. & Toxicol. (U.S.)* **16**, 546.

Thyroid tumours are the predominant morphological effect of ethylene thiourea (ETU) administered for a prolonged period to the rat, although in the hamster the liver is the chief target organ (*Cited in F.C.T.* 1977, **15**, 5). The concentration of ETU attained in the thyroid after oral administration to rats and guinea-pigs was far greater than that in other organs, and unchanged ETU was rapidly excreted in the urine, with only about 1% appearing in the faeces within 48 hr (*ibid* 1975, **13**, 584). ETU is also a teratogen in the rat (*ibid* 1977, **15**, 157), and evidence for its transplacental passage into the foetus has been reported (*ibid* 1977, **15**, 80). The latter study also showed that, in pregnant rats given a teratogenic dose of [4,5-^{14}C]ETU, radioactivity was evenly dispersed between the red blood cells and plasma, and reached fairly uniform levels in maternal liver, kidney, muscle and placenta. As much as 84% of the ^{14}C was eliminated in the urine within 48 hr, mostly as unchanged ETU, although traces of ethyleneurea and two unidentified metabolites were also found.

Further evidence of the metabolic transformation of ETU has now been obtained in pregnant rats given a teratogenic dose (100 mg/kg) of ETU, labelled with ^{14}C in either the 2- or 4,5-positions, on day 12 of gestation. Significant levels of radioactivity appeared in the blood within 5 min, and reached a maximum (0·48 μmol expressed as [2-^{14}C]ETU/g blood) within 2 hr. Somewhat lower maximum levels were similarly attained within 2 hr in the foetus and most maternal tissues, and these had decreased by more than 90% after a further 22 hr. The only exception was the thyroid, in which levels increased from 0·35 μmol/g at 2 hr to 0·65 μmol/g at 24 hr. Some 12% of the dose appeared in the urine within 3 hr, and this had risen to 80·2% by 24 hr and 82·5% by 48 hr. In contrast, only 0·53% was detected in the faeces over 48 hr. Expired carbon dioxide accounted for significant amounts (up to 7 nmol/2 min) of the [4,5-^{14}C]ETU, but for only traces of [2-^{14}C]ETU, a finding indicative of fragmentation of the imidazolidine ring and decarboxylation in the 4- and/or 5-positions. Radioactivity from [4,5-^{14}C]ETU became incorporated to a large extent in the maternal serum proteins and in the crude protein fraction of the foetal cells, whereas [2-^{14}C]ETU was found chiefly in the ethanol–water fraction and to a lesser extent in the chloroform fraction of the foetal homogenate. Thin-layer chromatography of foetal tissue revealed eight radioactive metabolites in addition to unchanged ETU from [2-^{14}C]ETU. The thyroxine level in treated rats did not differ significantly from that in controls, suggesting that the teratogenic effects of ETU were not due to a primary action on the thyroid.

PROCESSING AND PACKAGING CONTAMINANTS

3233. The cosmetic side of PVC bottles

Tester, D. A. (1976). The extraction of vinyl chloride from PVC containers. *J. Soc. cosmet. Chem.* **49**, 459.

Vinyl chloride (VC) monomer presents a considerable biological hazard (*Cited in F.C.T.* 1976, **14**, 347 & 498), and the possibility and extent of its migration into food, drinks and drugs when its polymers are used in containers has been the subject of concern.

The use of thermoplastics, and particularly of polyvinyl chloride (PVC), for packaging toiletries and cosmetics has increased rapidly. Residues of monomeric VC in PVC containers are minimal and their extraction by most solvents is slight. Maximum extraction levels for all the foodstuffs tested in the present study indicated a partition between the containers and their contents, the concentration of VC being much higher in the container. This effect overshadowed that of the weight ratio of the contents to the container. The rate of extraction increased with increasing temperature for all the systems studied and, under given conditions, the level of VC extracted was directly proportional to the initial level in the container. Studies of monomer extraction by corn (maize) oil, orange squash, water, 3% aqueous acetic acid, and 15 and 50% aqueous ethanol indicated a similar pattern for all the extractants except 50% ethanol. It may therefore be expected that other solvent systems, including reasonably dilute ethanolic solutions, will behave comparably. PVC containers holding 5 ppm or less of VC residue might be expected to yield 0·001–0·01 ppm of VC to the contained liquid after prolonged storage. Under normal conditions of cosmetic use, any contribution of VC to the human system from cosmetic products is likely to be introduced through the skin and to be of less toxicological significance than the contributions from ingestion of food and drinks, although there is as yet little information on the percutaneous absorption of VC from very dilute aqueous or oily solutions.

THE CHEMICAL ENVIRONMENT

3234. Beryllium hypersensitivity tests

Price, C. D., Jones Williams, W., Pugh, A. & Joynson, D. H. (1977). Role of *in vitro* and *in vivo* tests of hypersensitivity in beryllium workers. *J. clin. Path.* **30**, 24.

The likelihood that chronic beryllium disease is at least in part a manifestation of delayed hypersensitivity has prompted the search for tests that may be used both for diagnosis of the disease and for the long-term monitoring of potentially exposed beryllium workers. Studies have indicated that lymphocyte function tests are of great value in the detection of delayed hypersensitivity and preliminary work using the beryllium-induced macrophage-migration inhibition test (Be MIF) has proved promising. A subsequent investigation (cited above) compared the results of Be MIF testing in patients with chronic beryllium disease, in industrial workers potentially exposed to beryllium but showing no evidence of the disease, and in control subjects, who included both patients with sarcoidosis and normal healthy individuals.

White blood cells were separated from heparinized blood and adjusted to give final concentrations of 2·5 and 5·0 $\times 10^6$ cells/ml prior to culturing with three (10^{-7}, 10^{-8} and 10^{-9} M) concentrations of beryllium sulphate ($BeSO_4$). Peritoneal macrophages were obtained from paraffin-oiled guinea-pigs. Each culture supernatant was used in three wells of a migration plate. The areas of migration, measured by planimetry, were read at both 18 and 24 hr and a migration index was calculated by dividing the area of migration with antigen by the area of migration without antigen. Twelve readings for each subject were averaged to provide a mean MIF index.

Results revealed a positive inhibition in diseased patients, although steroid treatment, which the majority of patients were undergoing at the time, tended to increase the MIF index. Seven of the 50 beryllium-metal workers had MIF indices below 0·87, 16 below 0·93, 28 below 0·99 and 22 above 0·98. The only parameter possibly associated with sensitization was airborne exposure, to which 42·8% of the sensitized subjects but only 32·5% of the non-sensitized workers had been subjected. None of the control subjects appeared to be sensitized. The investigation, which also examined tuberculin reactivity (Mantoux test) revealed a trend towards tuberculin negativity in potentially exposed beryllium workers. Both this characteristic and the use of the Be MIF test in the long-term monitoring of potentially exposed workers should aid in the clarification of the important question of whether beryllium-sensitized individuals are more liable to develop chronic beryllium disease.

3235. Into the heart of lead poisoning

Khan, M. Y., Buse, M. & Louria, D. B. (1977). Lead cardiomyopathy in mice. A correlative ultrastructural and blood level study. *Archs Path.* **101**, 89.

The clinical problems commonly associated with lead intake in man and experimental animals are mainly gastro-intestinal, haemopoietic and central nervous system disorders. Few descriptions are available on the effects of lead on the cardiovascular system of experimental animals, although Asokan (*J. Lab. clin. Med.* 1974, **84**, 20) reported structural changes in the heart muscle of rats given 1% lead acetate as drinking-fluid over a period of 6 wk. The study cited above sets out to examine the ultrastructural effects of acute lead toxicity on the myocardium of mice and to relate any changes to blood-lead levels.

Three groups of 12 male mice were force-fed a lead acetate solution in distilled water on 5 days/wk for 2 wk, at doses of 20, 100 and 200 mg/kg body weight. The animals were killed and their hearts were excised 7 days after the last treatment. Small strips of myocardium were removed and prepared for electron microscopy. Blood-lead levels were determined by atomic absorption spectrophotometry.

At death no gross abnormalities were seen in any of the organs. Blood-lead levels in each group of mice varied, with a generally higher range in the animals that received higher doses of lead. To correlate the blood-lead levels with the observed myocardial structural changes, the data were organized into three groups with blood-lead levels of 4–16, 20–56 and 60–124 μg/100 ml, respectively.

Significant ultrastructural changes were observed only in animals with blood-lead levels over 20 μg/100 ml. Nuclear changes were substantial and their severity appeared to be related to lead levels. Peripheral and membranous condensation of nuclear chromatin was apparent and the nucleolus showed disorganization. Changes in mitochondria and the endoplasmic reticula were observed at blood-lead levels above 40 μg/100 ml, the frequency and severity of these again being related to the lead level. Characteristic features of the mitochondria were mild to moderate enlargement, disorientation of cristae, vacuolation and an increase in the intramitochondrial matrix. The endoplasmic reticulum showed mild to moderate dilation. Myofilamentous changes were restricted to animals with blood-levels above 60 μg/100 ml and severity was related to blood-lead level; they may have resulted from a specific metabolic disturbance or from a violent contraction of myofibrils. Of the organelles examined, the myofibrils appeared to be the most resistant to lead toxicity. Interstitial cell changes were restricted to mice with blood-lead levels above 40 μg/100 ml, and were characterized by an increase in cytoplasmic organelles, particularly polyribosomes and endoplasmic reticulum.

The nuclear and nucleolar changes seen in the study were perhaps related to disturbances of cellular RNA metabolism, and although some other compounds, such as ethionine, proflavine and aflatoxin, are known to complex with DNA to inactivate chromatin and produce clumping, it remains unclear whether the observed nuclear and nucleolar changes resulted from lead–DNA complexing or from some nonspecific cellular damage.

The applicability of these findings to lead intoxication in man requires further clarification. Although

the present study suggests a good correlation between myocardial effects and blood-lead levels, there is often a dichotomy between tissue and blood levels and between blood levels and tissue effects.

3236. A lead to the lead effect on globin synthesis

Ali, M. A. M. & Quinlan, A. (1977). Effect of lead on globin synthesis *in vitro*. *Am. J. clin. Path.* **67**, 77.

A disturbance in haem biosynthesis is one important aspect of lead toxicity as evidenced by anaemia and increased urinary excretion of coproporphyrin and δ-aminolaevulinic acid (Goldberg, *Br. J. Haemat.* 1972, **23**, 521). These changes have been attributed to the inhibition of several enzymes involved in haem synthesis or to interference with the transport of iron, as well as to changes in the erythrocyte membrane (*idem, Sem. Hemat.* 1968, **5**, 424). The present study examines the effects of lead, at various concentrations, on globin chain synthesis and the influence of haem on these effects, in an attempt to clarify the nature of the defect in haemoglobin synthesis associated with lead poisoning.

Reticulocyte-rich peripheral blood samples were incubated with lead acetate at concentrations of 10^{-6}, 10^{-5} and 10^{-4} M (equivalent to 20, 200 and 2000 μg/100 ml respectively) and the incorporation of tritiated leucine into globin was determined. To examine the effect of haem on the inhibition of globin synthesis, reticulocyte-enriched blood was incubated with lead in a concentration of 10^{-5} M (200 μg/100 ml) in the presence of haem at a concentration of 10^{-3} M or in its absence.

The depression of tritiated-leucine incorporation into globin chains was proportional to the concentration of lead acetate, leucine incorporation being reduced to about 85% of the control value with 10^{-6} M, 48% with 10^{-5} M and 15% with 10^{-4} M-lead acetate.

In the second study, lead alone depressed the incorporation of tritiated leucine to 45% of the control value, whilst globin synthesis with haem alone was 109% of the control. In the presence of both haem and lead, the mean incorporation of leucine into globin was 69% of the control value. However, the increase was not significantly different from that obtained with lead alone, confirming the previous observation of direct inhibition of globin synthesis as well as the haem-synthesis defect (Kassenaar *et al. J. biol. Chem.* 1957, **229**, 423). The mechanism of inhibition of globin synthesis by lead thus differs from its inhibition by ethanol, since the latter can be completely reversed by haem.

Lead is known to affect mitochondrial metabolism profoundly, both by disturbing ATP synthesis and by combining with sulphydryl-containing mitochondrial enzymes involved in cytochrome synthesis, and it may be that a similar effect accounts for the direct inhibition of globin synthesis by lead.

The authors remark on the inhibition of globin synthesis at a level of lead considered to be well below the accepted safe upper limit (70–80 μg/100 ml) for the blood concentration of persons exposed industrially. They suggest that even slight exposure to lead may be more dangerous to health than has previously been suspected and that further studies would aid in the clarification of subclinical lead poisoning.

3237. Injected EDTA and the kidney

Braide, V. B. (1976). Renal ultrastructural changes induced by calcium EDTA in rats. *Res. vet. Sci.* **20**, 295.

The injection of calcium disodium ethylenediaminetetraacetate ($CaNa_2EDTA$), which has been used to treat lead poisoning in man and domestic animals, has produced hydropic degeneration of the proximal convoluted tubules of the kidneys both under experimental conditions (*Cited in F.C.T.* 1967, **5**, 576) and as a side-effect of its therapeutic use (Holland *et al. Proc. Soc. exp. Biol. Med.* 1953, **84**, 359). Vacuolation of the tubular cells was shown to be associated with the uptake of ^{14}C-labelled $CaNa_2EDTA$ by the lysosomal fraction of the renal cortex, suggesting that vacuole formation involved pinocytosis and subsequent lysosomal activation (*Cited in F.C.T.* 1972, **10**, 698). Injected $CaNa_2EDTA$ has also produced haemorrhage and necrosis of the intestine, and the major ultrastructural changes in the duodenal epithelium of the rat have been shown to be mitochondrial swelling and dilatation of the endoplasmic reticulum (Braide & Aronson, *Toxic. appl. Pharmac.* 1974, **30**, 52). The ultrastructural changes produced in the kidney have now undergone a similar study.

Rats were infused iv for 24 hr with 0·1 M-$CaNa_2EDTA$ at a rate of 6 mmol/kg/24 hr, and the kidney tissue was then compared with that from saline-infused rats. $CaNa_2EDTA$ did not affect the renal mitochondria, except to the extent that they appeared to be reduced in number in the proximal tubular cells. These cells contained many large vacuoles filled with flocculations of electron-dense material, and showed evidence of increased lysosomal activity. The electron density of the glomerular basement membrane was decreased, and the space between it and the capillary endothelium was enlarged.

As infused $CaNa_2EDTA$ promotes collagen degradation in the rat (Aronson & Rogerson, *ibid* 1972, **21**, 440), it seems probable that the effects on the basement membrane were due to the degradation of the collagen of which it is largely composed. The consequent increased leakage of plasma albumin into the proximal tubular fluid, and/or the increase in collagen breakdown products, could then have been responsible for the increased number of pinocytotic vesicles in the proximal tubular epithelium.

3238. Expiry of expired methyl methacrylate

Derks, C. M. & d'Hollander, A. A. (1977). Some aspects of pulmonary excretion of methylmethacrylate monomer (MMM) in dogs. *J. surg. Res.* **22**, 9.

The effects of the iv administration of methyl methacrylate (MMA) have been studied extensively, largely as a result of speculation on the potential hazards

of MMA absorbed from the self-curing acrylic cement used in orthopaedic surgery. Homsy *et al.* (*Clin. Orthop. rel. Res.* 1972, no 83, p. 317) considered that the major route of excretion, following an iv injection of MMA to dogs, was through the lungs. However, thinking that the evidence for pulmonary excretion was unconvincing, the authors of the study cited above attempted to clarify the matter.

MMA was given to anaesthetized dogs by infusion through a vein in the paw. The total dose of 0·05 ml/kg, administered during a 4-min infusion period, was such that the cardiopulmonary function of the animals was only very slightly affected. MMA levels in the expired air were maximal within 2–4 min of the start of the infusion and, of the quantity of monomer excreted through the lungs, 50% was eliminated within 2·5 min and 93% was eliminated by the end of the perfusion period. Nevertheless, the total amount of pulmonary excretion was small, accounting for a maximum of 3% of the injected dose. Free MMA levels in arterial blood samples taken from the femoral artery 4 min after the beginning of the infusion were similar to those in the expired air, but after a further 5 min no free MMA could be detected. *In vitro* studies showed that equilibrium between MMA vapour and the blood, plasma or red cells was not established during a 165-min observation period, a finding due, it was suggested, to uptake of the monomer by the blood constituents.

Commenting on earlier work, these authors noted that from the expired air of a patient undergoing surgery, Homsy *et al.* (*loc. cit.*) recovered only some 0·5% of the MMA detected in the central venous circulation during the first 6 min after implantation of the cement. Furthermore, in contrast to the rapidity of the pulmonary excretion of MMA and of the fall in free MMA levels of the blood now reported, previous experiments in the dog using labelled MMA had detected radioactivity in the blood for comparatively long periods, suggesting the retention of polymers or metabolites. It is concluded that MMA is excreted in the expired air only to a very minor extent, the other metabolic pathways that must operate being aided by the high solubility of MMA in the blood.

3239. A potentiated breath of carbon tetrachloride

Folland, D. S., Schaffner, W., Ginn, H. E., Crofford, O. B. & McMurray, D. R. (1976). Carbon tetrachloride toxicity potentiated by isopropyl alcohol: Investigation of an industrial outbreak. *J. Am. med. Ass.* **236**, 1853.

Carbon tetrachloride is acknowledged to be one of the most toxic solvents in common use, and it is well known that its hepatotoxicity is enhanced by the consumption of alcohol (*Cited in F.C.T.* 1963, **1**, 282; *ibid* 1968, **6**, 808). The incident related here illustrates the potential hazard of its unauthorized use in a factory.

Eight of 43 workers packing isopropanol from bulk were exposed to the vapour of carbon tetrachloride, which was used on one occasion as a cleaning agent in place of the usual acetone. They became ill within 12 hr and a further six workers developed signs of toxicity within 48 hr. The signs included nausea (in 93%), vomiting (in 86%), headache (in 79%), weakness (in 86%) and abdominal pain (in 71%). Some workers also complained of dizziness, diarrhoea and blurred vision. The average duration of the illness was 7 days (range 2–21 days). Attack rates were highest among workers towards the north end of the plant, where the isopropanol-packaging line was only 12–15 m from open buckets containing 15 litres of carbon tetrachloride. Of the four who were taken to hospital, two had both renal damage and mild liver dysfunction, three required treatment for renal failure and one needed haemodialysis. Some 2 months after the incident, the isopropanol concentration in the air at the northern end of the plant was 410 ppm, compared with 140 ppm in the middle. Alveolar samples from the workers most heavily exposed to isopropanol contained a mean concentration of 100 ppm isopropanol with up to 19 ppm acetone, its metabolite, although no acetone could be detected in the ambient air. It seems probable that the relatively high acetone content of the workers' blood potentiated the toxicity of carbon tetrachloride, and the authors propose that stricter limits than the currently accepted 10 ppm should be set for the latter solvent in situations where concurrent exposure to isopropanol or acetone occurs.

3240. Ca DTPA strikes the foetus

Fisher, D. R., Calder, S. E., Mays, C. W. & Taylor, G. N. (1976). Ca-DTPA-induced fetal death and malformation in mice. *Teratology* **14**, 123.

Calcium trisodium diethylenetriaminepentaacetate (Ca DTPA) has been shown to chelate plutonium, yttrium and lanthanum and promote their excretion from the body (*Cited in F.C.T.* 1964, **2**, 93). However, the authors of the paper cited above reported earlier that Ca DTPA possessed considerable toxicity for the mouse foetus (*Cited in F.C.T.* 1977, **15**, 363), and they have now extended their original studies.

Female mice given five daily injections each of 720–2880 μmol Ca DTPA/kg on days 2–6, 7–11 or 12–16 of gestation were killed and examined on day 18. Foetal deaths showed an increase over the control level, particularly when Ca DTPA had been injected during early or mid-gestation, and resorption sites in the dams were commoner than dead foetuses. The frequency of gross malformations, which included exencephaly, ablepharia, spina bifida aperta, cleft palate and polydactyly, increased with dose, with the highest incidence of exencephaly and ablepharia appearing when Ca DTPA was given during early or mid-gestation and the highest incidence of polydactyly when it was given in late gestation. The incidence of polydactyly did not necessarily reflect the effect of Ca DTPA, however, since this condition tends to be spontaneous in the C57BL strain of animal used (but no polydactyly was observed in controls). In alizarin-stained specimens of foetuses, slightly retarded ossification of the skull-cap and an abnormally wide mid-line cranial fissure were common. The doses of Ca DTPA given produced no deaths among the dams, but haemorrhages in the

uterine walls and placenta were seen in some animals after the higher doses.

This further evidence supports the group's previous recommendation (*ibid* 1977, **15**, 363) that for the emergency removal of plutonium and other actinide elements from the human body, Ca DTPA should be replaced with the much safer zinc trisodium salt, which does not cause substantial depletion of body levels of zinc and manganese.

3241. Pectin lowers blood cholesterol

Kay, R. M. & Truswell, A. S. (1977). Effect of citrus pectin on blood lipids and fecal steroid excretion in man. *Am. J. clin. Nutr.* **30**, 171.

Several recent studies have shown that dietary fibre can protect against the toxic effects of certain food additives (amaranth, Tween 60 and sodium cyclamate), a finding attributed to its physico-chemical properties (*Cited in F.C.T.* 1975, **13**, 581; *ibid* 1976, **14**, 365 & 1977, **15**, 358) also appears that lack of fibre in the diet of affluent western man may contribute to the high incidence of colonic cancer, and possibly to other conditions such as cardiovascular disease. One of the many types of dietary fibre is pectin, which has been shown to increase the faecal excretion of lipids, cholesterol and bile acids in rats (Lin *et al. Am. J. Physiol.* 1957, **188**, 66) and, in gram quantities, to lower plasma cholesterol in man (Keys *et al. Proc. Soc. exp. Biol. Med.* 1961, **106**, 555; Palmer & Dixon, *Am. J. clin. Nutr.* 1966, **18**, 437; Jenkins *et al. Lancet* 1975, **I**, 1116). Further evidence that pectin may have a beneficial effect on blood-cholesterol levels in man is now available.

Four men and five women were fed for 3 wk on metabolically controlled diets and each day were given 15 g citrus pectin, mixed with fruit, sugar and orange juice to form a gel, in divided doses with meals. Except for some flatulence and abdominal distension, the pectin was well tolerated. During the 3-wk period, plasma cholesterol fell on average by 13% (range 5–26%), and in all but one case it rose again when pectin was discontinued. Plasma triglycerides, however, were unaffected. Mean intestinal transit time was unchanged, but there was a slight increase in the dry weight of the faeces and in the frequency of defaecation, accompanied by a decrease in faecal water content. When measured during the third week of pectin consumption, the faecal excretion of fat and neutral steroids (cholesterol and coprosterol) showed highly significant increases, of 44 and 17% on average respectively, and total bile acid excretion was also increased by a mean of 33%.

The effects on plasma cholesterol were greater than those reported by previous workers, perhaps because pectin was administered this time as a gel rather than in biscuit of capsule form. As pectin is largely degraded by colonic bacteria, it seemed probable that the effects observed were attributable to its physical properties in the upper intestine, and the authors suggest that it may interfere with the equilibrium between the micellar phase and the molecular phase which passes into the unstirred layer on the brush border.

3242. Aflatoxin rides again

Arseculeratne, S. N., Samarajeewa, U. & Welianga, L. V. (1976). Inhibition of aflatoxin accumulation in smoked substrates. *J. appl. Bact.* **41**, 223.

Flannigan, B. & Hui, S. C. (1976). The occurrence of aflatoxin-producing strains of *Aspergillus flavus* in the mould floras of ground spices. *J. appl. Bact.* **41**, 411.

Aflatoxin (AF) has produced acute liver damage, liver degeneration, liver tumours and teratogenic effects, and is possibly associated with Reye's syndrome (*Cited in F.C.T.* 1976, **14**, 151). Reports of its occurrence in vegetable foods and spices and attempts to prevent its production by various moulds continue.

The first paper cited here describes a study of the possible use of a smoking process to control mould growth and AF production in food materials. Both the growth of *Aspergiillus parasiticus* NRRL 2999 in grated coconut and in potato-dextrose broth and the production of AF in these cultures were inhibited to some degree by exposure of the substrate to coconut-shell smoke in a metal smoking chamber. On both substrates, AF production was significantly and sometimes totally inhibited, while mycelial growth was inhibited to a less marked degree. Treatment of the broth with liquid smoke again significantly reduced AF accumulation, but mycelial growth was not reduced. A strain of *Penicillium rubrum* isolated from mouldy copra was less susceptible than *A. parasiticus* to growth inhibition by direct smoking.

In view of suggestions that drying in electrical ovens might provide an alternative means of counteracting AF contamination of coconut kernels, it is of interest that in unsmoked coconut, moisture levels as low as 15% permitted significant toxin accumulation, while toxin production was totally inhibited in smoked substrates at moisture levels up to about 40%. The latter level is close to that of the fresh kernels (about 45%) and is unlikely to be reached during storage under all but the most adverse conditions. Pointing out the technical and economic advantages of smoking in this context, the authors claim that the main disadvantage of the process—the possible transmission of carcinogens in the smoke—may be counteracted by careful control of combustion temperatures or the use of liquid smoke. They recommend that studies on smoking processes should be extended to the industrial level and to other food crops, such as groundnuts, susceptible to AF contamination.

The second paper cited above describes an examination of the microflora of 20 samples of ground food spices and three samples of mixed-spice powder. Moulds were not detected in cloves, but in other spices propagules ranging in numbers from $0{\cdot}5 \times 10^2$/g in turmeric to $6{\cdot}4 \times 10^5$/g in black pepper were counted. Aspergillus species were the main contributors except in curry powder, fenugreek and gharum masala, in which Penicillia predominated. *A. glaucus* and *A. niger* occurred in 19 of the 23 samples, and there were low counts of *A. flavus* in 14 samples. Of 24 strains of *A. flavus* incubated *in vitro*, seven produced AF. These were isolated from whole ginger or from Jamaica red or white peppers.

3243. Is coffee a thiamine antagonist?

Somogyi, J. C. & Nägeli, U. (1976). Antithiamine effect of coffee (preliminary communication). *Int. Z. VitamForsch.* **46**, 149.

Roasted coffee contains 12–14% dry weight of chlorogenic acid, and smaller quantities of caffeic acid and other *o*-diphenols, a class of compounds known to have a pronounced antithiamine effect *in vitro* (Somogyi & Bonicke, *Int. Z. VitamForscn.* 1969, **39**, 65). The possible antithiamine effect of these constituents in man was studied in 25 subjects who were given 1 litre of coffee containing 0·2% chlorogenic acid in seven portions within 3 hr. Eight days later the same subjects received water as a control. In each experiment the urinary excretion of thiamine was measured, and in seven subjects blood concentrations of thiamine were also measured.

The decrease in thiamine excretion in urine was more rapid and considerably greater after coffee than after water. In 13 of 15 subjects, total urinary thiamine excretion was lower during the 8–10 hr after coffee consumption than after water consumption, with a mean difference of 45·5%. In a further experiment involving nine subjects studied 2 hr after the last portion of coffee or water was consumed, urinary excretion of thiamine was reduced in those imbibing coffee by a mean value of 35·8%. The thiamine content of blood, determined in some of the subjects in the first test, showed a small decrease in response to coffee consumption, but there were too few data for this result to be considered conclusive. However, the findings of this study are interpreted as an indication that the o-diphenols present in coffee do exert an antithiamine effect in man.

COSMETICS, TOILETRIES AND HOUSEHOLD PRODUCTS

3244. The price of a hair bleach

Fisher, A. A. & Dooms-Goossens, A. (1976). Persulfate hair bleach reactions: cutaneous and respiratory manifestations. *Archs Derm.* **112**, 1407.

Typical generalized urticarial skin reactions to ammonium persulphate (AP) have been described in hairdressers and their clients (*Cited in F.C.T.* 1964, **2**, 334). There have also been reports of allergic contact dermatitis from persulphates among bakers exposed to the chemical in 'flour correctors', an allergic sensitivity that has been shown to persist in some cases for at least 13 years without further exposures.

It has now been reported that the varied reactions to AP used as a booster for peroxide hair bleaches may include contact dermatitis, urticaria, rhinitis, asthma and syncope. The authors cited above present case histories of four women who were exposed to AP hair bleaches. One complained of a tingling and burning of the scalp, followed by reddening and oedema of the face, generalized urticaria and syncope. Patch tests with 2 and 5% AP in water provoked no reaction, but 5% AP solution rubbed into the skin of her forearm produced an urticarial wheal within 7 min. In another woman, rhinitis and asthma were troublesome only during the hours she worked as a hairdresser. A scratch test with 1% AP solution produced a local wheal immediately and a mild attack of asthma. A third woman who complained of burning of the scalp when a bleach was applied showed reddening and scaling of scalp and forehead skin next day. Patch testing with AP gave no reaction, however, and in this instance a primary skin irritation was thought to have resulted from too concentrated a solution of AP. The fourth woman, who worked as a hairdresser, suffered from acute eczematous dermatitis of both hands when she handled AP hair bleach. She gave a strong positive patch-test reaction to 2% AP solution.

3245. Nitrosamines from dentifrices?

Rao, G. S. & Osborn, J. C. (1977). Reaction of sodium nitrate with amino constituents of dentifrices: On formation of carcinogenic *N*-nitrososarcosine. *J. dent. Res.* **56**, 95.

Carcinogenic nitrosamines may be formed *in vivo* as a result of reaction between nitrites and secondary amines present in food and drugs (*Cited in F.C.T.* 1976, **14**, 205). The possibility has now been investigated that sodium *N*-lauroyl sarcosinate (LS), used as a detergent and antienzyme agent in dentifrices, could give rise to *N*-nitrososarcosine (NS), which has previously been shown in induce liver carcinomas in mice (*ibid* 1976, **14**, 363) and oesophageal tumours in rats (Druckrey, *Z. Krebsforsch.* 1967, **69**, 103).

When 50 mg LS was incubated at 37°C for 1 hr with 10 ml 1% sodium nitrite solution at pH 2–3, the yield of NS was in the range 2·5–4·2%. LS was then given to rats by stomach tube, alone or in combination with sodium nitrite (each at dose levels of 100 mg/kg in the form of 1% aqueous solutions) and the rats were killed 1 hr later. However, analysis of the stomach contents revealed no detectable NS. In contrast, incubation of sarcosine with nitrite *in vitro* yielded 43·7–61·4% NS, and in rats 10·5% of a sarcosine dose was converted to NS within 1 hr of co-administration with nitrite.

3246. Aftermath of alcalase exposure

Musk, A. W. & Gandevia, B. (1976). Loss of pulmonary elastic recoil in workers formerly exposed to proteolytic enzyme (alcalase) in the detergent industry. *Br. J. ind. Med.* **33**, 158.

Impaired pulmonary function has been described in men who had been occupationally exposed to

relatively high atmospheric levels of a proteolytic enzyme during the manufacture of enzyme detergents and who had developed signs of toxicity (*Cited in F.C.T.* 1976, **14**, 62). The follow-up paper cited above indicates that recovery may occur, at least partially, after termination of exposure to the enzyme, an alcalase derived from *Bacillus subtilis*.

Routine clinical and lung-function tests on 67 workers whose exposure to alcalase had ceased 3 yr before showed that 13 who had been heavily exposed still suffered significant loss of pulmonary elastic recoil, while 42 who had received only light to moderate exposure had no measurable residual disability. In the heavily exposed subjects, increased lung volume and increased pulmonary compliance were detected but no other parameters of lung function, including airways resistance, distinguished them from the mildly exposed subjects. Symptoms reported at the time of exposure had been similar in both groups, and both showed similar exercise tolerance, skin reactivity to alcalase, and trypsin-inhibitor capacity when re-examined after 3 yr. Complaints of breathlessness were associated with measurable airways obstruction but not with alterations in elastic recoil. Some subjects appeared to have recovered at least partially from the impairment of elastic recoil shown at the time of exposure.

3247. Odd alkyl sulphate metabolism

Burke, B., Olavesen, A. H., Curtis, C. G. & Powell, G. M. (1976). The biodegradation of the surfactant undecyl sulphate. *Xenobiotica* **6**, 667.

The alkyl sulphates used as detergents comprise a mixture of compounds of differing chain length. While the metabolism of those with an even number of carbons in the alkyl chain has been shown to proceed by a sequence of ω- and β-oxidation, the metabolism of analogous odd-numbered carbon-chain compounds has not been fully investigated, although preliminary studies indicated that ω- and β-oxidation were probably again involved (*Cited in F.C.T.* 1973, **11**, 346). The study cited above provides further evidence for this metabolic pathway.

Potassium undecyl [^{35}S]sulphate (UDS) was administered iv, ip or orally to rats in a dose of 5 mg/kg. Irrespective of the route of administration, most of the radioactivity was excreted in the urine, mainly as the ester sulphate (80% of the dose) and as inorganic sulphate (12%), indicating that the compound was readily absorbed from the gastro-intestinal tract.

In male rats with cannulated bile ducts and ureters, only a small amount (about 12·5%) of the iv-administered UDS (5 mg/kg) was detected in the bile, whereas in similarly treated females 14·9% of the dose was excreted by this route, a finding in agreement with previous work (*ibid* 1973, **11**, 346). On the evidence of electrophoresis, the biliary radioactivity was present mainly as unchanged UDS, together with minor amounts of inorganic sulphate. Less than 50% of the dose was recovered in the urine and bile within 6 hr, and at this time 17% was still associated with the liver. Confirmation that the administered sulphate accumulated in the liver was obtained from whole-body autoradiography of rats receiving 1 mg UDS per animal ip. Radioactivity levels in the liver were highest after 15 min and the kidney was the only other organ in which levels of radioactivity were higher than in the serum. Previous studies in the rat had indicated the importance of the liver in the metabolism of the even-numbered-carbon alkyl sulphates, but had demonstrated no biliary excretion of the 'even-carbon' compounds in either male or female rats. There was also a more rapid rate of excretion of the urinary metabolites than was found in the present experiment.

Thin-layer chromatography or electrophoresis demonstrated four radioactive metabolites in the urine of both free-ranging and cannulated animals receiving UDS. One fraction was identified as inorganic [^{35}S]sulphate and, although the other three fractions could not be totally separated, their relative proportions were observed to change radically with time. The major component, and the last of the three to appear in detectable amounts in the urine, was identified as propionic acid 3-[^{35}S]sulphate. When re-injected iv into the rat, this compound was mainly excreted unchanged in the urine, with 1·6% being converted to the inorganic sulphate. Of the other two metabolites, that appearing first in the urine was tentatively identified as pentanoic acid 5-[^{35}S]-sulphate. It was suggested that this acid could readily undergo lactonization with subsequent loss of inorganic [^{35}S]sulphate.

The observed metabolites indicated that the UDS was converted by ω-oxidation to a carboxylic acid, which then underwent sequential β-oxidation. The presence of the sulphate group did not appear to affect the catabolic breakdown of the fatty acid, except in the final stage, where chain-shortening was limited to the 3-carbon fragment, propionic acid 3-sulphate. While the earlier work on the 'even-carbon' alkyl sulphates demonstrated butyric acid 4-sulphate as the final metabolite in the rat, steric hindrance is possibly less important in other species, as glycollic acid 2-sulphate has been identified in the dog and man as a metabolite of hexadecyl sulphate (Merits, *Biochem. J.* 1975, **148**, 219).

METHOD FOR ASSESSING TOXICITY

3248. Eyewash in the rabbit irritancy test

Davies, R. E., Kynoch, S. R. & Liggett, M. P. (1976). Eye irritation tests—an assessment of the maximum delay time for remedial irrigation. *J. Soc. cosmet. Chem.* **27**, 301.

It has been suggested that irrigation of the rabbit eye after instillation of an irritant might give a result more applicable to the human situation and reduce the exaggerated response characteristic of this test organ. This possibility was investigated in a series of tests in which instillation of 0·1 ml of a 10% aqueous

solution of sodium lauryl sulphate into the rabbit eye was followed after a period varying between 4 and 120 sec by irrigation of the treated eye with either 20 or 100 ml water at 37 C. The eyes were examined 1 and 4 hr after the instillation and again at intervals between 1 and 35 days or until no further reaction was visible.

A delay of 20 sec or longer in irrigation resulted in corneal opacity or dulling of corneal lustre. A similar result was seen when irrigation was delayed for up to 2 min. Less severe and less persistent effects occurred when the time of exposure to the surfactant was less than 20 sec. Exposure for only 4 sec elicited no corneal opacity but resulted in dulling of lustre in four of 11 eyes. The critical exposure time before corneal damage is elicited by 10% sodium lauryl sulphate can therefore be taken as 4–10 sec. Although considerable conjunctival redness was caused by expsoure even for only 4 sec, recovery in this respect was quicker when the exposure to surfactant was shorter. There was no evident advantage in irrigating the eye with 100 ml water instead of 20 ml, but in relatively untrained hands adequate rinsing may be more easily effected by the use of the larger volume of water.

3249. Fishing for carcinogens

Stich, H. F. & Acton, A. B. (1976). The possible use of fish tumors in monitoring for carcinogens in the marine environment. *Prog. exp. Tumor Res.* **20**, 44.

High frequencies of skin papillomas have been reported in several fish species. These tumours could give an indication of the aquatic systems that are most seriously contaminated by carcinogens. The authors named above consicer the potential value of easily detected skin tumours in bottom-feeding fish as a monitoring device for carcinogens. If developed, this could present a much simpler method than the previously suggested use of mussels (*Citea in F.C.T.* 1977, **15**, 497).

According to the paper cited, fish species have been shown to develop tumours not only as a result of a particular combination of genes but also in response to chemical agents, physical factors and viruses. Although the tumour pathology of fish and experimental fish oncology are relatively new fields of study, the fish-tumour system has certain advantages. Large numbers of specimens from widespread locations can be obtained at relatively low cost, tumour incidence reaches epidemic proportions at times, providing adequate material for chemical or viral analysis, and hybrids and back-crosses are readily obtainable, permitting detailed genetic analysis of neoplastic transformation. Furthermore, large-scale experiments can be undertaken in hatcheries on an economic basis.

Similar virus-like particles have been observed in papillomas of the eel, goby and lemon sole, suggesting that these particles have an integral association with the neoplasms. The introduction of tissue cultures from various species of fish would aid the characterization and isolation of such virus-type particles.

Comparison of the sensitivity of fish to chemical carcinogens with the sensitivity of rodents and man must be a first priority if fish studies are to find widespread application. There is already much evidence that compounds carcinogenic to rodents are also carcinogenic to fish, and the DNA repair synthesis which indicates strand breakages of the DNA molecule is elicited in cultured fish cells by concentrations of carcinogens that also induce a comparable repair synthesis in cultured human cells. Another similarity between cultured fish cells and mammalian cells is their capacity to respond to dimethylnitrosamine only after its metabolic activation. The sensitivities of different species can be determined by comparing their capacities to activate chemical precarcinogens. Indeed fish-liver preparations are potent activators of DMNA and aflatoxin B_1. Such studies could be extended to assess the sensitivity of various fish species.

The use of bottom-dwelling fish as a built-in indicator organism for measuring the integrated effect of carcinogens or co-carcinogens is probably the most promising application of these ideas. Some flatfish, gobies and European eels seem to hold most potential, since they have high proportions of tumour-bearing fish and are relatively easy to collect and screen. That the frequency of tumours can vary with location has been demonstrated in lemon sole, which were shown to have a much higher tumour incidence in an area of urban discharge than in a similar but less polluted area.

Some complications of the fish-tumour system must be considered before it can provide a useful monitoring method. Detailed information will be needed on the developmental stage at which tumour induction occurs and on the location of the fish at this critical period. All tumour frequencies would also need to be age-adjusted to avoid the pitfall of simply comparing different age distributions within fish populations.

BIOCHEMICAL PHARMACOLOGY

3250. Liver enzyme induction and porphyrin synthesis

Ioannides, C. & Parke, D. V. (1976). The effect of allyl compounds on hepatic microsomal mixed function oxidation and porphyrogenesis. *Chemico-Biol. Interactions* **14**, 241.

We have recently reviewed the metabolism and hepatocarcinogenicity of safrole (Rostron, *Fd Cosmet. Toxicol.* 1977, **15**, 645). In the rat, this compound has been shown to stimulate the activity of several liver microsomal enzymes and to increase the synthesis of cytochrome *P*-450, the terminal component of the microsomal electron transport chain, and its 4-propenyl isomer, isosafrole, similarly increased the activity of biphenyl 2- and 4-hydroxylases in the liver (*Cited in F.C.T.* 1971, **9**, 294). Barbiturates, in addition to being potent inducers of the hepatic mixed-func-

tion oxidases (*ibid* 1971, **9**, 304), have been shown to increase the rate of prophyrin and haem synthesis (Moore *et al. Biochem. Pharmac.* 1970, **19**, 751) and to cause hepatic porphyria in animals and man (Whittaker & Whitehead, *Lancet* 1956, **i**, 543; Eales, *Anesthesiology* 1966, **27**, 703). Increased porphyrin biosynthesis has also resulted from administration of allylisopropyl-acetamide or allobarbital (Moore *et al. loc. cit.*; Abritti & De Matteis, *Chemico-Biol. Interactions* 1971/72, **4**, 281). The relationship between induction of the mixed-function oxidases and of 5-aminolaevulinate (ALA) synthetase, which controls the rate of porphyrin biosynthesis and can thus provide the haem required for cytochrome *P*-450 synthesis, has now been investigated further.

In rats killed 16 hr after a single sc dose (100 mg/kg) of safrole, isosafrole or secobarbital, there was an increase of 20–40% in ALA-synthetase activity, but this was not statistically significant. On the other hand, the same dose of alclophenac (4-allyloxy-3-chlorophenylacetic acid) or triallyl cyanurate almost doubled ALA-synthetase activity, and the known porphyrogens allylisopropylacetamide and allobarbital caused increases of 160 and 240% respectively. Three daily ip doses (75 mg/kg) of alclophenac or triallyl cyanurate produced no significant increases in liver weight, microsomal protein, cytochromes *P*-450 and b_5, NADPH–cytochrome *c* reductase, aniline hydroxylase or biphenyl 4-hydroxylase, whereas secobarbital produced a modest increase in the concentrations of the cytochromes, cytochrome *c* reductase and biphenyl 4-hydroxylase, and allobarbital gave greater increases in these and also increased the activity of *p*-nitrobenzoate reductase.

These results suggest that induction of cytochrome *P*-450 is not rate-dependent on the synthesis of haem and induction of porphyrin biosynthesis. In the case of compounds that induce the hepatic mixed-function oxidases but not ALA-synthetase activity, it seems that the haem required for the synthesis of cytochrome *P*-450 must be derived from the haem pool.

PATHOLOGY

3251. Macromolecular uptake by neonates

Martinsson, K. & Jönsson, L. (1976). The uptake of macromolecules in the ileum of piglets after intestinal "closure". *Zentbl. VetMed.* **23**, 277.

The problem of the uptake of macromolecules by the mammalian gut has important bearings on toxicology (*Cited in F.C.T.* 1977, **15**, 368). The authors of the paper cited above have previously described the mechanism of absorption of dextran blue (mean mol wt 2×10^6), in piglets less than 24 hr old, as a process of pinocytosis into the epithelial cells of the gut followed by lymphatic transmission, and have found that the second stage ceases in older piglets (*ibid* 1977, **15**, 87).

The dextran blue dye given by gastric intubation to 6-hr-old piglets was seen to be absorbed into the intestinal epithelium and to remain there for 5–7 days after the treatment. Almost no marker remained after that time. This could possibly have been due to the slow release of dye into the general circulation, but the colour of the lymph nodes gave no indication of such a release. It seems more likely that the dye remained in the mucosal cells until they were extruded into the intestinal lumen. Moon (*Proc. Soc. exp. Biol. Med.* 1971, **137**, 151) found that the complete replacement time of ileal epithelium in newborn piglets was 7–10 days which, considering the greater sensitivity of his method, roughly corresponds with the present results. In piglets treated when 8 days old, dextran blue was partially retained in the mucosa of the lower third of the intestine and was evident 1 day later, but there were considerable individual differences. In 10-day-old piglets most of the dye disappeared within the 4 days after its administration and clearance was complete in 7 days. These results are also in broad agreement with those of Moon (*loc. cit.*) who showed that the replacement time of the ileal epithelia was much shorter in 3-wk-old than in newborn piglets. In 16-day-old piglets some dye remained in the mucosa 1 day after treatment but in those 2 days older only a very small amount of dye was left in the mucosa 1 day after dosing, indicating that the absorptive capacity of the ileal epithelium is lost at about that age.

CANCER RESEARCH

3252. Urethane carcinogenesis quantified

Schmähl, D., Port, R. & Wahrendorf, J. (1977). A dose-response study on urethane carcinogenesis in rats and mice. *Int. J. Cancer* **19**, 77

Diethyl pyrocarbonate (DEPC), a preservative for wines, beer and soft drinks, reacts with ammonia to form urethane, a recognized carcinogen in several animal species. DEPC treatment produces a significant increase in the 'natural' concentration of urethane in alcoholic beverages, whilst its use in soft drinks increases the urethane content from normally undetectable levels to amounts up to 10 ppb ($b = 10^9$). DEPC is not approved for use in the UK, but in West Germany it is a permitted additive for soft drinks, although permission for its use in wine was rescinded in 1973 and further long-term studies were undertaken. These studies have now been reported.

Groups of 40 male and 40 female Sprague-Dawley rats and NMRI mice were given urethane in their drinking-water at levels providing daily doses of 0, 100, 500, 2500 and 12,5000 μg/kg body weight from 8 wk of age until their spontaneous death, except in the case of the last few survivors, which were killed.

The rats were treated for a maximum of 730 days, with the last control animal dying on day 680. The mouse experiment lasted 760 days, at which time the remaining control animals were killed.

The number of rats with malignant tumours exceeded the control incidence in the groups given 500 μg/kg/day or more and increased steadily with dose. An increase in benign tumours occurred at the two highest treatment levels. Female rats developed more malignant tumours than did males, although this may have been a consequence of their longer life. Nine of the 15 neoplasms observed at the highest dose level were mammary carcinomas, two were vascular tumours and two were neurogenic. In the mice, the number of animals with malignant and benign tumours increased steadily with dose, but there was no sex difference in the responses. Considered separately, the types of tumour showing a clear dose-dependence were lung tumours (adenomas and adenocarcinomas), malignant mammary tumours and haemangioendotheliomas (mainly in the liver).

Dose-dependence for urethane carcinogenesis was demonstrated statistically in both species. A modified Mantel-Bryan calculation indicated that doses of 0·7 and 0·14 μg urethane/kg/day, the amounts that would be ingested by a 70-kg man drinking 1 litre of liquid containing 50 or 10 ppb urethane, would produce a risk of cancer in the rat of 22 and 3·2 per 100,000, respectively. Analogous figures for the mouse were 1300 and 470 per 100,000. Although the authors stress that the susceptibility of man to urethane carcinogenesis has not been conclusively proven, similar human-risk factors cannot be discounted. They recommend, therefore, that some more acceptable alternative should be found to the use of DEPC as a preservative for soft drinks.

3253. Mutagenicity of malonaldehyde

Mukai, F. H. & Goldstein, B. D. (1976). Mutagenicity of malonaldehyde, a decomposition product of peroxidized polyunsaturated fatty acids. *Science, N.Y.* **191**, 868.

The initiating activity of malonaldehyde as a skin carcinogen has been described previously (*Cited in F.C.T.* 1976, **14**, 223). This three-carbon dialdehyde is formed during both the oxidative decomposition of polyunsaturated fatty acids and the metabolism of certain carcinogenic hydrocarbons, will cross-link the amino groups of DNA and apparently cross-links aminolipid following red-cell lipid peroxidation. Consideration of these facts has led to speculation about the mechanism by which the breakdown of cell-membrane fatty acids might damage genetic material. In the study cited above, malonaldehyde was examined for mutagenicity by the now-familiar Ames test.

Incubation of histidine-requiring auxotrophs of the bacterium *Salmonella typhimurium* with malonaldehyde increased the number of revertants in specific strains compared with the numbers in control plates. The effect was observed only on frameshift mutants with normal excision repair, strain sensitivity decreasing in the order hisD3052 > hisC207 > hisC3076. None of the base-pair substitution mutants tested showed any increase in revertant frequency.

The authors compare the observed pattern of mutagenic specificity with that of mitomycin C, a DNA cross-linking agent. It is thought that mutations produced by this antibiotic are due to errors in repair occurring as a result of excision repair-induced DNA damage. As indicated above, malonaldehyde is also a cross-linking agent, and its proclivity to react with guanine and cytidine is consistent with the expression of frameshift revertants in hisD3052 as a result of deletions of GC base-pairs.

3254. More studies of polycyclic aromatic hydrocarbon carcinogenesis

Schmidt, K. G., Schmähl, D., Misfeld, J. & Timm, J. (1976). Experimentelle Untersuchungen zur Syncarcinogenese. 7. Mitteilung: Syncarcinogene Wirkung von polycyclischen aromatischen Kohlenwasserstoffen (PAH) in Epicutantest an der Mäusehaut. *Z. Krebsforsch.* **87**, 93.

One of the gravest problems in investigating carcinogens is the impossibility of separating the interacting environmental factors that contribute to carcinogenesis in man (Schmähl, *Fd Cosmet. Toxicol.* 1977, **15**, 475). This paper reports an attempted study of the carcinogenicity of mixtures of hydrocarbons by their application to mouse skin.

Mixtures of 11 hydrocarbons, comprising the recognized carcinogens benzo[*a*]pyrene, dibenz[*a,h*]-anthracene, benz[*a*]anthracene and benzo[*b*]fluoranthene, as well as phenanthrene, anthracene, fluoranthene, pyrene, chrysene, benzo[*e*]pyrene and benzo[*g,h,i*]perylene, and mixtures of more restricted groups of these hydrocarbons, were applied in acetone solution to the shaven skin of female NMRI mice twice weekly in a life-time study. Some mice were treated with the entire mixture of carcinogens plus non-carcinogens, mixed in the proportions found in tobacco-smoke condensate, in automobile exhaust fumes and in food-curing smokes, and others with the carcinogen or non-carcinogen groups alone. Some tumours were induced at the application site by the mixtures of supposedly non-carcinogenic hydrocarbons. No inhibition of the tumour-inducing ability of the carcinogenic group was apparent when carcinogens and non-carcinogens were applied together; rather there appeared to be an additive effect. The numbers of animals with tumours were dose-related, and the types of tumour, mainly squamous-cell carcinomas, were similar in all those groups receiving carcinogen treatment, irrespective of the inclusion of non-carcinogens in the application.

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LETTER TO THE EDITOR

ABSENCE OF ACTIVITY OF AMARANTH (FD & C RED NO. 2) IN THE SALMONELLA/MICROSOME MUTAGENICITY TEST*

Sir,—Many countries have severely curtailed the use of amaranth (the trisodium salt of 3-hydroxy-4[(4-sulpho-1-naphthalenyl)azo]-2,7-naphthalenedisulphonic acid) as a colouring agent for foods, because of a publication by Andrianova (*Vop. Pitan.* 1970, **29** (5), 61) reporting the appearance of a wide range of tumours in amaranth-fed rats. It should be noted, however, that there is conflicting evidence about this point, as noted in an IARC report (*Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man.* Vol. 8, p. 41; International Agency for Research on Cancer, Lyon, 1975). As many pharmaceutical formulations still contain this dye, we thought it worthwhile to assay it for mutagenic potency, using the procedure described by Ames *et al.* (*Proc. natn. Acad. Sci., U.S.A.* 1973, **70**, 2281) and McCann *et al.* (*ibid* 1975, **72**, 979).

Amaranth was bought from R.A.L. (Pharmuka, France) or from Merck AG (Darmstadt, Germany). Samples were checked for purity by thin-layer chromatography on cellulose plates with ammoniacal sodium citrate buffer as solvent, and were free from aromatic amines (Sté Chimique Pointet-Girard, personal communication 1977). Both samples migrated as single spots and were indistinguishable in subsequent experiments.

Salmonella of strains TA98, TA100 and TA1538 were a gift from Dr. B. N. Ames (Berkeley, CA). These strains are histidine-requiring mutants, which can be reverted to prototrophy by different chemical mutagens. The procedure used was essentially that described by Ames *et al.* (*Mutation Res.* 1975, **31**, 347). Briefly, 0·1 ml of a log-phase bacterial culture was overlaid in 2·5 ml soft agar on minimal-medium Petri plates. The top agar contained a limiting amount of histidine and biotin, to enable the deficient bacteria to perform a few rounds of replication, in order to allow expression of mutagenesis. It also contained, where appropriate, a 9000-**g** supernatant of a homogenate of rat liver induced with Aroclor®, with NADP and glucose 6-phosphate as cofactors. Control plates exhibit a small number of spontaneous revertant colonies, while bacteria exposed to mutagens, having overcome the histidine requirement, show up as a numerically dose-dependent population. Controls are run in this type of study to check the phenotypic characteristics of the strains as well as to establish the spontaneous mutation rate (to be subtracted from plate counts), and positive controls using known mutagens are also included. Livers from Sprague–Dawley male rats weighing 200 ± 20 g were used for the metabolic-activation experiments in the study described here. An ip injection of 500 mg Aroclor 1254/kg (a gift from Monsanto Co. St. Louis, MO) in olive oil was used to induce hepatic monooxygenases; the rats were killed 5 days later, their livers were removed and hepatic microsomes were prepared as described previously (Lesca *et al. C.r. hebd. Séanc. Acad. Sci., Paris* 1976 **282D**, 1457).

No significant increase in the number of revertant colonies could be induced by amaranth in a dose range of 5 μg–2 mg/plate. (Higher concentrations produced a decrease in the number of revertants.) The threshold of significance is taken as twice the number of spontaneous revertants (about 20 for TA1538, 30 for TA98, and 140 for TA100). The control mutagens were methyl methanesulphonate for TA100 (with an activity of *c.* 0·5 revertants/nmol) and 2-nitrofluorene for TA98 and TA1538 (*c.* 50 and 40 revertants/nmol). Other controls were set up with benzo[a]pyrene, to check the activity of the microsomal preparations. Under the same conditions, we found that 5 μg benzopyrene/plate gave rise to about 2200 revertants. These experiments were repeated at least twice, with and without the rat-liver preparation.

It should be noted that the range of mutagenic potencies in Ames' test is roughly 10^{-2}–10^{+4} revertants/nmol (McCann & Ames, *Proc. natn. Acad. Sci., U.S.A.* 1976, **73**, 950). It follows that our results are not biased by comparison with inordinately potent mutagens.

Finally, we performed a spectral interaction study of amaranth with liver microsomes from Aroclor-pretreated rats, to ascertain whether such interaction contradicted the bacterial experiments. The microsomal cytochrome *P*-448 content was determined as described by Omura & Sato (*J. biol. Chem.* 1964, **239**, 2379). Difference spectra were recorded in 1-cm cuvettes containing 3 ml of a microsomal suspension (0·8 mg protein/ml, 2·4 nmol cytochrome *P*-448/mg protein) in 50 mM-tris HCl, 3 mM-$MgCl_2$ and 200 mM-sucrose buffer, pH 7.5. Upon addition of 10 μl of a 2 mM aqueous solution of amaranth to a suspension of oxidized microsomes, no difference spectrum occurred. This finding indicates that the product does not interact with the hydrophobic catalytic site of cytochrome *P*-448, and is consistent with the highly hydrophilic character of amaranth, and with

*Received 31 May 1977; revised version 1 August 1977.

our results showing a lack of mutagenicity in the presence (or absence) of a monooxygenase-dependent activation system.

The working group of IARC (*loc. cit.*) has reviewed ten studies on amaranth tumorigenicity, nine of them being negative. However, it has withheld its conclusion, owing to the paucity of data given in most of these reports. We are aware that the present results will not settle the debate, and only wish to make two comments. First, the Salmonella/microsome test, when applicable, has a record of about 90% reliability in the detection of carcinogens (McCann & Ames, *loc. cit.*) and our results support the view that amaranth is a dubious carcinogen, at least when assayed in a chemically pure form, a critical point about which no details were given by Andrianova (*loc. cit.*). Secondly, the studies of Roxon *et al.* (*Fd Cosmet. Toxicol.* 1967, **5**, 367) and Ryan *et al.* (*J. Pharm. Pharmac.* 1968, **13**, 492) show that amaranth undergoes reductive cleavage at the N=N double bond through the action of intestinal bacteria. The present work does not eliminate the possibility that some reactive intermediate could arise during this reaction and be reabsorbed from the gut into the host's bloodstream. We suggest that a modification of the Salmonella test ought to be devised, to assay the formation of mutagenic intermediates during the metabolism of drugs by the intestinal flora.

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ANNOUNCEMENT

INDUSTRIAL TOXICOLOGY TRAINING IN FINLAND

Following two earlier sessions in 1974 and 1977, the Institute of Occupational Health, Helsinki, is arranging a third advanced course in industrial toxicology. To be held from 7 to 12 August 1978 in the Institute's premises in Haartmaninkatu, Helsinki, this course is being sponsored by the Nordic Council of Ministers, and consequently Scandinavian participants will be given some priority and financial aid, although a limited number of research workers from other countries will be accepted. The course fee will be US $50 for Scandinavian participants and US $250 for others, and the deadline for applications is 1 May 1978.

The curriculum is scheduled to cover the following topics: concepts and definitions in industrial toxicology, exposure to chemicals via inhalation and skin, metabolism of industrial chemicals, active intermediates and tissue damage, chemical carcinogenesis, mutagenesis and teratogenesis, embryotoxic and teratogenic effects of industrial chemicals, metal toxicology and carcinogenicity, occupational metal exposure, neurotoxic syndromes in man, neurophysiological aspects in acute and chronic neurotoxicity, occupational lung and skin diseases, modern toxicity testing and the concept of TLV-MAC.

Further details may be obtained from Dr. Harri Vainio, Department of Industrial Hygiene and Toxicology, Institute of Occupational Health, Haartmaninkatu 1, SF-00290 Helsinki 29, Finland; telephone: 413 622.

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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*:

Saccharin: A possible example of an epigenetic carcinogen/mutagen. By J. Ashby, J. A. Styles, D. Anderson and D. Paton.

The thermal energy analysis of sodium saccharin. By I. S. Krull, U. Goff, M. Wolf, D. H. Fine and G. P. Arsenault.

Effet d'une charge en nitrate sur le nitrite salivaire et gastrique chez l'homme. Par D. Klein, N. Gaconnet, B. Poullain et G. Debry.

A sensitive method for the colorimetric determination of volatile nitrosamines in food products and air. By L. Čeh and F. Ender.

Early changes of cardiac function in rats on a high-fat diet. By G. Zbinden and B. Rageth.

Glucose tolerance and hyperkinesis. By L. Langseth and J. Dowd.

The suitability of simulants for foodstuffs, cosmetics and pharmaceutical products in migration studies. By K. Figge, J. Koch and W. Freytag.

High incidence of hepatic tumours in rats fed mouldy rice contaminated with *Aspergillus versicolor* containing sterigmatocystin. By K. Ohtsubo, M. Saito, H. Kimura and O. Tsuruta.

Effect of dietary indole-3-carbinol on the induction of the mixed-function oxidases of rat tissue. By J. G. Babish and G. S. Stoewsand.

On the aetiology of scombroid poisoning: Cadaverine potentiation of histamine toxicity in the guinea-pig. By L. F. Bjeldanes, D. E. Schutz and M. M. Morris.

Studies on the absorption and disposition of ^{3}H-labelled talc in the rat, mouse, guinea-pig and rabbit. By J. C. Phillips, P. J. Young, K. Hardy and S. D. Gangolli.

Problems involved in and a comparison of methods for the determination of total migration from packaging materials into fatty foods. By K. Figge, D. Cmelka and J. Koch. (Review paper)

Contribution a l'étude de la méthodologie d'évaluation de la toxicité cutanée. I. Toxicité percutanée —tests d'irritation. Par H. Dutertre-Catella, Nguyen Phu-lich, R. Truhaut et G. K. Dossou. (Review paper)

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CORRIGENDUM

Volume 15 (1977)

p. 492. line 2: *For* Schaumberg *read* Schaumburg.

[*Contents continued*]

Aims and Scope

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

Instructions to Authors

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

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

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

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

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

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

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

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

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

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

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

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

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Other Nomenclature, Symbols and Abbreviations. In general, authors should follow the recommendations published in the *Handbook for Chemical Society Authors* (1961), p. 164 and in the *I.U.P.A.C. Information Bulletin*, No. 13, p. 64, Appendix B (1961) In the title and summary, abbreviations should be avoided; in the Introduction, Results and Discussion they should be used sparingly.

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