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RESEARCH SECTION



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Fd Cosmet. Toxicol. Vol. 19, pp. 1 to 6

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*Research Section*

# THE METABOLIC FATE OF [$^{14}$C]COUMARIN IN BABOONS

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(*Received 24 June 1980*)

**Abstract**—Oral doses of [$^{14}$C]coumarin (4 mg/kg) were rapidly absorbed and eliminated by baboons. More than 90% of the dose appeared to be absorbed within 0·75 hr of administration. Mean plasma concentrations of radioactivity were highest (5·5 μg equivalents/ml) at 0·75 hr and declined biphasically with half-lives of about 0·7 and 24 hr, respectively. The former corresponds to the elimination half-life since a mean of more than 76% of the dose was excreted in the urine in 6 hr (81% in 24 hr). Urinary radioactivity (0–6 hr) mainly represented free and conjugated 7-hydroxycoumarin (accounting respectively for 14 and 49% of the dose). Less than 1% of the dose was excreted as *o*-hydroxyphenylacetic acid. Concentrations of radioactivity in most tissues were lower than those in blood and were only relatively high in excretory organs and fluids, such as the kidneys and bile. The disposition of [$^{14}$C]coumarin was unaffected by its co-administration with the flavonoid troxerutin (24 mg/kg).

## INTRODUCTION

The naturally occurring coumarin (2*H*-1-benzopyran-2-one) was first isolated from the tonka bean (*Dipteryx odorata*) more than 100 yr ago. It has since been used in the food and tobacco industries as a flavouring agent and also in cosmetics and toiletries (for reviews see Cohen, 1979; Feuer, 1974).

Although coumarin is the parent compound of the coumarin-type anticoagulants, it hardly possesses any such activity (Feuer, 1974). However, studies of coumarin administered alone or in combination with the flavonoid troxerutin have indicated that it is useful in increasing the flow of capillary blood (Kovách, Hamar, Dora, Marton, Kunos & Kun, 1970) and lymph (Casley-Smith, Földi-Börcsök & Földi, 1973), in reducing oedema (Casley-Smith, 1976) and in assisting in the repair of damaged blood-vessel walls (Hladovec, 1977).

Coumarin has been reported to be hepatotoxic to rats and dogs (Hagen, Hansen, Fitzhugh, Jenner, Jones, Taylor, Long, Nelson & Brouwer, 1967; Hazelton, Tusing, Zeitlin, Thiessen & Murer, 1956). However, the claim of Griepentrog (1973) that coumarin induced carcinomas in rats has been questioned (see Cohen, 1979).

The metabolism of coumarin has been studied in several laboratory animal species (for reviews see Cohen, 1979; Feuer, 1974) and rather extensively in man (Ritschel, Brady, Tan, Hoffmann, Yiu & Grummich, 1977; Shilling, Crampton & Longland, 1969). Metabolic data for the baboon have been reported only briefly (Gangolli, Shilling, Grasso & Gaunt, 1974), although an extensive toxicity study has been carried out in this species (Evans, Gaunt & Lake, 1979).

The objectives of the studies reported in this paper were to obtain more data on the fate of coumarin in the baboon and to determine whether the disposition of coumarin was affected when it was administered in combination with troxerutin, since such a combination is in therapeutic use.

## EXPERIMENTAL

*Materials.* [$^{14}$C]Coumarin (1,2-[3-$^{14}$C]benzopyrone) of specific activity 5·5 mCi/mmol was obtained from the Radiochemical Centre, Amersham, Bucks. Non-radioactive coumarin and troxerutin (7,3',4'-tris-[*O*-(2-hydroxyethyl)]rutin) were supplied by Schaper and Brümmer, Salzgitter (Ringelheim), FRG. *o*-Hydroxyphenylacetic acid, 7-hydroxycoumarin and β-glucuronidase (type H2) were obtained from Sigma Chemical Co., Poole, Dorset.

*Animals.* Adult male baboons (*Papio hamadryas*), weighing 5·6–6·6 kg and originally obtained from their natural habitat, were maintained on a complete dry diet. Drinking-water was available *ad lib.* and was supplemented at weekly intervals with blackcurrant juice and vitamin $B_{12}$. The former supplement was withheld during the experimental period because of its probable flavonoid content. The animals were housed in stainless-steel metabolism cages that permitted the separate collection of urine and faeces. Excreta were collected for 3 days after dosing.

*Dosing.* [$^{14}$C]Coumarin of adequate specific activity (1·67 μCi/mg) was prepared by co-crystallizing the radioactive and non-radioactive compound. The required [$^{14}$C]coumarin had a radiochemical purity exceeding 99% as shown by thin-layer chromatography in three solvent systems. [$^{14}$C]Coumarin (4 mg/kg) alone or in combination (1:6, w/w) with troxerutin (24 mg/kg) was administered in single doses in gelatin capsules.

*Measurement of radioactivity.* The radioactivity content of biological samples was measured by procedures described previously (Elsom, Chasseaud, Ray & Hawkins, 1979).

*Thin-layer chromatography.* Thin-layer chromatography (TLC) was carried out using prelayered silica-gel 60 $F_{254}$ plates of layer thickness 0·25 mm (E. Merck AG, Darmstadt, FRG) developed in the following solvent systems: (a) benzene–ethanol (9:1, v/v) and (b) toluene–ethyl acetate–acetic acid (5:4:1, by vol.). Radioactive areas of the silica-gel plate were

located by using either autoradiography on Kodak Kodirex X-ray film or a Berthold LB 2722 radiochromatogram scanner (Camlab Ltd., Cambridge). Areas of silica-gel were removed for measurement of radioactivity (Chasseaud, Hawkins, Cameron, Fry & Saggers, 1972).

*Isolation of radioactive components from urine.* Portions (5–10 ml) of urine samples collected during 0–6 and 6–24 hr after dosing from each animal were separately evaporated to dryness at 40°C under reduced pressure. The residues were triturated with a small volume of methanol and more than 95% of the radioactivity was recovered in the methanol extracts. These methanol extracts were evaporated to dryness and the residue was dissolved in distilled water. Portions of this aqueous extract were diluted with 1 M-acetate buffer, pH 3·8, to which β-glucuronidase (20,000 units/ml, containing some sulphatase activity) was added. The samples were incubated at 37°C for 16 hr and then evaporated to dryness. The residues were dissolved in methanol and portions of the methanol extracts were transferred quantitatively to TLC plates. Incubation of urine samples with acetate buffer alone did not appear to have any effect on the chromatographic pattern of metabolites.

*Mass spectrometry.* Mass spectra of the major radioactive component isolated from urine and of authentic 7-hydroxycoumarin were recorded on a Micromass 16F Spectrometer (V.G. Organic Ltd., Altrincham, Cheshire). Samples were introduced into the spectrometer by means of a direct insertion probe, and electron-impact spectra were obtained at an electron-beam energy of 70 eV, a trap current of 100 μA, a source temperature of 180°C and a chamber temperature of 100°C.

## RESULTS

### *Excretion*

After administration of a single 4-mg/kg dose of [$^{14}$C]coumarin to three baboons, the radioactivity was well absorbed and rapidly excreted. A mean (± 1 SD) of 76·5 ± 5·3% of the dose was excreted in the urine during 6 hr and a further 5·0 ± 2·6% thereafter up to 72 hr. A mean of 3·4 ± 0·4% was excreted in the faeces.

### *Plasma concentrations*

Peak mean concentrations of radioactivity of 5·5 μg equivalents/ml occurred at 0·75 hr and declined with a half-life of about 0·7 hr (Fig. 1) when measured up to 5 hr after dosing (Fig. 1). In view of the rapid urinary excretion of radioactivity, this half-life probably represented the elimination phase. However, inspection of the plasma concentration–time curve (Fig. 1) shows that a slower 'terminal' phase was present (half-life about 24 hr). This probably represented a slow distribution phase.

### *Urinary metabolites*

Radioactivity in extracts of urine following the administration of single oral doses of [$^{14}$C]coumarin could be resolved by TLC into several radioactive components. Most of the radioactivity, however, was associated with two components. One of these was chromatographically similar to 7-hydroxycoumarin,

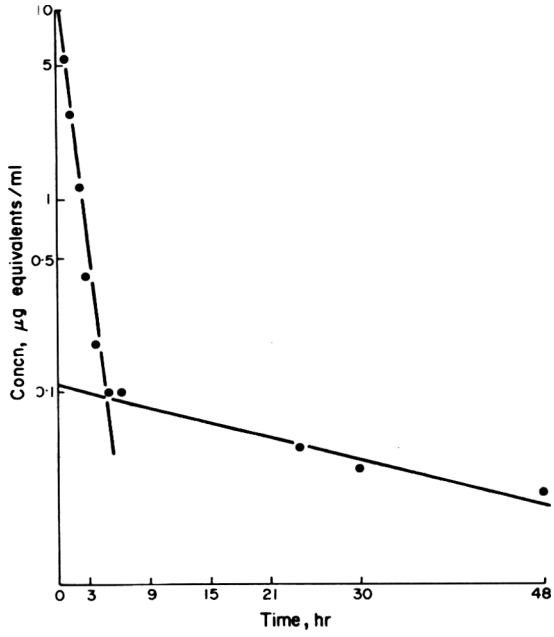


Fig. 1. Mean plasma concentrations of radioactivity following administration of a single oral dose of [$^{14}$C]coumarin (4 mg/kg) to baboons.

and exhibited fluorescence properties similar to those of 7-hydroxycoumarin when exposed to ultraviolet radiation at 360 nm. The second major component was present at the origin and presumably represented a more polar conjugate. In urine extracts treated with β-glucuronidase/sulphatase, the component at the origin decreased in amount and the component corresponding to 7-hydroxycoumarin increased accordingly (Fig. 2). After treatment of the urine

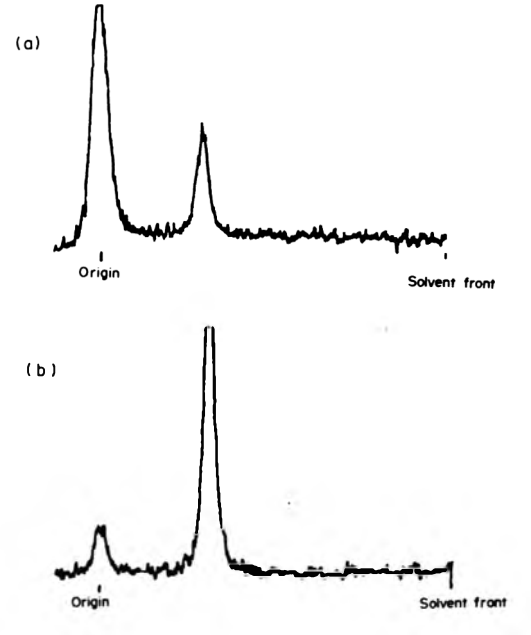


Fig. 2. Radiochromatograms of an extract of 0–6-hr urine from a baboon given a single oral dose of 4 mg [$^{14}$C]coumarin/kg: (a) before and (b) after treatment of the extract with β-glucuronidase/sulphatase.

Table 1. *Metabolites of [$^{14}$C]coumarin in the 0–6-hr urines of baboons given a single oral dose of [$^{14}$C]coumarin (4 mg/kg) alone or mixed with troxerutin (24 mg/kg)*

| Metabolite | Proportions (%*) of coumarin metabolites | | | | |
|---|---|---|---|---|---|
| | In urine of baboon no. | | | Mean ± 1 SD | |
| | 1 | 2 | 3 | As % of urinary radioactivity | As % of dose |
| **After [$^{14}$C]coumarin administration** | | | | | |
| 7-Hydroxycoumarin (free) | 21·9 | 16·4 | 18·0 | 18·8 ± 2·8 | *14·4* |
| 7-Hydroxycoumarin conjugate | 61·7 | 68·0 | 63·5 | 64·4 ± 10·9 | *49·2* |
| Total 7-hydroxycoumarin† | *68·7* | *60·4* | *61·8* | | *63·6 ± 4·4* |
| *o*-Hydroxyphenylacetic acid | 1·4 | 1·6 | 0·3 | 1·1 ± 0·7 | |
| | *1·13* | *1·11* | *0·25* | | *0·8 ± 0·5* |
| **After [$^{14}$C]coumarin + troxerutin administration** | | | | | |
| 7-Hydroxycoumarin (free) | 54·0 | 38·7 | 16·7 | 36·5 ± 18·9 | *28·1* |
| 7-Hydroxycoumarin conjugate | 39·0 | 51·2 | 67·8 | 52·7 ± 14·6 | *40·6* |
| Total 7-Hydroxycoumarin† | *66·9* | *74·5* | *64·4* | | *68·6 ± 5·3* |
| *o*-Hydroxyphenylacetic acid | 0·2 | 0·2 | 0·1 | 0·2 ± 0·1 | |
| | *0·17* | *0·17* | *0·08* | | *0·14 ± 0·05* |

*Values expressed as a percentage of the administered dose are printed in italics; other results are expressed as a percentage of urinary radioactivity.

†Total 7-hydroxycoumarin determined by TLC in the 6–24-hr urine samples was 2·8 and 4·8% for the coumarin-treated and coumarin + troxerutin-treated groups respectively.

extracts with β-glucuronidase/sulphatase, 83% of the urinary radioactivity (64% of the dose) was associated with 7-hydroxycoumarin (Table 1).

A radioactive component chromatographically similar, after two-dimensional TLC, to *o*-hydroxyphenylacetic acid accounted for only a small proportion of the dose (Table 1). No unchanged [$^{14}$C]coumarin was detected in any urine sample.

The identity of the major urinary metabolite, 7-hydroxycoumarin was confirmed by mass spectroscopy (Fig. 3), the only notable fragmentation being the successive loss of CO (*m/e* 134) + CHO (*m/e* 105) units from the molecular ion ($M^+$, *m/e* 162).

*Disposition of [$^{14}$C]coumarin administered together with troxerutin*

The data presented in Tables 1 & 2 show that the concomitant administration of the flavonoid troxerutin with [$^{14}$C]coumarin, did not affect the disposition of the latter.

*Tissue distribution*

In view of the foregoing results, this study was carried out after administration of [$^{14}$C]coumarin (4 mg/kg) with troxerutin (24 mg/kg). At 0·75 hr after dosing, only about 6% of the administered dose remained in the gastro-intestinal tract, indicating that absorption of [$^{14}$C]coumarin was extremely rapid. Other tissues, except the kidneys, contained lower concentrations than the blood, indicating that there was little tissue uptake of [$^{14}$C]coumarin and/or its metabolites (Table 3). Elimination of radioactivity from some tissues was slower than from blood, and at 4 and 24 hr after dosing these tissues (e.g. liver and

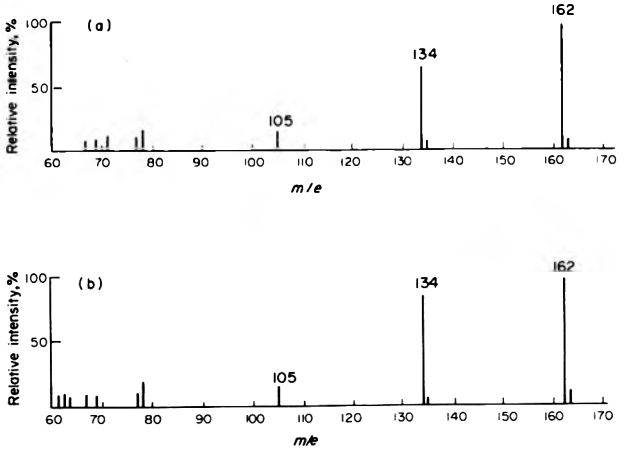


Fig. 3. Mass spectra of (a) the major metabolite isolated from the urine of baboons given a single oral dose of 4 mg [$^{14}$C]coumarin/kg and (b) authentic 7-hydroxycoumarin.

Table 2. *Pharmacokinetic parameters of radioactivity after single oral doses of [$^{14}$C]-coumarin (4 mg/kg) or [$^{14}$C]coumarin admixed with troxerutin (24 mg/kg)*

| Parameter | Values for baboons dosed with [$^{14}$C]Coumarin | Values for baboons dosed with [$^{14}$C]Coumarin + troxerutin |
|---|---|---|
| Peak concn of radioactivity in plasma (μg/ml) | 5·5 | 5·8 |
| Time of peak concn (hr) | 0·75 | 0·75 |
| Plasma half-life (hr) | 0·69 | 0·73 |
| Excretion (% of dose) | | |
| In urine 0–6 hr | 76·5 | 77·0 |
| 0–3 days | 81·6 | 83·6 |
| In faeces 0–3 days | 3·4 | 2·6 |

lungs) contained higher concentrations than did the blood.

Concentrations of radioactivity in bile were relatively high (Table 3) and some ratios of bile:blood concentrations exceed 1000, suggesting that some of the dose was eliminated by this route. However, in view of the rapid elimination of coumarin in the baboon, enterohepatic circulation is unlikely to be an important feature of coumarin disposition in this species.

The measured tissues, including the gut but excluding bile and urine, were calculated to contain about 23% of the dose at 0·75 hr, 3·9% at 4 hr and 1·0% at 24 hr, indicating again that coumarin was rapidly eliminated in the baboon.

## DISCUSSION

The large proportion of the dose of [$^{14}$C]coumarin eliminated in the urine of baboons (*c.* 81·6%) was similar to that (*c.* 82%) reported for man (Shilling *et al.* 1969). The rate of excretion was greatest during the first 6 hr after dosing in baboons, but comparable data have not been reported for man (Shilling *et al.* 1969).

Much (*c.* 66%) of an oral dose of [$^{14}$C]coumarin

Table 3. *Distribution of radioactivity in the tissues of baboons following a single dose of [$^{14}$C]-coumarin (4 mg/kg) mixed with troxerutin (24 mg/kg)*

| Tissue | Distribution of radioactivity, 0·75, 4 and 24 hr after dosing: 0·75 hr ng/g* | 0·75 hr % * | 4 hr ng/g* | 4 hr % * | 24 hr ng/g* | 24 hr % * |
|---|---|---|---|---|---|---|
| Liver | 3696 | 2·21 | 361 | 0·19 | 201 | 0·12 |
| Lungs | 2144 | 0·36 | 211 | 0·03 | 41 | 0·006 |
| Kidneys | 12681 | 1·13 | 883 | 0·10 | 166 | 0·02 |
| Heart | 561 | 0·06 | 86 | 0·01 | 23 | 0·003 |
| Adrenals | 713 | 0·002 | 66 | 0·0003 | 60 | 0·0002 |
| Spleen | 755 | 0·04 | 47 | 0·003 | 26 | 0·001 |
| Thymus | 541 | 0·03 | 40 | 0·002 | 10 | 0·0003 |
| Thyroid | 655 | 0·002 | 34 | 0·0001 | 15 | 0·0001 |
| Testes | 802 | 0·004 | 61 | 0·0003 | 19 | 0·0001 |
| Eyes | 320 | 0·01 | 110 | 0·004 | 82 | 0·002 |
| Brain | 46 | 0·03 | 16 | 0·01 | 110 | 0·06 |
| Pancreas | 798 | 0·04 | 38 | 0·002 | 27 | 0·001 |
| Stomach | 5705 | 1·24 | 130 | 0·03 | 61 | 0·014 |
| Small intestine | 741 | 0·41 | 1130 | 0·65 | 46 | 0·03 |
| Large intestine | 429 | 0·22 | 223 | 0·13 | 64 | 0·05 |
| Lymph nodes† | 787 | 0·20 | 38 | 0·01 | 12 | 0·003 |
| Muscle† | 232 | 2·50 | 28 | 0·30 | 9 | 0·09 |
| Fat† | 400 | 1·40 | 84 | 0·30 | ND | ND |
| Blood† | 4192 | 8·06 | 99 | 0·19 | 20·3 | 0·04 |
| Bile | 422982 | — | 175267 | — | 26964 | — |
| Intestinal contents | — | 5·61 | — | 1·92 | — | 0·58 |
| Stomach contents | — | 4·43 | — | 0·06 | — | 0·03 |

ND = Radioactivity at or below twice the background

*Results are expressed as ng equivalents/g tissue (specific activity 12,907 dpm/μg) and as the percentage of the dose present in each organ or tissue.

†These values were calculated assuming that lymph nodes, muscle, fat and blood represented 1, 42·3, 13·8 and 7·5% of the body weight, respectively.

was eliminated in the urine of baboons as free and conjugated 7-hydroxycoumarin. This finding is similar to that (*c.* 60%) reported in an earlier study (Gangolli *et al.* 1974). Humans may excrete a slightly greater proportion of the dose (*c.* 79%) in this form (Shilling *et al.* 1969), but these small differences may merely be the consequence of the use of different methods in the various studies. Certainly, rodents excrete very much less of a dose of coumarin as the 7-hydroxy metabolite (Gangolli *et al.* 1974; Kaighen & Williams, 1961).

The production of the metabolite *o*-hydroxyphenylacetic acid has been associated with the hepatotoxic potential of coumarin in certain animal species (see review by Cohen, 1979). This compound is a minor metabolite in man (*c.* 2·8% of the dose; Shilling *et al.* 1969) and in baboons (< 1% of the dose; Table 1).

Ritschel (1978) and co-workers (Ritschel, Brady & Tan, 1979; Ritschel *et al.* 1977; Ritschel, Hoffmann, Tan & Sanders, 1976) carried out detailed studies of coumarin pharmacokinetics in man. They reported that coumarin was subjected to extensive first-pass metabolism after oral doses and that the derivatives in the plasma consisted primarily of 7-hydroxycoumarin glucuronide. The proportions of coumarin and free 7-hydroxycoumarin appeared to be about 5 and 1% respectively of that of 7-hydroxycoumarin glucuronide. A similar distribution of coumarin and its metabolites could well occur in baboon plasma, but this aspect was not examined in this study. Active tubular secretion of 7-hydroxycoumarin glucuronide has been claimed to occur in man (Ritschel *et al.* 1977) and such a mechanism would need to be evoked to account for the rapid excretion of this metabolite in baboons.

The metabolism of coumarin in laboratory animal species exemplifies species differences (Cohen, 1979; Gangolli *et al.* 1974). Notable differences in the production of the various coumarin metabolites occur between certain species. Rats, for example, excrete about 1% of a dose of coumarin as 7-hydroxycoumarin and about 20% as *o*-hydroxyphenylacetic acid (Kaighen & Williams, 1961). The corresponding figures in the rabbit are about 12 and 20%, respectively (Kaighen & Williams, 1961) and dogs excrete only about 10% of a dose of coumarin in the urine as the 7-hydroxy metabolite (Gangolli *et al.* 1974; Ritschel & Grummich, 1980). Strain differences in coumarin metabolism have been reported in mice. Coumarin 7-hydroxylase activity (Wood & Conney, 1974) and 7-hydroxycoumarin excretion (Lush & Arnold, 1975) have been shown to be greater in the DBA/2J strain of mice than in other strains. The chicken is another species that eliminates coumarin mainly as conjugated 7-hydroxycoumarin (Cacini & Ritschel, 1979).

Thus, as has been stressed elsewhere (Cohen, 1979; Evans *et al.* 1979; Gangolli *et al.* 1974), the baboon metabolizes coumarin in a very similar fashion to man, and for this reason the baboon must be considered the species of choice for toxicity studies of coumarin, the results of which could then be more confidently extrapolated to man than those obtained in species such as rodents.

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Fd Cosmet. Toxicol. Vol. 19, pp. 7 to 11

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# STUDIES ON THE METABOLISM OF CALCIUM STEAROYL-2-LACTYLATE IN THE RAT, MOUSE, GUINEA-PIG AND MAN

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**Abstract**—The absorption, metabolism, tissue distribution and excretion of $^{14}C$-labelled calcium stearoyl-2-lactylate (CSL) was compared with that of [U-$^{14}C$]lactic acid in the mouse and the guinea-pig. A comparison was also made of the rates of hydrolysis of CSL by various tissue preparations from the rat, mouse, guinea-pig and man. In both mice and guinea-pigs, the metabolism and tissue distribution of radioactivity from [U-$^{14}C$]lactate-labelled CSL were similar to those of an equivalent dose of free [U-$^{14}C$]lactate, with the majority of the radioactivity rapidly excreted as $^{14}CO_2$. Homogenates of liver and intestinal mucosa from the rat, mouse and guinea-pig rapidly hydrolysed CSL to lactic acid and stearic acid, and whole blood from rats and mice also hydrolysed the compound, but at a much slower rate. Although no significant hydrolysis of CSL was detected using human blood, the single sample of human duodenal mucosa rapidly hydrolysed the compound to stearic and lactic acids. These studies suggest that the biological fate of CSL is similar in all the species investigated and that the compound is unlikely to present a hazard to man in terms of its metabolic fate.

## INTRODUCTION

Calcium stearoyl-2-lactylate (CSL) is used as a dough-conditioner in yeast-leavened bakery products, and as an emulsifier by the cosmetic and pharmaceutical industries. The material was evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA; 1974) which established an acceptable daily intake (ADI) for man of 20 mg/kg body weight.

The metabolism of CSL has been studied in the rat by H. C. Hodge (unpublished data 1955, cited by JECFA, 1974), who showed that there was no difference in the distribution and excretion of radioactivity from groups of rats given either CSL labelled with [$^{14}C$]lactic acid or an equivalent mixture of stearic acid and [$^{14}C$]lactic acid. Furthermore, it was shown that *in vitro* hydrolysis of CSL with lipase proceeded readily to form stearic and lactic acids (H. C. Hodge, unpublished data 1961, cited by JECFA, 1974).

The results presented in this report compare the *in vivo* metabolism of [U-$^{14}C$]lactic acid with that of CSL labelled with [U-$^{14}C$]lactic acid, in the mouse and guinea-pig. A comparison has also been made *in vitro* of the rates of hydrolysis of CSL by gastrointestinal mucosal homogenate of the rat, mouse, guinea-pig and man, by liver homogenate of the rat, mouse and guinea-pig and by whole blood from the rat, mouse and man.

## EXPERIMENTAL

*Materials.* Calcium stearoyl-2-[U-$^{14}C$]lactylate ([$^{14}C$]CSL) was supplied by A/S Grindstedverket, Denmark. The material had a specific activity of 2·01 mCi/mmol. D- and L-[U-$^{14}C$]lactic acid, sodium salts, were obtained from the Radiochemical Centre Ltd., Amersham, Bucks., and had specific activities of 21 and 100 mCi/mmol, respectively. Unlabelled CSL was supplied by Food Industries Limited, Bromborough Port, Merseyside, and complied with The Emulsifiers and Stabilisers in Food Regulations 1975 (Statutory Instrument 1975, no. 1486). Precoated Cellulose MN 300 thin-layer plates were obtained from Camlab Ltd., Cambridge, and precoated silica-gel GFHL plates from Anachem Ltd., Luton, Beds.

*Animals and dosing.* The studies were carried out using male Wistar albino rats (120–150 g body weight), male Tuck TO strain mice (30–50 g body weight) and male Dunkin–Hartley guinea-pigs (250–350 g body weight), all supplied by OLAC (1976) Ltd., Bicester, Oxon. The rats and mice were maintained on Spratts' Laboratory Diet No. 1 and the guinea-pigs on Oxoid Diet SG1 with a vitamin C supplement in the drinking-water. All animals were given food and water *ad lib.* and kept at 20 ± 1°C. For *in vivo* experiments, [$^{14}C$]CSL was administered in aqueous suspension by oral gavage at a dose level of either 90 or 900 mg/kg, and [$^{14}C$]lactate by oral gavage as an aqueous solution, equimolar with respect to D- and L-lactate, at a dose level equivalent to 900 mg CSL/kg. For *in vitro* hydrolysis studies, [$^{14}C$]CSL was dissolved in 2-ethoxyethanol (40 mg/ml; $5 \times 10^7$ dpm/ml).

In vivo *metabolic studies.* Groups of four male guinea-pigs were given, by oral intubation, 5·0 ml [$^{14}C$]CSL/kg (8 μCi/kg), in concentrations to provide a dose level of the material of either 90 or 900 mg/kg. Animals were housed in all-glass metabolism cages (Jencon Scientific Limited, Hemel Hempstead, Herts.) and air was drawn through the cage at a constant rate of 250 ml/min. Exhaled $^{14}CO_2$ was trapped in ethanolamine–2-ethoxyethanol (1:4, v/v), at intervals up to 48 hr, and urine and faeces were collected at 24 and 48 hr. At this time the animals were killed and the liver, kidneys, heart, lungs, spleen, testes and gastrointestinal tract were removed. The radioactivity

excreted in expired $CO_2$, urine and faeces and present in the organs at post-mortem examination was determined.

Groups of four male mice were given, by oral intubation, 2·0 ml [$^{14}$C]CSL/kg (40 $\mu$Ci/kg) in concentrations to provide dose levels of the material of 90 and 900 mg/kg. Excretion of radioactivity over 48 hr and tissue levels at post-mortem examination were determined as described for guinea-pigs.

In addition, groups of four male mice and four male guinea-pigs were given DL-[U-$^{14}$C]lactate by oral intubation at a dose level (325 mg/kg) equivalent to 900 mg CSL/kg. As in the previous experiments, excretion of radioactivity during 48 hr and tissue levels of radioactivity were determined.

In vitro *hydrolysis studies.* These studies were carried out using either washed livers or intestinal mucosal scrapings from rats, mice and guinea-pigs killed by cervical dislocation. Liver was homogenized in ice-cold 0·154 M-KCl containing 50 mM-tris-HCl buffer (pH 7·4) and 1 mM-EDTA, and 5·9 ml of the homogenate (0·1 g tissue/ml) was incubated with 0·1 ml [$^{14}$C]CSL solution in a shaking water-bath at 37°C. Aliquots (5 $\mu$l) were taken at 0, 10, 20, 30, 45 and 60 min and applied to a thin-layer plate.

Control incubations were carried out using boiled homogenate, aliquots being taken at the same time intervals. After the plates had been developed, the areas corresponding to lactate and CSL were scraped off and counted.

Sections of small intestine were removed from the animals, washed through with oxygenated Krebs–Ringer bicarbonate buffer (pH 7·4) and cut open and the mucosal layer was scraped off onto a microscope slide. A 10% (w/v) homogenate was prepared in Krebs–Ringer bicarbonate buffer and 5·9 ml of this homogenate was incubated at 37°C with 0·1 ml [$^{14}$C]CSL solution.

Aliquots (5 $\mu$l) were assayed for $^{14}$C-labelled lactate and [$^{14}$C]CSL by thin-layer chromatography as described above.

The sample of human duodenal mucosa, obtained at surgery, was snap-frozen in liquid nitrogen and then stored at −20°C until used. The thawed sample was homogenized in Krebs–Ringer bicarbonate buffer (3% w/v) and incubated at 37°C with [$^{14}$C]CSL. The hydrolysis was followed as in the other experiments.

Whole blood (2·5 ml) from rats, mice and human volunteers was incubated with Krebs–Ringer bicarbonate buffer (pH 7·4) and [$^{14}$C]CSL solution (0·1 ml) at 37°C for up to 1 hr. Aliquots (5 $\mu$l) were assayed for $^{14}$C-labelled lactate and [$^{14}$C]CSL as described above.

*Chromatography.* Urine and aliquots of homogenate were chromatographed on either Cellulose MN 300 or silica-gel GFHL and developed in butan-1-ol–water (86:14, v/v). Unlabelled lactate was visualized by spraying with bromocresol green (0·04% w/w in ethanol) and unlabelled CSL by spraying with 2,7-dichlorofluorescein (0·15% w/w in ethanol). Radioactivity on thin-layer plates was visualized using a Radiochromatogram Spark chamber (Birchover Instruments, Letchworth, Herts.) and was quantitated by counting the silica-gel or cellulose scraped from sections of the plates and suspended in toluene scintillant containing Cab-O-Sil (4% w/v) and 2,5-diphenyloxazole (0·4% w/v).

*Radioactivity determinations.* Radioactivity was measured in a Packard 2650 liquid scintillation counter and efficiency was determined by the external channels-ratio method. Urine (0·5 ml) and $^{14}CO_2$ trapping solution (3 ml) was counted in a scintillation fluid of toluene–2-ethoxyethanol (1:1, v/v) containing 2,5-diphenyloxazole (0·4% w/v). Faeces and tissues were oxidized in a Packard 306 sample oxidizer and the $^{14}CO_2$ produced was trapped in Carbosorb and

Table 1. *Excretion of radioactivity by male mice and male guinea-pigs given a single oral dose of $^{14}$C-labelled calcium stearoyl-2-lactylate ([$^{14}$C]CSL) or* DL-*[U-$^{14}$C]lactate*

| Route of excretion | Time from dosing | Dose (mg/kg)... Animals/group... | Radioactivity excreted (% of dose)* after intubation with: [$^{14}$C]CSL 90 / 4 | [$^{14}$C]CSL 900 / 4 | [$^{14}$C]Lactate 325 / 3 |
|---|---|---|---|---|---|
| | | | **Mice** | | |
| $CO_2$ | 0–7 | | 69·7 (68·4–71·0) | 57·5 (50·7–65·7) | 81·3 (75·8–84·9) |
| | 7–24 | | 7·1 (4·2–10·4) | 22·3 (12·1–32·9) | 8·0 (4·9–12·1) |
| | 24–48 | | 3·4 (1·8–5·8) | 2·8 (2·5–3·2) | 2·9 (2·6–3·3) |
| Urine | 0–24 | | 14·8 (11·8–17·5) | 14·1 (11·9–16·2) | 3·4 (3·0–4·5) |
| | 24–48 | | 0·7 (0·3–1·1) | 2·1 (0·8–4·1) | 0·6 (0·5–0·7) |
| Faeces | 0–24 | | 2·4 (2·0–3·3) | 1·8 (1·3–2·1) | 0·9 (0·8–1·1) |
| | 24–48 | | 0·3 (0·1–0·6) | 0·3 (0·3–0·4) | 0·2 (0·2) |
| | | Total... | 98·4 (97·8–99·5) | 100·9 (97·2–103·6) | 97·3 (96·8–97·8) |
| | | | **Guinea-pigs** | | |
| $CO_2$ | 0–7 | | 63·1 (54·4–67·2) | 60·5 (50·6–67·2) | 77·6 (74·8–80·4) |
| | 7–24 | | 12·2 (8·3–22·7) | 18·1 (13·2–24·4) | 4·0 (3·9–4·2) |
| | 24–48 | | 3·5 (2·4–4·4) | 3·3 (2·8–4·3) | 2·5 (2·0–3·0) |
| Urine | 0–24 | | 9·2 (8·2–11·0) | 8·1 (6·4–9·8) | 3·3 (3·1–3·6) |
| | 24–48 | | 0·8 (0·6–1·3) | 1·0 (0·3–2·7) | 0·4 (0·3–0·4) |
| Faeces | 0–24 | | 3·0 (2·7–3·7) | 2·3 (1·8–2·6) | 1·6 (1·5–1·6) |
| | 24–48 | | 0·8 (0·6–1·3) | 0·6 (0·4–0·7) | 0·5 (0·4–0·5) |
| | | Total... | 92·6 (88·8–93·9) | 93·9 (89·0–96·0) | 89·9 (87·0–92·7) |

*Results are expressed as means for the numbers of animals shown, with the range of values in brackets.

Table 2. *Tissue distribution of radioactivity in male mice and male guinea-pigs 48 hr after oral administration of $^{14}$C-labelled calcium stearoyl-2-lactylate ([$^{14}$C]CSL) or DL-[U-$^{14}$C]lactate*

| | | Recovery of radioactivity (%) administered as: | | |
|---|---|---|---|---|
| | | [$^{14}$C]CSL | | [$^{14}$C]Lactate |
| | Dose (mg/kg)... | 90 | 900 | 325 |
| Tissue | Animals/group... | 4 | 4 | 3 |
| | | **Mice** | | |
| G-i. tract | | 0·67 ± 0·15 | 0·79 ± 0·08 | 0·84 ± 0·02 |
| Liver | | 0·79 ± 0·18 | 0·91 ± 0·05 | 0·98 ± 0·08 |
| Kidney | | 0·22 ± 0·03 | 0·26 ± 0·03 | 0·21 ± 0·03 |
| Lung | | 0·04 ± 0·02 | 0·04 ± 0·01 | 0·04 ± 0·01 |
| Testes | | 0·03 ± 0·01 | 0·03 ± 0·01 | 0·04 ± 0·02 |
| Heart | | 0·05 ± 0·04 | 0·02 ± 0·01 | 0·03 ± 0·01 |
| Spleen | | 0·03 ± 0·01 | 0·02 ± 0·01 | 0·02 ± 0·01 |
| | Total... | 1·82 ± 0·55 | 2·07 ± 0·14 | 2·14 ± 0·09 |
| | | **Guinea-pigs** | | |
| G-i. tract | | 3·05 ± 0·12 | 2·01 ± 0·17 | 1·87 ± 0·04 |
| Liver | | 2·40 ± 0·79 | 4·11 ± 3·06 | 7·87 ± 4·13 |
| Kidney | | 0·26 ± 0·04 | 0·24 ± 0·01 | 0·18 ± 0·01 |
| Lung | | 0·20 ± 0·05 | 0·16 ± 0·04 | 0·10 ± 0·01 |
| Testes | | 0·06 ± 0·02 | 0·03 ± 0·01 | 0·07 ± 0·02 |
| Heart | | 0·06 ± 0·01 | 0·06 ± 0·01 | 0·05 ± 0·02 |
| Spleen | | 0·04 ± 0·01 | 0·05 ± 0·02 | 0·03 ± 0·01 |
| | Total... | 6·07 ± 0·77 | 6·66 ± 3·03 | 10·17 ± 4·07 |

Results are expressed as means ± 1 SD for the numbers of animals shown.

counted in Permafluor V (Packard Instruments, Des Plaines, IL, USA). Recovery of $^{14}$C was between 97 and 99%.

## RESULTS

### In vivo *studies*

The excretion of radioactivity from mice during the 48 hr following administration of a single oral dose of [$^{14}$C]CSL at 90 or 900 mg/kg or of [$^{14}$C]lactic acid at a dose level equivalent to the higher dose of CSL is shown in Table 1. Following administration of CSL at the lower dose level, more than 97% of the radioactivity was eliminated within 48 hr, most (approximately 77%) being excreted as $^{14}CO_2$ within 24 hr. Most of the remaining radioactivity was excreted in the urine in 24 hr, with only low levels of activity in the faeces and the 48-hr urine. At the higher dose level, the rate of metabolism to $^{14}CO_2$ over the first 7 hr was less, although the total excreted in 24 hr was similar. The percentage of radioactivity excreted in urine and faeces was similar at both dose levels.

The overall excretion of radioactivity following administration of [$^{14}$C]lactate was also rapid, about 90% of the administered radioactivity being eliminated as $^{14}CO_2$ in 24 hr. However, significantly less activity was excreted in the urine (approximately 4% of the dose) and less was excreted in the faeces than after CSL.

The total residual activity and the distribution of radioactivity in the tissues were similar 48 hr after administration of either [$^{14}$C]CSL or [$^{14}$C]lactate (Table 2). Approximately 2% of the administered dose remained in the tissues, mainly in the liver, gastro-intestinal tract and kidney. Only traces of activity (less than 0·05% of the dose in each case) were found in the other tissues.

The excretion of radioactivity from guinea-pigs during the 48 hr following oral intubation with 90 or 900 mg [$^{14}$C]CSL/kg or 325 mg [$^{14}$C]lactic acid is shown in Table 1. Although the rate and extent of conversion of $^{14}$C-labelled CSL to $^{14}CO_2$ was similar to those in the mouse, the percentage of the dose excreted in the urine of the guinea-pig was less (approximately 9%) and the total excreted by all routes in 48 hr was also less. The excretion of radioactivity in urine and faeces following [$^{14}$C]lactate administration to guinea-pigs was similar to that in the mouse; however the overall conversion to $^{14}CO_2$ was less, and the total amount of radioactivity excreted by all three routes was also less (approximately 90% of the dose).

The total residual activity and distribution of radioactivity in the tissues of guinea-pigs was similar 48 hr after administration of either [$^{14}$C]CSL or [$^{14}$C]lactate (Table 2). As in the mouse, most of the activity was in the liver, gastro-intestinal tract and kidney, with only traces of radioactivity in the other tissues. However, the activity in these tissues was significantly greater than in the mouse, and the total activity in all the tissues examined exceeded 6% of the administered dose.

Thin-layer chromatography of urine from mice and guinea-pigs showed that all of the radioactivity co-chromatographed with lactic acid.

### In vitro *studies*

The appearance of lactate and the disappearance of stearoyl-2-lactylate during the hydrolysis of [$^{14}$C]-CSL by liver homogenates from the rat, mouse and guinea-pig is shown in Fig. 1a,b. The livers of all three species readily hydrolysed the compound, the initial rate being greatest in the guinea-pig (24·7 μmol/g liver/hr) and least in the mouse (7·5 μmol/g liver/hr).

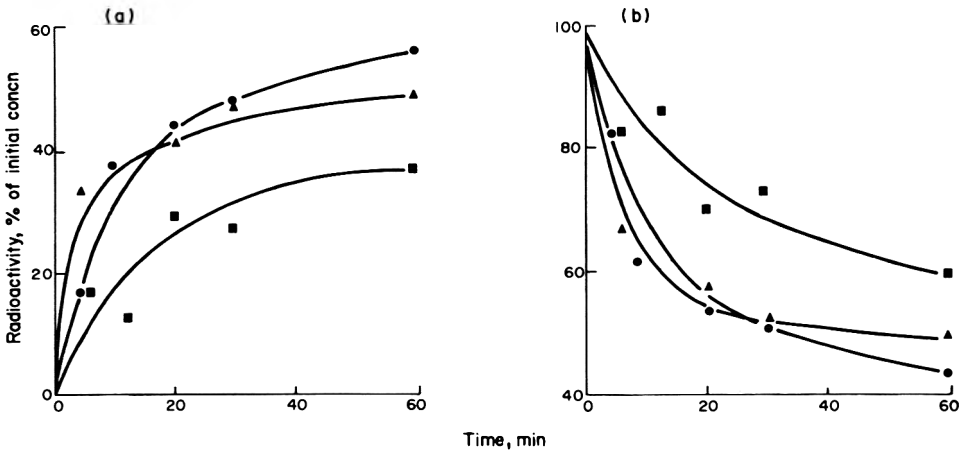


Fig. 1. Hydrolysis of $^{14}$C-labelled calcium stearoyl-2-lactylate by liver homogenates of the mouse (■), rat (●) and guinea-pig (▲): (a) appearance of [$^{14}$C]lactate; (b) disappearance of calcium stearoyl-2-[$^{14}$C]-lactylate. All values are corrected for non-enzymic breakdown.

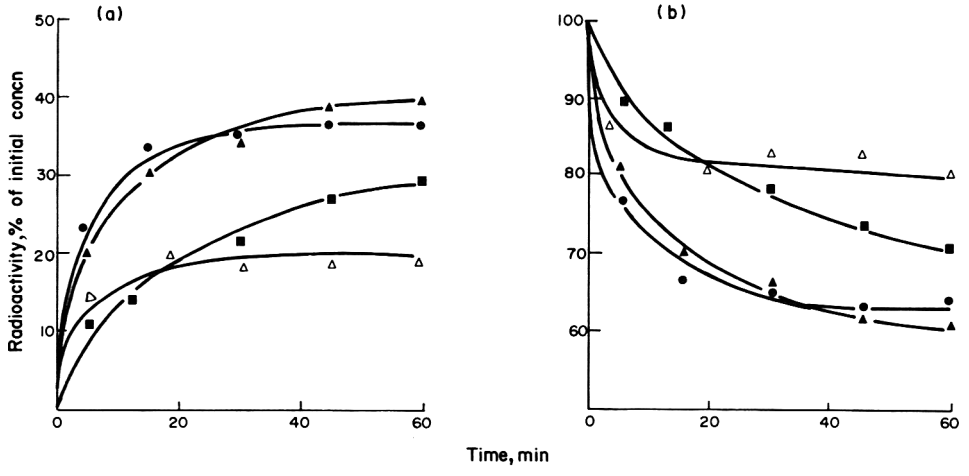


Fig. 2. Hydrolysis of $^{14}$C-labelled calcium stearoyl-2-lactylate by gastro-intestinal mucosal homogenates of the mouse (■), rat (●), guinea-pig (▲) and man (△): (a) appearance of [$^{14}$C]lactate; (b) disappearance of calcium stearoyl-2-[$^{14}$C]lactate. All values are corrected for non-enzymic breakdown.

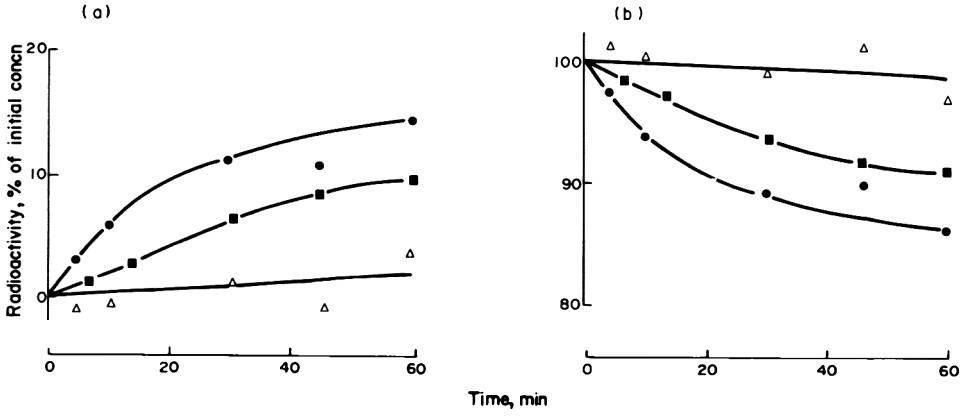


Fig. 3. Hydrolysis of $^{14}$C-labelled calcium stearoyl-2-lactylate by whole blood from rat (●), mouse (■) and man (△): (a) appearance of [$^{14}$C]lactate; (b) disappearance of calcium stearoyl-2-[$^{14}$C]lactylate. All values are corrected for non-enzymic breakdown.

In all three species, the overall extent of hydrolysis in 1 hr was similar, with between 40 and 60% of the compound hydrolysed.

The hydrolysis of [$^{14}$C]CSL by homogenates of gastro-intestinal mucosa from the rat, mouse and guinea-pig, and by a mucosal homogenate from human duodenum is shown in Fig. 2a,b. The initial rates of hydrolysis by rat and guinea-pig mucosa are similar and are significantly greater than that of mouse mucosa. The sample of human duodenal mucosa also hydrolysed CSL, although the extent of hydrolysis in 1 hr was less than that achieved by the rat, mouse or guinea-pig mucosa.

The hydrolysis of [$^{14}$C]CSL by whole blood from rats, mice and one human volunteer is shown in Fig. 3a,b. Whole blood from rodent species hydrolysed the compound slowly, the initial rates being 0·8 $\mu$mol/g blood/hr for the rat and 0·27 $\mu$mol/g blood/hr for the mouse. However, there was no measurable hydrolysis by human blood.

## DISCUSSION

The *in vivo* experiments using mice and guinea-pigs showed that the metabolism and tissue distribution of radioactivity demonstrated with [$^{14}$C]lactate-labelled CSL were similar to those for an equivalent dose of free [$^{14}$C]lactate, although in the former case the initial rate of $^{14}CO_2$ evolution was somewhat less in both species and the urinary excretion was significantly greater. The additional urinary radioactivity in CSL-treated animals may have been lactoyllactic acid, derived from enzymic de-esterification of the parent compound. It has been suggested that lactoyllactate and higher polymers may escape intermediary oxidation and be excreted in the urine (Giesecke & Fabritius, 1974).

The tissue distribution of radioactivity 48 hr after a single dose of either [$^{14}$C]lactate or [$^{14}$C]CSL was found to be similar in the mouse and in the guinea-pig, although the total amount of label retained in the guinea-pig was greater.

The results are similar to those in the rat reported by H. C. Hodge (unpublished data 1955, cited by JECFA, 1974). Although this author found a lower rate of metabolism to $^{14}CO_2$ than was demonstrated in the present study (60% of the administered $^{14}$C excreted in 24 hr, compared to 80% in the mouse and guinea-pig), he reported that the levels of urinary radioactivity were similar in animals given either [$^{14}$C]lactate or [$^{14}$C]CSL and that the tissue levels of radioactivity were also similar, although neither the proportion of the dose excreted nor the total activity retained was given.

Homogenates of liver and intestinal mucosa from all three species studied rapidly hydrolysed CSL to lactic and stearic acids. Whole blood from rats and mice also hydrolysed CSL, although at a much slower rate than the liver and gut mucosa.

Thus the results of the metabolic studies lead to the conclusion that the biological fate of CSL is comparable in both rodent (rat and mouse) and non-rodent (guinea-pig) species and that the metabolism both *in vivo* and *in vitro* proceeds by the hydrolysis of the compound to stearic and lactic acids by the non-specific carboxylic ester hydrolases. The products of metabolism are subsequently disposed of along normal physiological routes.

Despite the failure to detect any significant hydrolysis of CSL by human blood, the single sample of human duodenal mucosa readily hydrolysed this compound to stearic acid and lactic acid. It seems likely, therefore, that the metabolism of CSL would be similar in man to that in the rodent and non-rodent species examined.

Although these experiments were conducted only with male animals, similar results are likely with female animals. The toxicological studies, both short-term and long-term, have not revealed any differences in the response of male and female rats or dogs to CSL administration (JECFA, 1974) and there are no qualitative differences in the tissue distribution and substrate specificity of the carboxylic ester hydrolases in male and female animals (Albro & Thomas, 1973; Frisch, 1971).

As CSL is hydrolysed to normal physiological components, it is unlikely to present a toxicological problem in terms of its metabolic fate in man at the dose levels encountered in the human diet.

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# STUDIES ON LIVER MICROSOMES OF FEMALE RATS FED PURIFIED DIETS VARYING IN FAT CONTENT AND WITH AND WITHOUT PROPYL GALLATE

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**Abstract**—This work was undertaken to determine whether or not propyl gallate, added as a 0·3% (w/w) supplement to purified diets containing various types and amounts of fat, modifies the function of the hepatic microsomal mixed-function oxidase system. The criteria used included measurements of liver weight, liver to body-weight ratio and hepatic microsomal-protein level as well as analyses of specific components and functions, such as total microsomal cytochrome *P*-450 content, and the levels of activity of aniline hydroxylase, aminopyrine *N*-demethylase and NADPH-cytochrome *c* (*P*-450) reductase. In contrast to butylated hydroxytoluene, propyl gallate at a level of 0·3% in the diet did not alter the function of the mixed-function oxidase system, even when administered in a diet high in polyunsaturated fat, which has a permissive effect on the induction of hepatic cytochrome *P*-450 levels by phenobarbital and 3-methylcholanthrene.

## INTRODUCTION

The administration of certain drugs and other xenobiotics to rats and mice increases the activity of the hepatic microsomal mixed-function oxidase (MFO) system (Conney, 1967). The capacity of some dietary antioxidants to effect induction is of special interest. In addition, many antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) also inhibit tumorigenesis when fed to animals on commercial laboratory rations and exposed to chemical carcinogens (King, Bailey, Gibson, Pitha & McCay, 1979; Wattenberg, 1979a,b), although this has not been demonstrated in animals fed purified diets (King & Otto, 1978). Supplementation of commercial rations with BHT causes a dramatic increase in rat liver weight, an increase in liver weight to body-weight ratio, and a decrease in growth rate (Botham, Conning, Hayes, Litchfield & McElligott, 1970; Creaven, Davies & Williams, 1966; Johnson & Hewgill, 1961). In addition, BHT produces changes in various MFO activities, including an increase in biphenyl-4-hydroxylase and 4-methoxybiphenyl demethylase (Creaven *et al.* 1966) and a decrease in enzyme-catalysed lipid peroxidation (Tappel, Lundbert & Boyer, 1953; Vainio, 1974). BHA supplementation has little (Johnson & Hewgill, 1961) or no (Creaven *et al.* 1966) effect on liver weight but still causes an increase in MFO activities (Creaven *et al.* 1966). BHT and BHA do not accumulate in significant amounts in the body (Stuckey, 1968).

Propyl gallate (PG) is an efficient dietary antioxidant in terms of the stabilization of unsaturated fat in foods, but its effect on liver composition and *in vivo* metabolism has been evaluated only to a small extent. It is more efficient as an antioxidant than either BHA or BHT (Vanderhoek & Lands, 1973). At supplementation levels up to 0·5% (24 mM), PG had no effect on growth rate, survival time or liver and adrenal weights in rats fed a diet containing 15 or 20% lard (Creaven *et al.* 1966; den Tonkelaar, Verschuuren, Kroes & van Esch, 1968).

According to Torrielli & Slater (1971), adding PG at a level of 68 μM resulted in 50% inhibition of aminopyrine *N*-demethylase activity in female rat-liver microsomes. These workers also showed that 61 μM-PG inhibited oxygen uptake associated with lipid peroxidation by 95% in microsomal suspensions, while 9·9 μM PG decreased the activity of NADPH-cytochrome *c* reductase by approximately 50%. On the other hand, Yang & Strickhart (1974), using 10 or 25 μM-PG found no inhibition of NADPH-dependent reduction of cytochrome *P*-450 or cytochrome *c* by liver microsomes and suggested that there is no inhibition of cytochrome c-reductase activity by PG *in vitro*. These workers also observed that cytochrome *c*, but not cytochrome *P*-450, was reduced non-enzymically by 100 μM-PG *in vitro* (Yang & Strickhart, 1974).

Feeding purified diets containing various levels and types of fat, especially polyunsaturated fat and linoleic acid, has been shown by McLean (1977) and co-workers to have a permissive influence on the induction of hepatic cytochrome *P*-450 by inducers such as phenobarbital and 3-methylcholanthrene, but PG has not been tested in this context. Depending on the composition of the diet, PG reduces the incidence of mammary carcinomas in female rats given a single oral dose of 7,12-dimethylbenz[a]anthracene (DMBA) to a greater or lesser extent (King & McCay, 1980). In female rats fed purified diets containing 20% corn oil, or 18% coconut oil plus 2% linoleic acid, or 2% linoleic acid only, 0·3% PG provided some protection against mammary-tumour development in all three dietary groups in comparison with the unsupplemented controls, but the extent of protection differed according to the type and amount of fat in the diet.

Table 1. *Composition of diets fed, with or without added antioxidant, to female Sprague–Dawley rats for 1 month*

| Constituent | Concn (%) in High-polyunsaturated fat diet | High-saturated fat diet | Low-fat diet |
|---|---|---|---|
| Casein | 23 | 23 | 23 |
| Fat | 20* | 20† | 2‡ |
| Sucrose | 46 | 46 | 64 |
| Salt mixture§ | 4 | 4 | 4 |
| Alphacel‖ | 6 | 6 | 6 |
| Vitamin mixture¶ | 1 | 1 | 1 |

*Stripped corn oil.
†Stripped hydrogenated coconut oil (18%) and linoleic acid (2%).
‡Linoleic acid.
§Mixture according to Hubbell, Mendell & Wakeman (1937), modified to contain 0·03% zinc chloride.
‖Non-nutrient bulk.
¶Vitamin fortification mixture (ICN Life Sciences Co., Cleveland, OH).

This report is concerned with the effect of PG on hepatic mixed-function oxidases, as influenced by various levels of dietary fat.

## EXPERIMENTAL

*Animals, diets and treatment.* Weanling (21-day-old) outbred female Sprague–Dawley rats weighing 50–60 g were obtained from a colony maintained at the University of Oklahoma Health Sciences Center. The animals were housed in stainless-steel suspended wire-mesh cages and tap-water was supplied *ad lib.* by a Hardco Automatic Watering System. The three experimental diets (Table 1) were prepared to our specifications by ICN Pharmaceuticals, Inc., Life Sciences Group, Cleveland, OH. The type and content of fatty acids were monitored in all batches of diet to ensure that fat composition was consistent. The low-fat diet was designed to contain sufficient linoleic acid to prevent a deficiency of essential fatty acid (Guarnieri & Johnson, 1970). Diets supplemented with antioxidants contained 0·3% (w/w) PG or BHT (Sigma Chemical Co., St. Louis, MO). Diets were stored for no more than 4 wk at −17°C in sealed containers to minimize autoxidation of polyunsaturated fatty acids.

Animals were fed the experimental diets from weaning until they were 50 (±1) days old, and during this time they were weighed weekly on a Mettler automatic-tare animal-weighing balance. At the end of the feeding period, they were fasted for 18 hr and then killed. Their livers were quickly removed, rinsed twice in normal saline, blotted dry on filter paper and weighed.

*Liver microsomal preparation.* Liver from each rat was finely minced and homogenized with three passes of a motor-driven teflon pestle and Potter–Elvehjem-type homogenizer in a 0·05 M-Tris–0·25 M-sucrose buffer, pH 7·4, using 1 g liver/5 ml buffer. The homogenate was centrifuged at 9500 rpm (10,000 g) for 15 min in a Sorval SS-34 using 50-ml Oak Ridge-type screw-cap tubes. The supernatant was decanted into 30-ml Oak Ridge-type screw-cap tubes and centrifuged in a type 30 head and Beckman L5-75 ultracentrifuge at 30,000 rpm (105,000 g) for 90 min. The resulting supernatant was discarded and the pellet was re-homogenized in Tris-sucrose buffer, transferred to a clean 30 ml tube and centrifuged at 30,000 rpm for 60 min. The microsomal pellet obtained was kept on ice and used immediately or stored at −20°C and thawed on ice for use within 2 days. Microsomes to be assayed were suspended in 0·15 M-phosphate buffer, pH 7·5, so that 1 ml of suspension contained the microsomes from 2 g liver.

*Analyses and enzyme assays.* Microsomal protein was determined by the method Lowry, Rosebrough, Farr & Randall (1951). Cytochrome *P*-450 was determined from the reduced carbon monoxide difference spectrum (Omura & Sato, 1964) using a Pye Unicam SP8-100 spectrophotometer.

Aniline hydroxylation was measured by assay of *p*-aminophenol formation using a modification of the method of Imai, Ito & Sato (1966). The reaction system consisted of 0·1 ml of microsomal suspension (approximately 1 mg protein), 11 mM-$MgCl_2$, 43 mM-KCl, 21 mM-nicotinamide, 8 mM-aniline and an NADPH-generating system (5 mM-glucose-6-phosphate, 0·16 mM-NADP and 0·40 IU glucose-6-phosphate dehydrogenase, all from Sigma Chemical Co.), in 0·15 M-phosphate buffer, pH 7·5, the final volume being 2 ml. The reaction was initiated by the addition of microsomes and carried out at 37°C in a Precision Dubnoff metabolic shaker. Reactions were stopped at intervals of 0, 5, 10, 15 and 30 min by the addition of 0·5 ml 35% trichloroacetic acid and the tubes were centrifuged for 10 min at 2000 g. *p*-Aminophenol was determined spectrophotometrically at 639 nm in 1-ml aliquots of the resulting supernatant.

Aminopyrine *N*-demethylation was determined by measuring the production of formaldehyde by the method of Nash (1952). The reaction system was the same as that described for aniline hydroxylation, except that it contained 2·5 mM-aminopyrine instead of aniline. Incubations were carried out for 0, 5, 10, 15 and 30 min at 37°C. Reactions were stopped by the addition of 0·2 ml 20% $ZnSO_4$ and 0·5 ml saturated $Ba(OH)_2$. After centrifugation for 10 min at 1000 g, 1-ml aliquots were assayed for formaldehyde formation, optical densities at 412 nm being compared with a standard formaldehyde series run simultaneously with each assay.

Cytochrome *c*-reductase activity was measured in

high ionic-strength buffer solution, as described by Vermilion & Coon (1978).

## RESULTS

The feeding of diets varying in type and amount of fat for 1 month caused no measurable differences in animal weights, liver weights or liver- to body-weight ratios in the 50-day-old female Sprague–Dawley rats (Table 2), or in microsomal-protein values (mg/g liver). Furthermore, the addition of a 0·3% (w/w) PG supplement to the various diets resulted in no detectable differences in any of these parameters (Table 2) compared with values for liver taken from animals eating non-supplemented diets. The addition to each diet of an equivalent amount of BHT (0·3%, w/w), however, resulted in significant increases in both liver weights and microsomal-protein levels per gram of liver. This was reflected also in higher liver-weight to body-weight ratios in all three groups on BHT-supplemented diets compared to the non-supplemented groups (Table 2). The low-fat diet supplemented with BHT was associated with slightly lower microsomal-protein values, followed by the high-saturated fat/BHT-supplemented diet with the group fed the high-polyunsaturated fat + BHT diet showing the highest values. However, the differences between each of the BHT-supplemented groups were not statistically significant.

The activities of the two hepatic microsomal MFO systems, aniline hydroxylase and aminopyrine *N*-demethylase also showed no change as a function of diet or with the addition of PG to each of the three diets (Table 2). In contrast, the inclusion of BHT in the diet at the same level as the PG (0·3%, w/w) resulted in significant increases in both activities. There were, however, no differences between diets in the extent of induction of either enzyme by BHT.

Further evidence for a lack of induction of the microsomal MFO system by PG was the absence of any effect on the specific content of cytochrome *P*-450 and the activity of NADPH-cytochrome *c* reductase (Table 2). Furthermore, these parameters of the MFO system showed no significant variation with changes in the lipid content of diets either with or without PG supplementation.

PG's lack of influence on cytochrome *P*-450 contrasts with a marked induction of total cytochrome *P*-450 in animals fed BHT at the same level under identical conditions. Neither antioxidant, however, had any influence on hepatic NADPH-cytochrome *c* (*P*-450) reductase levels, nor did dietary fat have any significant influence on this MFO component.

## DISCUSSION

The results of these studies suggest that regardless of the dietary fat content, PG has no influence on the rat-liver microsomal drug-metabolizing system *in vivo*. In contrast, several studies in which rats have been fed diet supplemented with BHT have shown that this antioxidant causes a marked increase in liver weight, microsomal protein level per gram of liver, and the total cytochrome *P*-450 level (Botham *et al.* 1970; Creaven *et al.* 1966; Johnson & Hewgill, 1961; Kahl & Wulff, 1979). The results of this study confirmed these observations and have further demonstrated that BHT has no apparent effect on the activity of NADPH-cytochrome *c* (*P*-450) reductase, regardless of the level or type of dietary fat being consumed. Unlike PG, dietary BHT does result in significant increases in total cytochrome *P*-450, an effect that is magnified in animals consuming a diet high in either saturated or polyunsaturated fat. Although the difference was not statistically significant, there was a slight increase in total cytochrome *P*-450 as a result of BHT consumption in a low-fat (2%) diet. Neither of the high-fat diets resulted in an induction on their own, but both permitted increases in cytochrome *P*-450 by BHT. Marshall & McLean (1971) have reported that in male rats the concentration of cytochrome *P*-450 resulting from phenobarbital induction is determined by the nature of the dietary fat, with the greatest level of induction occurring in rats fed more highly unsaturated fat. However, Caster, Wade, Norred & Bargmann (1970), who fed varying concentrations of eight saturated fatty acids in diets containing 1·8% safflower oil to provide optimal levels of essential fatty acids, concluded that the major effect upon the drug-metabolizing capabilities of male rat liver was associated with the absolute amount of fat in the diet and was independent of fatty acid chain length. This appears to be the case for BHT induction in the female rat since in our study no differences were seen between the induction capabilities of either of the high-fat dietary groups. Since almost all drug-metabolizing activities are depressed in animals consuming a fat-free diet (Marshall & McLean, 1971), a key factor appears to be the availability of essential fatty acids. It appears that the type and level of dietary fat fed in excess of the essential fatty acid requirement further modulate the influences of various inducers on the drug-metabolizing system in a very complicated manner. More studies are needed to unravel the complex interaction between dietary lipids and liver microsomal metabolism.

Even when total microsomal-protein levels do not vary, it is possible that some enzymes are increasing while others are decreasing, keeping the overall level of protein constant. Measurements of two MFO functions (aminopyrine *N*-demethylation and aniline hydroxylation) showed no changes as a result of PG supplementation or variations in the fat component of the diet.

A general indicator of MFO induction is an increase in total cytochrome *P*-450. The animals supplemented with PG showed no significant change in total liver cytochrome *P*-450, in contrast to the increases caused by BHT (mentioned above). It is possible that individual *P*-450 proteins could have been increased and others decreased as a function of PG-mediated modulation of cytochrome *P*-450 heterogeneity, but this seems very unlikely in view of the constancy of the *N*-demethylase and aniline-hydroxylase activities referred to in Table 2.

The fact that dietary supplementation with PG did not inhibit MFO activitiy *in vivo* while other authors (Torrielli & Slater, 1971; Yang & Strickhart, 1974) have found that addition of PG to MFO assay systems *in vitro* did cause inhibition, suggests that the levels of PG attained *in vivo* may be much lower.

Table 2. *Effects of dietary fat and antioxidants on growth, liver measurements and the hepatic microsomal mixed-function oxidase system in female Sprague Dawley rats*

| Parameter | Mean values ± SEM following feeding of: | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Diet high in polyunsaturated fat | | | Diet high in saturated fat | | | Diet low in fat | | |
| | Without PG, BHT | With PG† | With BHT† | Without PG, BHT | With PG† | With BHT† | Without PG, BHT | With PG† | With BHT† |
| No. of rats/group‡ . . . | 5 | 5 | 6 | 6 | 6 | 4 | 6 | 6 | 5 |
| Body weight (g) | 147 ± 16 | 138 ± 12 | 132 ± 17 | 136 ± 11 | 142 ± 13 | 130 ± 18 | 138 ± 9 | 131 ± 15 | 132 ± 13 |
| Liver weight (g/100 g body weight) | 3·4 ± 0·12 | 3·4 ± 0·14 | 5·6 ± 0·33* | 3·6 ± 0·12 | 3·7 ± 0·21 | 5·3 ± 0·32* | 3·6 ± 0·11 | 3·5 ± 0·10 | 5·1 ± 0·25* |
| Microsomal protein (mg/g liver) | 7·59 ± 0·37 | 8·23 ± 0·29 | 10·82 ± 0·38* | 7·99 ± 0·22 | 7·72 ± 0·31 | 10·23 ± 0·32* | 8·10 ± 0·20 | 8·11 ± 0·24 | 10·01 ± 0·29* |
| Aniline hydroxylase§ | 0·44 ± 0·03 | 0·43 ± 0·02 | 0·88 ± 0·05* | 0·49 ± 0·04 | 0·48 ± 0·03 | 0·92 ± 0·04* | 0·43 ± 0·03 | 0·44 ± 0·04 | 0·87 ± 0·05* |
| Aminopyrine *N*-demethylase ‖ | 5·41 ± 0·12 | 5·58 ± 0·19 | 9·76 ± 0·63* | 5·40 ± 0·21 | 5·65 ± 0·16 | 9·54 ± 0·48* | 5·38 ± 0·18 | 5·35 ± 0·19 | 10·02 ± 0·39* |
| Cytochrome *P*-450 (nmol/mg protein) | 0·79 ± 0·04 | 0·80 ± 0·04 | 1·18 ± 0·05* (7) | 0·85 ± 0·06 (5) | 0·77 ± 0·03 | 1·10 ± 0·08* (9) | 0·81 ± 0·04 | 0·74 ± 0·03 (5) | 0·92 ± 0·07* (10) |
| Cytochrome-*c* reductase (μmol/mg protein/min) | 0·17 ± 0·01 | 0·18 ± 0·01 | 0·15 ± 0·02 (7) | 0·17 ± 0·01 (5) | 0·18 ± 0·01 | 0·15 ± 0·01 (9) | 0·19 ± 0·01 (5) | 0·18 ± 0·01 (5) | 0·18 ± 0·01 (10) |

PG = Propyl gallate BHT = Butylated hydroxytoluene

†Both PG and BHT were added to the appropriate diets at 0·3% (w/w).

‡Except where indicated otherwise in brackets.

§In nmol *p*-aminophenol formed/mg protein/min.

‖In nmol formaldehyde formed/mg protein/min.

Values marked with an asterisk differ significantly from that for the group on the corresponding non-supplemented diet: $*P < 0·05$ by Student's *t* test.

Some of the PG may be lost during microsomal preparation, but this is not a likely explanation. Another possibility is that *in vivo* partitioning between intracellular compartments may play a role in the distribution of PG to a site or sites of metabolic interaction other than at the microsomal membrane. This seems likely in view of the fact that PG added to the diet at the same level (0·3%) as in the present study has been shown *in vivo* to inhibit chemical carcinogenesis caused by DMBA (King & McCay, 1980).

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*Fd Cosmet. Toxicol.* Vol. 19, pp. 19 to 24

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# PURIFICATION AND SOME PROPERTIES OF CHICKEN-LIVER AFLATOXIN $B_1$ REDUCTASE

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**Abstract**—Aflatoxin $B_1$ reductase was isolated from the livers of 1- to 2-day-old chickens. The enzyme was purified more than 800-fold with a yield of 29% by the following steps: 100,000-g ultracentrifugation; acid (pH 5·0) precipitation; 40–75% ammonium sulphate fractionation; DEAE-cellulose column chromatography; hydroxyapatite column chromatography; Sephadex G-100 column chromatography; second hydroxyapatite column chromatography. The final enzyme preparation was colourless and contained a trace amount of impurity. The mol wt of the enzyme was estimated to be 46,500 by Sephadex G-100 gel filtration. The 17-ketosteroids androsterone, dehydroisoandrosterone, and oestrone inhibited the activity of $AFB_1$ reductase by 50–70% under the described assay conditions. The inhibition by oestrone appeared to be noncompetitive.

## INTRODUCTION

The potent hepatocarcinogenic mycotoxin aflatoxin $B_1$ ($AFB_1$) can be converted into various metabolites in animals (Masri, Booth & Hsieh, 1974; Patterson & Allcroft, 1970). It requires metabolic activation to exert its carcinogenic and mutagenic effects (Fahmy, Fahmy & Swenson, 1978; Gurtoo & Bejba, 1977; Gurtoo, Dahms & Paigen, 1978; Martin & Garner, 1977; Patterson & Roberts, 1972a). In the liver, the cyclopentenone ring of $AFB_1$ can be reduced by cytoplasmic reductase to aflatoxicol (AFL; Patterson & Roberts, 1972b; Salhab, Hsieh, Wong & Ruebner, 1975; Schoenhard, Lee, Howell, Pawlowski, Libbey & Sinnhuber, 1976). AFL has been identified *in vivo* as the major aflatoxin metabolite in the plasma of rats administered $AFB_1$ orally or iv (Wong & Hsieh, 1978). The activity of liver $AFB_1$ reductase was shown to be related to the sensitivity of an animal species to acute aflatoxicosis and to aflatoxin-induced carcinogenesis (Edwards, Rintel & Parker, 1975; Hsieh, Wong, Wong, Micas & Ruebner, 1977).

Our preliminary studies established that the liver of a 1–2-day-old chicken possesses much higher $AFB_1$ reductase activity than the liver of a duckling or a rat (Chen, 1979). The enzyme requires NADPH as cofactor for the reduction and is fairly stable in crude liver preparations. The present report describes the purification of this enzyme from chicken liver and the determination of some of its properties.

## EXPERIMENTAL

### *Animals and chemicals*

The chickens (*Gallus gallus*, Cochins), 1–2-days old, were purchased from the market at Shih-Lin, Taipei, Taiwan. NADP, glucose-6-phosphate (G6P), G6P-dehydrogenase (Torula yeast), tris(hydroxymethyl) aminomethane (Tris) and the reagents for disc gel electrophoresis were products of Sigma Chemical Co. (Saint Louis, MO). Sephadex G-100 was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden), DEAE-cellulose (Whatman DE 52) from Whatman Inc. (Springfield Mill, England), hydroxyapatite from Bio-Rad Laboratories (Richmond, CA), Liquiflor from New England Nuclear Co. (Boston, MA), crystalline $AFB_1$ from Calbiochem Co. (San Diego, CA), and ammonium sulphate and silica-gel (Type 60) from E. Merck Co. (Darmstadt, FRG). Ring-labelled [$^{14}$C]$AFB_1$ was purified from the chloroform extract of *Aspergillus parasiticus* incubated in the presence of sodium 1-[$^{14}$C]acetate (Hsieh & Mateles, 1971).

### *Assay for $AFB_1$ reductase*

The concentrations of the components of the assay mixture were as follows: 50 mM-potassium phosphate buffer (pH 6·0), 2·5 mM-G6P, 2·5 mM-NADP and 35-$\mu$M $AFB_1$ containing 0·02 $\mu$Ci [$^{14}$C]$AFB_1$ which was predissolved in DMSO–methanol (1:1, v/v). The assay mixture also contained 1 U G6P dehydrogenase/ml. The reaction was started by the addition of the $AFB_1$ reductase solution. After incubation at 37 C for 40 min in the dark, the reaction was stopped by adding 1 ml chilled methanol. The mixture was spun briefly to remove the denatured proteins. The supernatant was then extracted twice with 2 ml chloroform. The AFL in the extracts was separated from $AFB_1$ by silica-gel thin-layer chromatography and the radioactivities were quantitated by liquid scintillation counting as previously described (Chen, 1979). One enzyme unit was defined as the amount required to reduce 1 nmol $AFB_1$ to AFL in 40 min.

*Protein determination*

Protein concentration was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), using bovine serum albumin as standard.

*Purification of $AFB_1$ reductase*

(All of the purification procedures were carried out at 0–4°C.)

*Preparation of 100,000-g supernatant.* The chickens were killed by decapitation. The livers (30 g) were excised immediately and homogenized in a Waring blender with 3 volumes of chilled 0·15 M-KCl–20 mM-potassium phosphate (KP) buffer (pH 6·8). The homogenate was centrifuged at 100,000 g for 1 hr.

*Acid precipitation.* The 100,000-g supernatant was diluted with an equal volume of 20 mM-KP buffer (pH 6·8), and the pH was slowly adjusted to 5·0 with 1 N-acetic acid. The suspension was spun at 20,000 g for 30 min to remove the protein precipitate. The pH of the supernatant was then brought back to neutral with 1 N-$NH_4OH$.

*Ammonium sulphate fractionation.* To the enzyme-containing supernatant solid ammonium sulphate was added slowly to 40% saturation under continuous stirring. The mixture was spun at 20,000 g for 30 min. The supernatant was brought to 75% saturation with ammonium sulphate. The protein precipitate was collected by centrifugation, dissolved in a small volume of buffer A (5 mM-Tris-HCl pH 8·0, 1 mM-EDTA) and used for DEAE-cellulose column chromatography.

*DEAE-cellulose column chromatography.* A DEAE-cellulose column (2·6 × 40 cm) was pre-equilibrated with two bed volumes of buffer A. The enzyme solution was applied to the column and washed with 400 ml buffer A. It was followed by a linear continuous gradient of 0–170 mM-KCl in buffer A (total volume 1200 ml). The flow rate was 0·6 ml/min. Fractions of 14 ml were collected and assayed for enzyme activity. Only fractions with high enzyme activity were combined and concentrated by precipitation with ammonium sulphate to 80% saturation. The concentrated protein was dissolved in a minimum volume of buffer B (10 mM-KP, pH 6·8, 2 mM-EDTA, 5 mM-2-mercaptoethanol) and dialysed overnight against the same buffer.

*First hydroxyapatite column chromatography.* The dialysed enzyme solution was applied to an equilibrated hydroxyapatite column (1·5 × 12 cm) and washed with 100 ml buffer B. The column was then eluted with a linear gradient formed from 200 ml buffer B and 200 ml of a mixture containing KP (pH 6·8), EDTA and 2-mercaptoethanol at concentrations of 200 mM, 2 mM and 5 mM respectively. The flow rate was 0·45 ml/min. Fractions of 12 ml were collected and assayed for $AFB_1$ reductase activity. The fractions with high enzyme activity were combined and concentrated by ammonium sulphate precipitation to 80% saturation.

*Sephadex G-100 column chromatography.* The concentrated enzyme solution was chromatographed on an equilibrated Sephadex G-100 column (2·6 × 65 cm) in buffer C (40 mM-KP, pH 6·8, 2 mM-EDTA, 5 mM-2-mercaptoethanol). The flow rate was 20 ml/hr at a constant hydrostatic pressure of 35 cm $H_2O$. Fractions of 10 ml were collected and assayed for $AFB_1$ reductase activity.

*Second hydroxyapatite column chromatography.* The active fractions from the Sephadex G-100 column were combined, diluted with an equal volume of de-

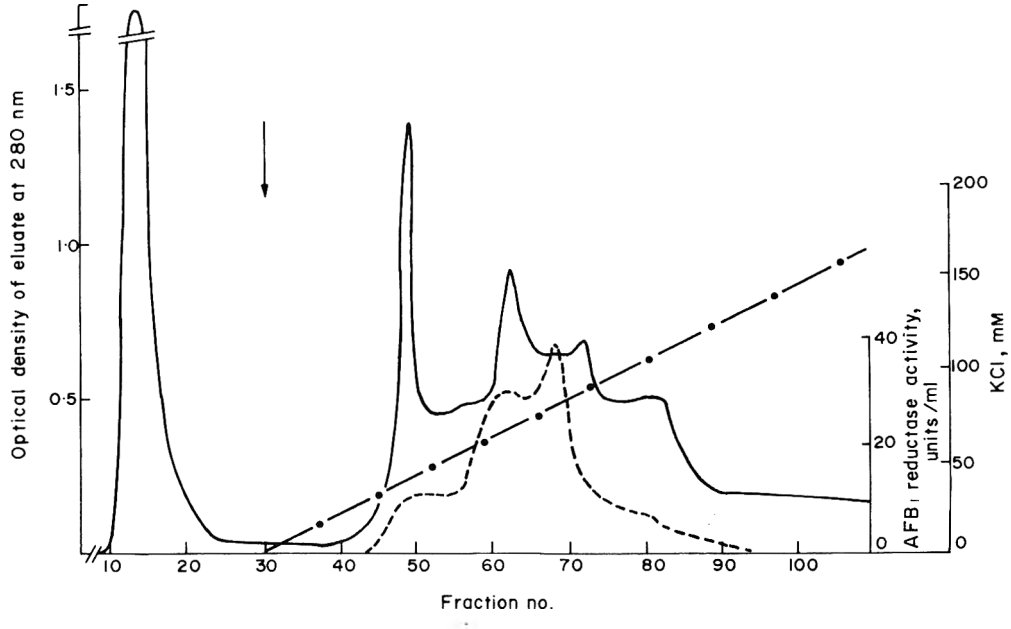


Fig. 1. Separation by DEAE-cellulose column chromatography of the 40–75% ammonium sulphate fraction: enzyme activity (---); protein concentration, estimated by the optical density of eluate at 280 nm (——); KCl (eluent) gradient (—●—). The arrow shows the start of the KCl gradient. One enzyme unit is defined as the amount required to reduce 1 nmol $AFB_1$ to aflatoxicol in 40 min. The volume of each fraction was 14 ml. Only the fractions with high enzyme activity (55–75) were combined for further purification. For details, see experimental section.

ionized water, and then rechromatographed on the hydroxyapatite column. The sample was eluted with a linear gradient formed from 200 ml 30 mM-KP (pH 6·8) and 200 ml 200 mM-KP (pH 6·8). The flow rate was 10 ml/hr. Fractions of 7 ml were collected.

*Disc gel electrophoresis*

Using the final enzyme preparation, disc gel electrophoresis was carried out on 7% acrylamide gel at pH 9·5 according to the methods of Ornstein (1964) and Davis (1964). A current of 5 mA/gel was applied for approximately 1 hr. The gel was stained with 0·25% (w/v) aqueous Coomassie Brilliant Blue R-250 in 45% (v/v) aqueous methanol and 9% (v/v) aqueous acetic acid and destained in a solution of 7·5% (v/v) aqueous acetic acid and 5% (v/v) aqueous methanol.

*Determination of molecular weight*

The molecular weight of the enzyme was estimated from the data obtained from Sephadex G-100 gel filtration. Aliquots (20 mg) of the standard proteins bovine serum albumin (mol wt 67,000), chymotrypsinogen A (mol wt 25,000) and cytochrome c (mol wt 12,000) were used for calibration. The values of $K_{av} = v_e - v_0/v_t - v_0$ ($v_e$ = elution volume; $v_t$ = total volume of the column; $v_0$ = void volume) were plotted against the logarithms of the known molecular weights.

*Effect of 17-ketosteroids on enzyme activity*

To investigate the previous report that $AFB_1$ reductase activity in crude liver preparations is inhibited by 17-ketosteroids, the activity of purified $AFB_1$ reductase was assayed in the presence of androsterone, dehydroisoandrosterone or oestrone. To study the type of inhibition, the enzyme was assayed at various concentrations of $AFB_1$ in the presence of 20 μM- and 40 μM-oestrone. The data were presented as Lineweaver–Burk plots.

## RESULTS AND DISCUSSION

Figure 1 shows the results of separation of the 40–75% ammonium sulphate fraction on the DEAE-cellulose column. The enzyme activity was distributed over several peaks. Only fractions with high enzyme activity (55–75) were combined for further purification. The apparent activity exhibited by the discarded trailing peaks may be attributed to some less specific liver reductases, since various cytoplasmic NADPH-dependent aldo–keto reductases with broad specificities have been reported (Bachur, 1976). Figure 2 shows the distribution of protein and of enzyme activity after separation on the first hydroxyapatite chromatographic column. The active fractions (16–27) were combined and subjected to Sephadex G-100 filtration. The pooled active fractions from this filtration (those eluting between 175 and 205 ml; Fig. 3) were applied to a second hydroxyapatite column, from which a broad single peak (fractions 28–36) containing the enzyme activity was eluted (Fig. 4). The molecular weight of the purified $AFB_1$ reductase was estimated to be 46,500, by Sephadex G-100 gel filtration (Fig. 5).

Table 1 shows that the specific activity of $AFB_1$ reductase in the fractions collected from the final stage of purification was more than 800 times greater than that of the chicken-liver homogenate. About 29% of the enzyme could be recovered. The final enzyme preparation was colourless and was shown by

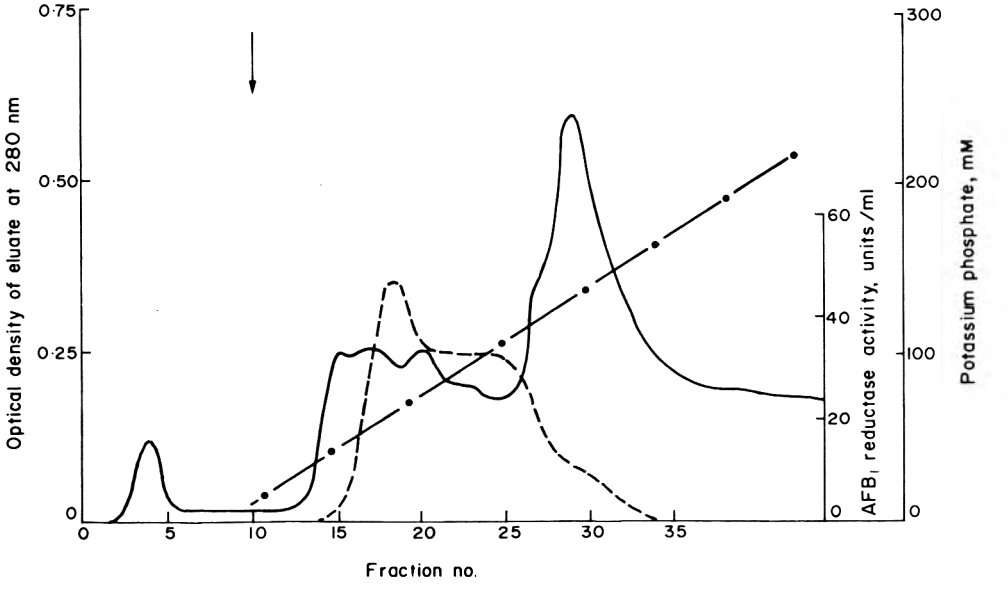


Fig. 2. Separation on a hydroxyapatite chromatographic column of the enzyme preparation obtained from the DEAE-cellulose column: enzyme activity (---); protein concentration estimated by the optical density of eluate at 280 nm (——); potassium phosphate (eluent) gradient (—●—). The arrow indicates the start of the potassium phosphate gradient. One enzyme unit is defined as the amount required to reduce 1 nmol $AFB_1$ to aflatoxicol in 40 min. The volume of each fraction was 12 ml. Only fractions with high enzyme activity (16–27) were combined for further purification. For details, see experimental section.

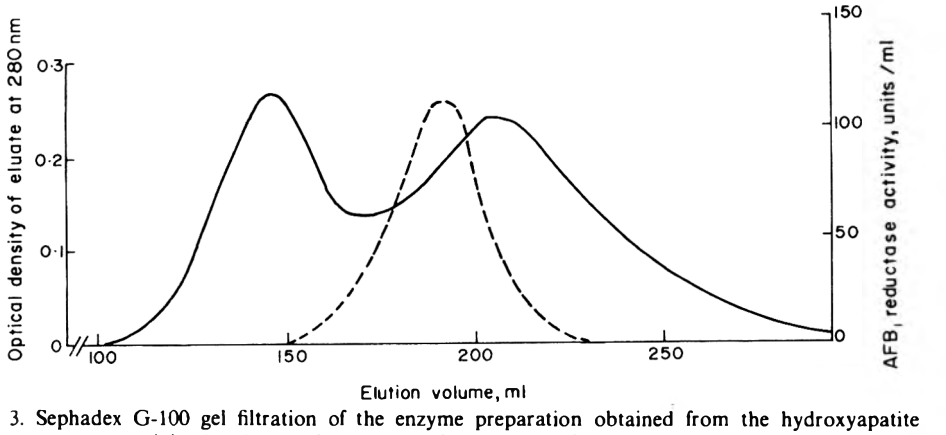


Fig. 3. Sephadex G-100 gel filtration of the enzyme preparation obtained from the hydroxyapatite column: enzyme activity (---); protein concentration (——), estimated by the optical density of the eluate at 280 nm. One enzyme unit is defined as the amount required to reduce 1 nmol $AFB_1$ to aflatoxicol in 40 min. Only the fractions with high enzyme activity (those eluting between 175 and 205 ml) were combined for further purification.

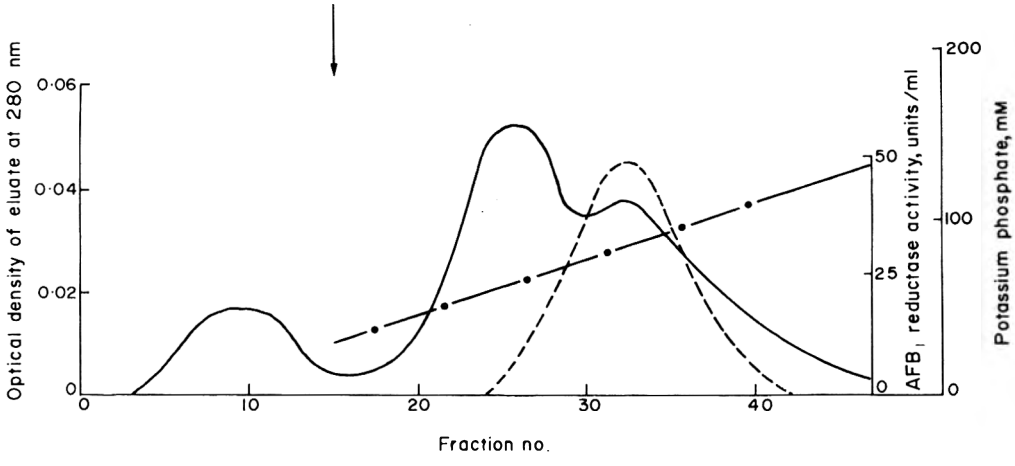


Fig. 4. Separation on the second hydroxyapatite chromatographic column of the enzyme preparation obtained from the Sephadex G-100 filtration: enzyme activity (---); protein concentration (——) estimated by the optical density of the eluate at 280 nm; potassium phosphate (eluent) gradient (—●—). The arrow indicates the start of the eluent gradient. One enzyme unit is defined as the amount required to reduce 1 nmol $AFB_1$ to aflatoxicol in 40 min. The volume of each fraction was 7 ml. A broad single peak (fractions 28–36) containing the enzyme activity was eluted. For details, see experimental section.

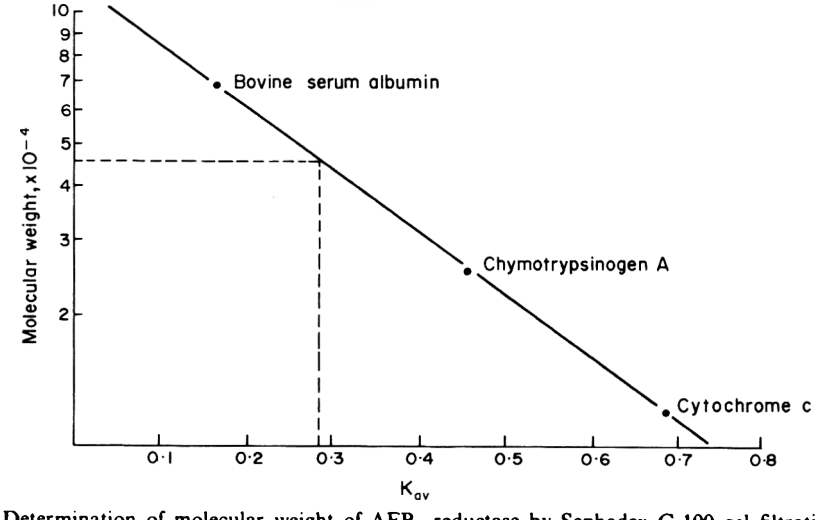


Fig. 5. Determination of molecular weight of $AFB_1$ reductase by Sephadex G-100 gel filtration. The enzyme had a $K_{av}$ value of 0·279, corresponding to a mol wt of 46,500. $K_{av} = V_e - V_0/V_t - V_0$ where $V_e$ = elution volume, $V_0$ = void volume, and $V_t$ = total volume of the column.

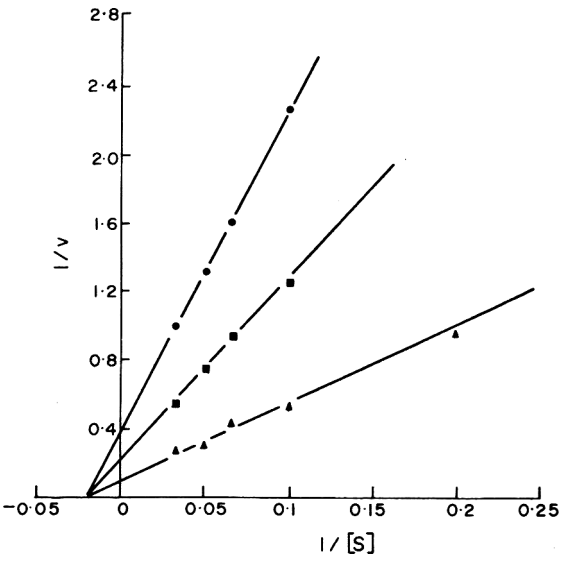


Fig. 6. Lineweaver–Burk plot for the reduction of $AFB_1$ to aflatoxicol catalysed by $AFB_1$ reductase in the presence of 0 (▲), 20 (■) or 40 (●) μM-oestrone at 37°C and pH 6. v = rate of reaction (nmol aflatoxicol formed/mg protein/min); [S] = $AFB_1$ concentration (μM).

Table 1. *Purification of aflatoxin $B_1$ ($AFB_1$) reductase from chicken liver*

| Stage of purification | Protein (mg) | Specific activity (enzyme units*/mg protein) | Recovery (%) | Purification factor† |
|---|---|---|---|---|
| Homogenate | 5400 | 1·91 | 100·0 | 1·00 |
| Supernatant, 100,000 g | 2320 | 4·05 | 91·1 | 2·12 |
| Acid precipitation | 1512 | 6·2 | 90·9 | 3·25 |
| Ammonium sulphate fractionation (40–75%) | 901 | 10·6 | 87·4 | 5·55 |
| DEAE-cellulose column | 150 | 56·3 | 81·8 | 29·48 |
| Hydroxyapatite column | 27 | 192·9 | 50·5 | 101·00 |
| Sephadex G-100 column | 6·22 | 535·1 | 32·3 | 280·15 |
| Second hydroxyapatite column | 1·95 | 1534·5 | 29·0 | 803·40 |

*1 enzyme unit is defined as the amount required to reduce 1 nmol $AFB_1$ to aflatoxicol in 40 min.
†The purification factor is the specific activity of a purified sample as a fraction of the specific activity of the homogenate.

Table 2. *Effect of 17-ketosteroids on $AFB_1$ reductase activity*

| Addition to enzyme assay mix* | Relative $AFB_1$ reductase activity (%) |
|---|---|
| None | 100 |
| Androsterone, 100 μM | 36·2 |
| Dehydroisoandrosterone, 100 μM | 28·5 |
| Oestrone, 20 μM | 49· |
| 40 μM | 28·0 |

*Androsterone and dehydroisoandrosterone were predissolved in dimethylsulphoxide and oestrone was predissolved in methanol before addition to the incubation medium.

analytical disc gel electrophoresis to contain a trace amount of impurity. The purified enzyme was unstable and storage at 0°C for 1 wk resulted in 50% less activity. Compared with other cytoplasmic aldo–keto reductases (Bachur, 1976; Chen, 1979; Culp & McMahon, 1968; Felsted, Gee & Bachur, 1974), the purified $AFB_1$ reductase showed similar physicochemical characteristics such as sulphydryl sensitivity, pH optimum (pH 6–8), and low molecular weight.

Patterson & Roberts (1972) reported that 17-ketosteroids were able to inhibit $AFB_1$ reductase activity in the high-speed supernatant of avian livers and that

these livers also possessed NADPH-linked 17-ketosteroid reductase activity. The three 17-ketosteroids tested in the present study inhibited the activity of purified chicken-liver $AFB_1$ reductase by 50–70% (Table 2). The inhibition by oestrone was noncompetitive (Fig. 6), which implies that $AFB_1$ and oestrone bind at different sites on the enzyme. Whether the inhibition by the other 17-ketosteroids is also noncompetitive requires further studies.

The substrate of the purified enzyme *in vivo* is not clear. Patterson & Roberts (1972) suggested 17-ketosteroids as the natural substrates. A direct assay of the enzyme with these steroids and a comparison of the $K_m$ values are necessary to answer this question.

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# COMPARATIVE METABOLISM OF ZEARALENONE AND TRANSMISSION INTO BOVINE MILK

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**Abstract**—The metabolism of zearalenone in the urine of the cow, the pig, the rabbit, the rat and man was studied. Both free and conjugated zearalenone (63%), α-zearalenol (32%) and β-zearalenol (5%) were present in the urine of the pig; α-zearalenol was the predominant metabolized species. In the cow, free and conjugated (glucuronic and sulphate) zearalenone (29%), α-zearalenol (20%) and β-zearalenol (51%) were found; in contrast to swine, β-zearalenol was the predominant species. In rat urine, the major species was free zearalenone, which constituted over 90% of the total zearalenone and metabolites. In the rabbit, 46% of the urinary metabolites took the form of zearalenone conjugates, 29% were conjugates of α-zearalenol and 25% were conjugates of β-zearalenol. The distribution of metabolites in the faeces was similar to that in the urine. In man, zearalenone and α-zearalenol were the major metabolites followed by β-zearalenol; all were in the glucuronide form.

Free and conjugated forms of zearalenone and diastereomeric zearalenols were present in cows' milk. The total concentration of zearalenone and its metabolites was 1·3 ppm (after receiving 25 ppm dietary zearalenone for 7 days); zearalenone constituted 35%, α-zearalenol, 31% and β-zearalenol, 34% of the free metabolites. α-Zearalenol is three times more oestrogenic than zearalenone.

## INTRODUCTION

Zearalenone is an oestrogenic metabolite produced by *Fusarium roseum* and other fusarium species, which causes hyperoestrogenism in pigs and various laboratory animals (Mirocha, Pathre & Christensen, 1976). It has a profound effect on the fertility of pigs (Chang, Kurtz & Mirocha, 1979) and only a moderate effect on bovine fertility (H. L. Whitmore, unpublished data, 1980).

Preliminary studies on the metabolism of zearalenone by Mirocha *et al.* (1976) showed that free zearalenone was the predominant species found in the urine of a rat given zearalenone. Pathre, Mirocha & Hagler (1978) reported that zearalenol was the principal urinary metabolite in pigs intubated with zearalenone. Tashiro, Nishimura & Ueno (1980) also found zearalenol as a metabolic derivative of zearalenone fed to rats. Kiessling & Pettersson (1978) using crude preparations of rat-liver enzymes, found two isomers of zearalenol as products of zearalenone metabolism.

In this paper we describe the *in vivo* metabolism of zearalenone in laboratory and farm animals.

## EXPERIMENTAL

*Materials.* Zearalenone was obtained from International Minerals Corporations, Terre Haute, IN, and recrystallized to a constant melting point of 163–164°C. An isomer of zearalenol identified as α-zearalenol (Hagler, Mirocha, Pathre & Behrens, 1979) was the generous gift of Dr. Shipchandler.

β-Glucuronidase (GRD; Mollusk), lyophilized with 20% sorbitol, grade A, and GRD/aryl sulphatase (ARS; *Helix pometia*) grade B, were obtained from Calbiochem–Behring Corp., San Diego, CA. The GRD and ARS were standardized with phenolphthalein glucuronide and phenolphthalein disulphate (Sigma Chemical Co., St. Louis, MO), respectively, according to the procedure described by Talalay, Fishman & Huggins (1946) with some minor modifications. For hydrolysis of the urine (2 ml) and milk (10 ml) conjugates, approx. 10,000 Fishman units of GRD and 5000 Whitehead units of ARS were used.

*Administration of zearalenone*

*Swine.* Zearalenone (1·0 g) was administered by stomach intubation as a slurry in water to a 3-wk-old piglet weighing 8 kg. The residual slurry in the tube was recovered and was analysed for zearalenone in order to estimate zearalenone actually administered (726 mg or 90 mg/kg). The piglet was kept in a metabolic cage and the urine and faeces were collected every 24 hr for 4 days and were then immediately frozen.

*Cows.* Two cows were fed dietary zearalenone. These animals were part of a pilot study designed to measure the long-term effects of zearalenone on lactating cows. Only one cow was lactating during this study. A diet containing 25 ppm zearalenone was fed from day 0 to day 8 and milk was collected on day 7. There was no further zearalenone in the diet until day 29 when a diet containing 250 ppm zearalenone was started and continued until day 50. Milk was collected on days 30 and 31.

The other cow was not lactating, but received 100 ppm of dietary zearalenone from day 0 to day 8. The urine and faeces were collected on day 7 for analysis. The above protocol was not designed for these metabolism studies, but the advantage was taken of available material.

*Rats.* Twenty-one-day-old, white virgin female rats (Holtzman Co., Madison, WI) were used in this study. The rats were selected for uniformity of size and

vigour, and were kept in separate metabolic cages. In one experiment 1·25 mg zearalenone/rat was administered to four rats in 50% aqueous ethanol by stomach intubation. The urine was allowed to accumulate for 72 hr before analysis. In a second experiment zearalenone (5 mg on 5 g feed mixture) was incorporated into a nutritionally balanced diet and was offered to each rat. When they had consumed all of the feed containing zearalenone, they were given control diet. The urine and faeces were collected daily. The drainage-ports were rinsed with 5 ml of 95% ethanol after collection of the urine, which was analysed together with the ethanol washes.

*Rabbits.* Zearalenone (1 g) was dissolved in 2 ml of 2 N-NaOH solution Two rabbits (Oke–Crest Rabbitary, Edina, MN) were injected intramuscularly with zearalenone solution (0·5 g of zearalenone/ml per rabbit). The urine from each rabbit was collected every 24 hr for 5 days.

*Man.* An adult male was given a single oral dose of 100 mg zearalenone and the urine was collected during the following 24 hr.

*Analytical gas chromatography*

Trimethylsilyl ether (TMS) derivatives used in gas chromatography (GC) were prepared by adding 50–100 μl of "Tri Sil BT" (Pierce Chemical Co., Rockford, IL) to a dry extract in a 0·5-dram (1·85-ml) vial with a polyethylene lined screw cap. The analysis of the sample was achieved by injecting 1 μl of the reaction mixture onto a 1 m × 3 mm stainless steel column packed with 3% OV-17 or QF-1 on 100–120 mesh Gas-Chrom Q. The carrier gas was nitrogen with a flow rate of 30 ml/min. All separations were performed on a Hewlett Packard 5710 Chromatograph (Hewlett–Packard Corp, Palo Alto, CA) equipped with a flame-ionization detector and column temperature programmed from 150° to 290°C at 8°/min.

*Gas chromatography–mass spectrometry*

An LKB-9000 gas chromatography–mass spectrometry (GC–MS) system (LKB-Produkter-AB, Bromma, Sweden) with a PDP-8E on-line computer (Digital Equipment Corp., Maynard, MA) was used to confirm the identification of metabolites. Gas chromatographic conditions were similar to those above, but helium was used as carrier gas. All analyses were run under selected ion monitoring mode; the ions of TMS ethers of zearalenone and zearalenols monitored were as follows: zearalenone—$m/e^+$: 462 ($M^+$), 447, 350, 333, 305, 260, 151; zearalenol—$m/e^+$: 536 ($M^+$), 521, 446, 350, 333, 307, 305, 260.

*High-pressure liquid chromatography*

High-pressure liquid chromatographic (HPLC) separations were carried out on a Waters Associates Inc. (Milford, MA) liquid chromatograph equipped with a Model 440 absorbance detector (280 nm) and a Model 420 fluorescence detector (254 nm excitation; 440 nm emission) connected synchronously. All analyses were run isocratically on μ Bondapak Phenyl (Waters Associates Inc.) using water: methanol (35:65), 0·7 ml/min or water: acetonitrile (65:35), 0·5 ml/min.

For actual analysis, the samples were reconstituted in 100–200 μl of the appropriate solvent system, and an aliquot of 5–20 μl was injected into the system. The fractions corresponding to zearalenone and zearalenols were collected to confirm their identification by GC–MS.

The milk extracts were purified by HPLC because they were too complex to analyse directly by GC or GC–MS. The fractions collected from HPLC were concentrated to dryness, silylated and then analysed by GC–MS.

*Hydrolysis of conjugates*

An aliquot (2–10 ml) of the urine or milk sample was placed in a test tube and boiled briskly. The pH of the substrate was adjusted to 4·5 with 0·1 M-acetate buffer (5·785 g of sodium acetate and 3·25 ml glacial acetic acid made up to 1 litre with distilled water, pH 4·5). This buffered substrate was incubated with GRD or GRD-ARS at 37°C for 2 hr.

*Isolation of metabolites*

The sample (hydrolysed or unhydrolysed) was passed through an XAD-2 column (1 g purified XAD-2 resins presaturated with distilled water). The column was then eluted with 15 ml of isopropanol–chloroform (1:3). The solvents from the eluate were removed and the residue was dissolved in 2 ml of methanol. This methanol solution was filtered through a 0·5 μm Millipore filter (Sample Clarification Kit, Waters Associates Inc.). The filtered methanol solution was then analysed by either GC or HPLC as described above.

*Analysis of urine*

For the determination of total metabolites, a 2-ml aliquot of urine was boiled and then incubated with 10,000 Fishman units of GRD for 2 hr at 37°C, pH 4·5. The hydrolysed urine was then passed through an XAD-2 column as described above and analysed by GLC, GC–MS and HPLC. For the determination of the free metabolites only a 2-ml sample of urine was boiled and then passed through an XAD-2 column before analysis.

The metabolites in the urine were quantified by GC and/or HPLC using external standards. The recoveries on control urine samples amended with zearalenone and zearalenols in the concentration range of 10–50 μg/ml of urine indicated that recoveries approached 100% with a coefficient of variation of 2·5%. The concentration of the metabolites in the urine were therefore reported without recovery corrections.

Similar results were obtained for the analysis of faeces.

*Analysis of milk*

A 10-ml sample of milk was boiled and cooled and then incubated with glucuronidase (10,000 Fishman units) and aryl sulphatase (5000 Whitehead units) for 2 hr at 37°C, pH 4·5. Acetone (10-ml) was added and the mixture was centrifuged at 9000 rpm for 20 min. The acetone was evaporated from the supernatant which then passed through an XAD-2 column as described above. The solvents were evaporated from the eluate and the residue was partitioned in 10 ml acetonitrile–petroleum ether (1:1, v/v). The petroleum ether layer was discarded and the solvent was evapor-

ated from the acetonitrile layer and the residue was dissolved in methanol and separated into fractions (by HPLC) which were then analysed by GC–MS in the selected ion monitoring (SIM) mode.

*Analysis of faeces*

A sample of 1–5 g of faeces was extracted three times with 20 ml ethyl acetate, filtered and evaporated to dryness. The residue was taken up in 25 ml acetonitrile and partitioned with 25 ml petroleum ether. The petroleum ether layer was discarded and the solvent was evaporated from the acetonitrile layer. This residue was taken up in 25 ml chloroform and partitioned with 6 ml 1 N-NaOH. The chloroform layer was discarded and the NaOH layer was acidified with 2 N-$NH_3PO_4$ to pH 7·5. This was then partitioned with two lots of 25 ml chloroform and the acid layer was discarded. The chloroform layer was concentrated and then analysed by HPLC and GLC.

## RESULTS AND DISCUSSION

The urine was analysed for metabolites of zearalenone in the free and conjugated (glucuronides and sulphate) forms. The hydrolysed urine yielded total zearalenone and its metabolites whereas the metabolites in their free form were determined by analysing the urine without enzyme treatment. Samples were analysed either in duplicate or triplicate. In general, the glucuronic acid adduct was measured by calculating the concentration of zearalenone and and zearalenol in the hydrolysate after subtraction of the value for the free form; the sulphate form was determined by measuring the total hydrolysate resulting from the action of GRD/ARS and subtracting the values obtained from the GRD treatment and the free form.

The purified urinary metabolites were analysed by HPLC on reverse-phase columns such as Waters' $\mu$ Bondapak Phenyl and $\mu$ Bondapak CN. These columns give excellent separation of zearalenone, $\alpha$-zearalenol and $\beta$-zearalenol with MeOH/$H_2O$ (65:35) and $CH_3CN/H_2O$ (35:65) mobile solvents, respectively, in the isocratic mode. We chose $\mu$ Bondapak CN columns for quantification because they give better resolution of the free metabolites. A typical separation of zearalenone and the isomeric zearalenols is shown in Fig. 1. A dual detector system was used on the HPLC, i.e., fluorescence and absorbance. The ratio of the value obtained by fluorescence and absorbance provides a diagnostic method of identifying and quantifying the derivatives. In some cases, the ratios were inconsistent and when this occurred, the sample was further analysed by GC–MS.

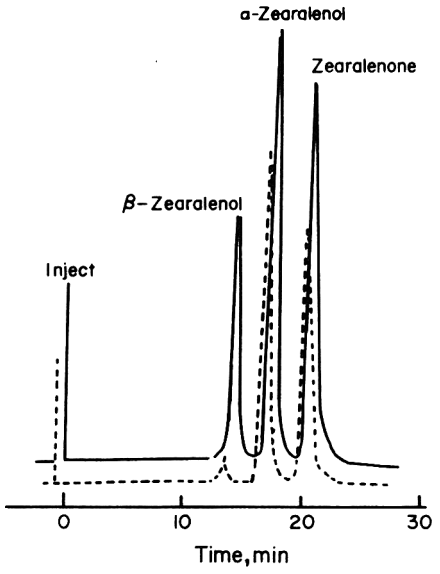


Fig. 1. Resolution of zearalenone and the $\alpha$ and $\beta$ isomers of zearalenol by high-pressure liquid chromatography. The components were resolved on a Waters Instrument on $\mu$ Bondapak CN using acetonitrile–water (35:65, v/v) at 0·5 ml/min with simultaneous UV absorption (280 nm; ——) and fluorescence detection (254, 440 nm; – – –).

The distribution of metabolites in the urine collected from the piglet is presented in Table 1. Most of the metabolites were excreted during the first 48 hr after the administration of zearalenone. Free zearalenone was found to be a major constituent of the urine; 31% of the total urinary zearalenone was in the glucuronide form. Diastereomeric zearalenols, appearing mainly as glucuronides (>80%) were presented and constituted 37% of the total concentration of zearalenone and zearalenols in the urine; more than 90% of the diastereomeric zearalenol was $\alpha$-zearalenol. However, less than 7% of the total zearalenone administered was recovered as urinary zearalenone and its metabolites. No sulphate form of the metabolites could be detected.

The distribution of zearalenone metabolites in the urine collected randomly from the lactating cow is shown in Table 2. Zearalenone and zearalenols in free and conjugated forms were found: 84% of the total urinary zearalenone metabolites were glucuronides and sulphates. The ratio of the concentrations of total zearalenone:$\alpha$-zearalenol:$\beta$-zearalenol was about 2:1:3; $\beta$-zearalenol was higher in concentration in all analyses. There was an appreciable amount of the sulphate adduct in bovine urine.

Table 1. *Distribution of zearalenone and its metabolites in pig urine after administration of 90 mg zearalenone/kg body weight by stomach intubation*

| Time after dosing (hr) | Volume of urine collected (ml) | Zearalenone | | | | α-Zearalenol | | | | β-Zearalenol | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Free | | Glucuronide | | Free | | Glucuronide | | Free | | Glucuronide | |
| | | mg | (μg/ml) | mg | (μg/ml) | mg | (μg/ml) | mg | (μg/ml) | mg | (μg/ml) | mg | (μg/ml) |
| 0–48 | 350 | 12·6 | (36) | 5·6 | (16) | 1·33 | (3·8) | 8·9 | (25) | 0·22 | (0·6) | 1·16 | (3·3) |
| 48–72 | 195 | 1·74 | (8·9) | 0·55 | (2·8) | 0·16 | (0·8) | 0·72 | (3·7) | 0·14 | (0·7) | 0·23 | (1·2) |
| 72–96 | 250 | 0·7 | (2·3) | 0·7 | (2·8) | ND | | ND | | ND | | ND | |

ND = not detected

Table 2. *Distribution of zearalenone and its metabolites in the urine of a cow after 7 days on a diet containing 100 ppm zearalenone*

| Form of metabolite | Zearalenone | | α-Zearalenol | | β-Zearalenol | |
|---|---|---|---|---|---|---|
| | Concn in urine (μg/ml) | (Percentage of total zearalenone) | Concn in urine (μg/ml) | (Percentage of total α-zearalenol) | Concn of urine (μg/l) | (Percentage of total β-zearalenol) |
| Free | 0·207 | (10·7) | 0·472 | (35·7) | 0·376 | (11·1) |
| Glucuronide conjugate | 1·420 | (73·7) | 0·698 | (52·9) | 2·370 | (70·0) |
| Sulphate conjugate | 0·3 | (15·6) | 0·150 | (11·4) | 0·630 | (18·6) |
| Total | 1·927 | | 1·320 | | 3·376 | |

Table 3. *Distribution of zearalenone and its metabolites in the urine of rats collected during the 96 hr following dietary administration of 5 mg zearalenone/rat*

| Rat no. | Zearalenone (μg/ml) | | α-Zearalenol (μg/ml) | | β-Zearalenol (μg/ml) | |
|---|---|---|---|---|---|---|
| | Free | Glucuronide | Free | Glucuronide | Free | Glucuronide |
| 1 | 13·7 | 35 | ND | ND | 1·25 | 2·5 |
| 2 | 47·5 | 0 | ND | ND | 1·25 | Trace |
| 3 | 5·0 | Trace | 4·1 | Trace | 0·9 | Trace |
| 4 | 14·1 | Trace | 0·9 | 0·9 | 0·9 | Trace |

ND = Not detected Trace = Concentration less than 20 ng/ml
Values are expressed in μg/ml of urine (ppm).

Table 4. *Distribution of zearalenone and its metabolites in rabbit urine during the 96 hr following a single intramuscular dose of 500 mg zearalenone*

| Time after dosing (hr) | Percentage of dose excreted during period | Zearalenone (mg) | α-Zearalenol (mg) | β-Zearalenol (mg) |
|---|---|---|---|---|
| 0–24 | 64·2 | 159·8 | 98·5 | 83·7 |
| 24–48 | 8·96 | 18·5 | 13·2 | 13·2 |
| 48–96 | 0·62 | 0·89 | 1·0 | 1·2 |

Values are means for two rabbits; all metabolites were excreted as conjugates.

No conjugate was detected in the 72-hr urine of rats given 1·25 mg zearalenone/rat by gastric intubation. About 60% of the administered zearalenone was excreted as zearalenone and zearalenols (data not shown); the latter constituted less than 8% of the total metabolites.

Other rats were given zearalenone (5 mg) in the diet and the urine and faeces were collected over 96 hr. Although all rats consumed zearalenone-treated feed, there was considerable variation in the amount of feed and water consumed by each rat. Free zearalenone and its glucuronic acid adduct were the principle metabolites; only a trace of the isomeric zearalenols was found (Table 3). The concentration of metabolites found varied considerably, presumably because of biological variation, in spite of the fact that all rats received an identical dose. The fraction of the total dose excreted in the urine as total metabolites average about 30% and ranged from 20 to 60%.

In an attempt to isolate large quantities of conjugates of zearalenone, two rabbits were injected with 500 mg zearalenone as the sodium salt. The urine of the rabbit was collected over a period of 96 hr. The rabbit excreted zearalenone, α-zearalenol and β-zearalenol in the ratio of 2:1:1; more than 95% of each metabolite was in the glucuronide form (Table 4).

The metabolism of zearalenone by man was similar to that of swine with respect to the production of a large proportion of α-zearalenol and like the rabbit in exclusive production of glucuronide adducts (Table 5).

Table 5. *Distribution of zearalenone and its metabolites in the urine of man after a single oral dose of 100 mg zearalenone*

| Time (hr) | Zearalenone (μg ml) | α-Zearalenol (μg ml) | β-Zearalenol (μg ml) |
|---|---|---|---|
| 6 | 3·71 | 2·97 | ND |
| 12 | 6·87 | 6·00 | 2·66 |
| 24 | 2·69 | 4·02 | 1·97 |

ND = Not detected
Values are expressed in μg/ml of urine or ppm. All metabolites were in the glucuronide form; no free or sulphate forms were detected.

Table 6. *Distribution of zearalenone and its metabolites in the milk of a cow after dietary administration of zearalenone*

| Dose of zearalenone (mg/kg diet)* | Time after start of treatment (days) | Form of metabolite | Concn of metabolites in milk | | | Total metabolites (ng/ml) |
|---|---|---|---|---|---|---|
| | | | Zearalenone (ng/ml) | α-Zearalenol (ng/ml) | β-Zearalenol (ng/ml) | |
| 25† | 7 | Free | 210 | 186 | 189 | |
| | | Glucuronide conjugate | 152 | 322 | 64 | |
| | | Sulphate conjugate | 119 | ND | 117 | |
| | | | | | | 1359 |
| 250 | 1§ | Free | 45 | 50 | 64 | |
| | | Total conjugates‖ | ND | ND | ND | |
| | | | | | | 159 |
| | 2§ | Free | 62 | 45 | 63 | |
| | | Total conjugates‖ | 42 | 44 | 184 | |
| | | | | | | 440 |

ND = Not detected

*See experimental for details of dietary regime.
†At this dose the cow consumed approx 8 kg zearalenone-treated grain/day (200 mg zearalenone/day).
‡At this dose the cow consumed approx. 4 kg zearalenone-treated grain/day (1 g zearalonone/day).
§Days 1 and 2 at this dose are referred to as days 30 and 31 in the experimental.
‖The amount of sulphate conjugates found at this dose was insignificant. All samples were analysed in triplicate.

Zearalenone and α-zearalenol were found in roughly equal abundance after hydrolysis. β-Zearalenol was also present, but its concentration was about one-third of that of α-zearalenol.

Analysis of the cow's milk samples presented special problems owing to the low amounts of the metabolites and the lack of a suitable analytical method. A workable procedure was developed and was tested for precision using control milk with added zearalenone and zearalenols in the concentrations 1–10 μg/ml. The average percentage recoveries obtained for zearalenone and α-zearalenol were 67·5 ± 6·4 and 57 ± 2·8% respectively. We did not attempt any study to determine the recovery percentage and precision of our procedure at concentrations of less than 500 ng/ml. The distribution of the metabolites in milk is presented in Table 6.

The estimates of metabolites given in Table 6 are not corrected for recovery losses. The milk collected from the cow given 25 ppm dietary zearalenone for 7 days contained a sufficient concentration of zearalenone and its metabolites to determine them with reasonable precision (±10%) by HPLC and GC–MS. The milk collected from the cow 24 and 48 hr after administration of 250 ppm zearalenone had such low levels of zearalenone and its metabolites that they were only detected in the concentrated fractions collected by HPLC followed by analysis *via* selected ion monitoring. The estimates reported in the table were solely computed from the selected ion monitoring profile. No attempt was made to determine the recovery during fraction collection. We failed to notice any significant difference between the glucuronidase and aryl sulphatase hydrolysis estimates for these two milk samples. In order to determine possible artefacts, five control milk samples were run through an exactly identical procedure, and it was found that the selected ion monitoring profile on these control samples was devoid of zearalenone or zearalenols.

The MY-1 cow consumed about 8 kg of corn containing 25 ppm of zearalenone every day for 7 days before the milk was collected and yielded an average of 8 litre of milk per day during that period. Our calculations indicate that the milk obtained 7 days after initial administration contained a total of 2·9 mg zearalenone, 4·1 mg α-zearalenol and 3·8 mg β-zearalenol. The cow had consumed about 1·4 g of total zearalenone in 7 days, and about 0·7% of the consumed zearalenone was found in the milk as total metabolites.

A summary of the distribution of total zearalenone and its metabolites in the urine of bovine, porcine and rat species is shown in Table 7. Essentially, the cow excretes more than 50% of urinary zearalenone derivatives as total β-zearalenol whereas other species (swine, rat) excrete a greater percentage of zearalenone. In contrast to the cow, swine excrete α-zearalenol as their major metabolite and they hold this characteristic in common with man. The analysis of the faecal material obtained from the cow showed the same distribution of metabolite species as found in the urine. The milk samples, however, as shown in Table 6, do not show total β-zearalenol as the

Table 7. *Comparative metabolism of zearalenone: distribution of urinary and faecal metabolites*

| Species | Percentage distribution of | | |
|---|---|---|---|
| | Zearalenone | α-Zearalenol | β-Zearalenol |
| | | **In urine** | |
| Cow | 29 | 20 | 51 |
| Swine | 63 | 32 | 5 |
| Rat | 93 | 4 | 3 |
| | | **In faeces** | |
| Cow | 25 | 12 | 58 |
| Swine | 91 | 9 | ND |
| Rat | 97 | ND | 3 |

ND = Not detected

major zearalenol species, but rather α-zearalenol. The amount of total zearalenone in cows' milk was roughly equal to the total amount of the alpha and beta isomers of zearalenol. The metabolism of zearalenone in the cow appears to differ from that in swine.

In rats, zearalenone appears to be a principal constituent of the urinary metabolites. Mirocha *et al.* (1977) reported preliminary experiments in the study of metabolism of zearalenone by rats and detected free zearalenone and the glucuronide adduct of zearalenone. They recovered about 70% of the total zearalenone administered to rats by stomach intubation. We noted a fairly consistent pattern of excretion of zearalenone when administered to rats by intubation; however, considerable variation was observed repeatedly when zearalenone was fed as dietary zearalenone. The distribution of these metabolites in faeces was similar to their distribution in urine (Table 7).

The synthesis of α-zearalenol by the various animals is significant because this metabolite is three to four times more active oestrogenically than the parent compound zearalenone (Hagler *et al.* 1979). However, this derivative is often found as the glucuronic acid derivative and the biological activity of such conjugates is not known.

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# METABOLIC FATE OF T-2 TOXIN IN A LACTATING COW

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**Abstract**—Excretion, transmission and metabolism of T-2 toxin, a trichothecene mycotoxin produced by Fusarium species, was studied in a lactating Jersey cow. After daily oral administration of unlabelled T-2 toxin by capsule for 3 consecutive days, tritium-labelled T-2 toxin was administered orally on day 4. Maximum levels of radioactivity in excreta and plasma were reached at the following times after dosing (with the concentration, expressed as T-2 toxin, in parenthesis): faeces at 44 hr (9·2 ppm), urine at 16 hr (5·5 ppm), milk at 16 hr (37 ppb) and plasma at 8 hr (64 ppb). By 72 hr almost all of the radioactivity had been eliminated in the urine and faeces, in a ratio of 3:7 respectively, and 0·2% of the dose given to the cow had been transmitted into the milk. Insignificant accumulation of the toxin in specific organs was observed. Chromatographic analyses of the tritium residues in cow tissues and excreta revealed that T-2 toxin was rapidly metabolized, yielding principally three major unknown metabolites (designated TC-1, TC-3 and TC-6) in addition to very polar metabolite(s) designated TC-8. Within the first 24 hr, the three major metabolites accounted for 30–40% of the extractable radioactivity in urine, 60–70% in milk and 50–60% in plasma. Minor amounts of unmetabolized T-2 toxin and of the metabolites HT-2 toxin, neosolaniol, and 4-deacetylneosolaniol were also detected in the cow.

## INTRODUCTION

T-2 toxin (4$\beta$,15-diacetoxy-8$\alpha$-(3-methylbutyryloxy)-3$\alpha$-hydroxy-12,13-epoxytrichothec-9-ene) is a toxic metabolite produced by species of Fusarium (Bamburg & Strong, 1971) and has various toxic effects on laboratory and farm animals. This toxin, as well as diacetoxyscirpenol (Mirocha, Pathre, Schauerhamer & Christensen, 1976), deoxynivalenol and nivalenol (Yoshizawa & Morooka, 1977), are some of the most important trichothecene mycotoxins occurring naturally in agricultural products (Ghosal, Biswas & Chattopadhyay, 1978; Ghosal, Biswas, Srivastava, Chakrabarti & Chaudhary, 1978; Hsu, Smalley, Strong & Ribelin, 1972; Mirocha, Schauerhamer, Christensen & Kommedahl, 1979; Rukmini & Bhat, 1978). Moreover, the toxin is possibly involved in sublethal and lethal toxicoses of man (Joffe, 1971; Szathmary, Mirocha, Palyusik & Pathre, 1976; Ueno, Sato, Sakai & Enomoto, 1972b) and of farm animals, including cattle, pigs, horses and poultry (Hibbs, Osweiler, Buck & Mcfee, 1974; Hsu *et al.* 1972; Puls & Greenway, 1976; Ueno, Ishii, Sakai, Kanaeda, Tsunoda, Tanaka & Enomoto, 1972a). Consequently, there is a possibility of human consumption of animal products contaminated with T-2 toxin and its metabolites, and distribution and metabolism studies of the toxin in animals could provide important information both for evaluating and for controlling human exposure to residual T-2 metabolites in foods of animal origin.

T-2 toxin orally administered to mice and rats was rapidly eliminated into the faeces and urine without specific accumulation in any organ (Matsumoto, Ito & Ueno, 1978). The edible portions of the carcases of broiler chickens contained 0·06 and 0·04 ppm equivalents of T-2 toxin 24 and 48 hr, respectively, after dosing with 0·5 mg [$^3$H]T-2/kg body weight (Chi, Robison, Mirocha, Swanson & Shimoda, 1978b). About 0·9 $\mu$g [$^3$H]T-2 equivalents were transmitted into an egg by laying hens intubated daily with 1 mg [$^3$H]T-2/kg body weight for 8 consecutive days (Chi, Robison, Mirocha, Behrens & Shimoda, 1978a). The percentages of administered radioactivity found 18 hr after dosing in swine intubated with 0·1–0·4 mg [$^3$H]T-2/kg body weight were 0·7% in muscle and 0·29–0·43% in liver (Robison, Mirocha, Kurtz, Behrens, Weaver & Chi, 1979b).

In addition to these distribution studies, several authors have reported on the metabolism of T-2 toxin in animals. In liver homogenates and intestinal strips of rats, T-2 toxin was rapidly metabolized to HT-2 toxin, which was then converted into T-2 tetraol *via* 4-deacetylneosolaniol (Yoshizawa, Swanson & Mirocha, 1980a). The rats receiving T-2 toxin excreted HT-2 toxin, neosolaniol and several unknown metabolites in the excreta (Matsumoto *et al.* 1978). T-2 tetraol and 4-deacetylneosolaniol were found in the excreta of broiler chickens administered T-2 toxin, but the major metabolites were unidentified compounds (Yoshizawa, Swanson & Mirocha, 1980b).

In the lactating cow, T-2 toxin was transmitted into the milk at concentrations ranging from 10 to 160 ppb after intubation with 182 mg unlabelled T-2/day for 15 consecutive days (Robison, Mirocha, Kurtz, Behrens, Chi, Weaver & Nystrom, 1979a), but the metabolic fate of the toxin in lactating cows was unknown. This report describes the excretion, transmission and metabolism of [$^3$H]T-2 toxin in a lactating cow.

## EXPERIMENTAL

*T-2 toxin.* [$^3$H]T-2 toxin was synthesized by oxidation of T-2 toxin in the C-3 position with dimethyl-

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sulphite-*N*-chlorosuccimide, followed by reduction with sodium [$^3$H]borohydride as described by Wallace, Pathre, Mirocha, Robison & Fenton (1977). Prior to dilution with unlabelled T-2 toxin, the labelled toxin was identified by gas–liquid chromatography (GLC) and thin-layer chromatography (TLC), by comparison with a standard, and by proton magnetic resonance spectroscopy. The specific activity of the administered toxin was 190·1 mCi/mmol (408 $\mu$Ci/mg). The radiopurity, as determined by TLC followed by liquid scintillation spectrophotometry, was over 99%. The purity of unlabelled T-2 toxin used for pre-intubation was 98·2%, based on the GLC of its trimethylsilyl ether derivatives.

*Animal treatment.* A 375-kg lactating Jersey cow in good milk production was placed in the isolation stall of the College of Veterinary Medicine, University of Minnesota. A catheter (Foley retention catheter, size 24 FR) was inserted into the bladder for urine collection. The cow was provided with hay and a commercial pelleted 11%-protein dairy ration and water *ad lib.*

The cow was pre-intubated with finely crystalline T-2 toxin at 180 mg/day for 3 days by gelatine capsule, and during this period urine, faeces and milk samples were collected every 12 hr. On day 4, the cow received a single oral dose of powdered [$^3$H]T-2 toxin (156·9 mg, 64 mCi, equivalent to 0·171 mCi/kg body weight). These treatments were equivalent to dietary levels of 31–36 ppm T-2 toxin.

*Sampling.* After administration of the labelled toxin, urine and faeces samples were collected every 4 hr for 48 hr, and at 12-hr intervals thereafter until the cow was killed. An aliquot of each sample of urine or faeces was saved for radioassay, the remaining excreta being combined for urinary and faecal analysis of tritium-labelled T-2 metabolites. Blood samples were taken from the jugular vein 2 and 4 hr after administration of [$^3$H]T-2 toxin, then at 4-hr intervals up to 48 hr, and thereafter every 12 hr until termination. About 20 ml milk was collected every 4 hr for 48 hr after treatment with the labelled toxin, and the cow was machine-milked every 12 hr throughout the experiment. Although the cow showed a good appetite, milk and urine production decreased by about 38 and 50%, respectively, during the experimental period. The cow was killed 3 days after treatment with [$^3$H]T-2 toxin, and the following tissues and organs were collected for analysis of tritium residues: muscle, liver, kidney, fat, heart, bile, ovaries and mammary gland.

*Radioassay.* Tissues, faeces and whole blood were digested with perchloric acid–hydrogen peroxide and counted as previously described (Chi *et al.* 1978b). Aliquots of milk, urine and plasma were counted in Aquasol 2® (New England Nuclear, Boston, MA) without digestion. The radioactivity was counted on a Beckman LS-8000 liquid scintillation spectrophotometer. All samples were corrected for quench, and the background activity was subtracted.

*Extraction of milk.* The milk sample (200 ml) was homogenized with acetone (800 ml) and filtered by suction (Buchner). The residue was rinsed with acetone (50 ml × 2), dried at room temperature and digested for radioassay of the unextractable tritium in accordance with the procedure previously described (Yoshizawa *et al.* 1980b). The combined filtrate was evaporated to dryness *in vacuo*, and the remaining residue was redissolved in water (50 ml) and partitioned with petroleum ether (b.p. 60–70°C, 50 ml × 2). The petroleum ether layer was back-extracted with acetonitrile (30 ml). The acetonitrile was combined with the aqueous layer and concentrated *in vacuo* to yield about 30 ml of aqueous solution, which was introduced onto a pre-conditioned Amberlite XAD-2 resin column (1 × 14 cm, 20–50 mesh; Mallinckrodt Inc., Paris, KY). The column was eluted sequentially with 100 ml water and 100 ml methanol–water (9:1, v/v). The methanol eluate was concentrated and introduced onto a column containing Florisil (10 g, 1·5 × 12 cm, 60–100 mesh; Fisher Scientific Company, Fair Lawn, NJ) packed in chloroform–methanol (3:1, v/v) and topped with a layer of anhydrous sodium sulphate (5 g). The column was eluted with 100 ml chloroform–methanol (3:1, v/v) and then with 100 ml methanol. After evaporation of the solvent, each eluate was dissolved in an aliquot of methanol. The main stages of this procedure are outlined in Fig. 1.

*Extraction of excreta.* A 50-ml portion of each urine sample was chromatographed successively on Amberlite XAD-2 and Florisil columns as described above (Fig. 1). The water eluate from the XAD-2 column was evaporated to dryness, and the resulting distillate and the residue were counted for radioassay. Faeces were extracted in accordance with a procedure previously described (Yoshizawa *et al.* 1980b).

*Extraction of plasma.* Each plasma sample (10 ml) was diluted with an equal volume of water and introduced onto an Amberlite XAD-2 (1 × 14 cm) column (Fig. 1). The column was rinsed with 50 ml water and then eluted with 100 ml methanol–water (9:1, v/v). The methanol eluate was evaporated to dryness and analysed for T-2 metabolites.

*Quantification of T-2 metabolites by thin-layer radiochromatography.* The criteria for tentative quantification of T-2 metabolites were generally those of Yoshizawa *et al.* (1980b). An aliquot of each individual Florisil-chloroform/methanol eluate (for urine, faeces and milk) and XAD-methanol eluate (for plasma) was applied on a high-performance silica-gel TLC plate (10 × 10 cm; gel thickness 200 $\mu$m; Whatman Inc., Clifton, NJ) and developed in chloroform–methanol (9:1, v/v). After development the plate was scraped in a 2 mm-wide zone into a scintillation vial containing water (200 $\mu$l). Forty zones were collected for each sample. Ethanol (1 ml) was added to the vial, followed by Aquasol 2 (10 ml). Radioactive T-2 metabolites that appeared in the radiochromatogram of an individual sample were quantitated using the specific activity of the administered [$^3$H]T-2 toxin (9·058 × $10^5$ dpm/$\mu$g T-2 toxin).

## RESULTS

### *Tritium excretion and tissue residues*

The cumulative excretion of radioactivity in the urine, faeces and milk of a cow up to 72 hr after a single intubation of [$^3$H]T-2 toxin is shown in Fig. 2. Urinary excretion was rapid and substantially complete by 48 hr. Faecal excretion, however, was slow up to 24 hr, but more rapid between 24 and 48 hr and

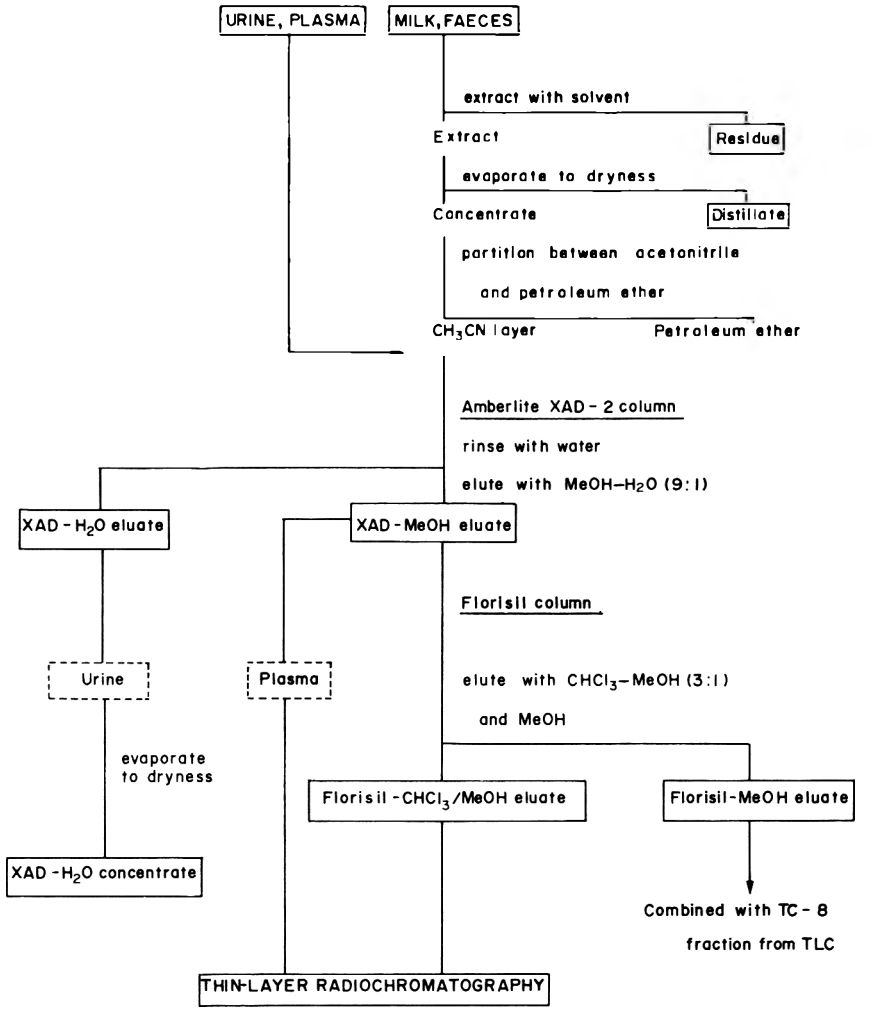


Fig. 1. Procedure for analysis of metabolites in the excreta, milk and plasma of a lactating cow intubated with [$^3$H]T-2 toxin.

substantially complete by 60 hr. By 72 hr, the cow had eliminated nearly 72% of the administered tritium in the faeces and almost 29% in the urine, so that almost all of the administered dose had been eliminated by the two routes. Only 0·2% of the radioactivity given to the cow was transmitted into the milk by 72 hr.

The pharmacodynamics of tritium elimination are shown in Figs 3 and 4. Maximum levels of radioactivity in excreta and plasma were reached at the following times after dosing, the relevant concentration, expressed in equivalents of T-2 toxin, being given in parenthesis: faeces at 44 hr (9·2 ppm), urine at 16 hr (5·5 ppm), milk at 16 hr (37 ppb), and plasma at 8 hr (64 ppb), and then the radioactivity in urine, milk and plasma decreased with half-lives of 12, 24 and 16 hr after dosing, respectively.

Analysis of radioactive residues in tissue samples, excreta, blood and milk 3 days after treatment revealed appreciable levels of tritium in bile, liver and kidney (equivalent, respectively, to 27·2, 18·5 and 13·9 ppb [$^3$H]T-2 toxin). These levels were higher than those in whole blood (13·3 ppb) and plasma (10·2 ppb) and in other tissues, including the spleen (9·4 ppb), heart (10·1 ppb), mammary gland (11·3 ppb), ovaries (10·7 ppb), muscle (8·8 ppb) and fat (4·7 ppb). The levels in faeces, urine and milk at this time were 136·9, 212·0 and 11·4 ppb [$^3$H]T-2 toxin equivalents.

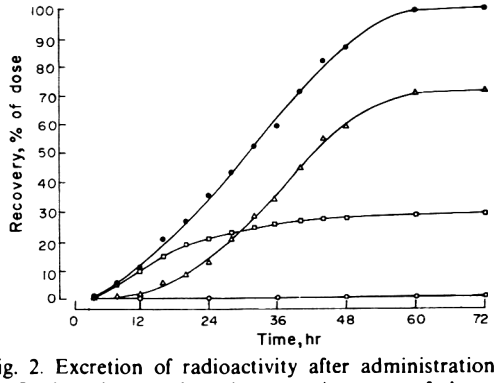


Fig. 2. Excretion of radioactivity after administration of [$^3$H]T-2 toxin to a lactating cow, in terms of the total excretion (●) and excretion in the urine (□), faeces (△) and milk (○), accounting respectively for 100·8, 71·8, 28·9 and 0·2% of the administered dose by 72 hr.

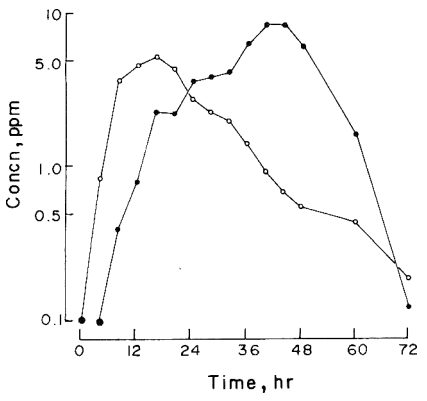


Fig. 3. Concentration of [$^3$H]T-2 equivalents in the urine (○) and faeces (●) of a lactating cow after administration of [$^3$H]T-2 toxin.

The tritium residues found in liver, kidney, spleen and heart were equivalent, respectively, to 0·0798, 0·0110, 0·0062 and 0·0166% of the administered radioactivity in the whole organ.

*Urinary metabolites of T-2 toxin*

By chromatography on XAD-2 columns, tritium residues in urine samples were concentrated in the XAD-90% methanol eluate in yields of 72% (12 hr), 54% (24 hr), 35% (48 hr) and 24% (72 hr). The remaining activity, however, was not adsorbed on the column resin and was eluted in the XAD-$H_2O$ fraction (Table 1). When this fraction was evaporated to dryness *in vacuo*, about 6–15% of the original radioactivity in urine was collected in the distillate, while most of the radioactivity remained in the concentrate in a yield of 24–54% of the original radioactivity in urine (440–2150 ppb equivalents of T-2 toxin), suggesting that very polar metabolites, including conjugates of T-2 metabolites, were eliminated in the cow urine.

After chromatography of Florisil columns, individual chloroform–methanol eluates, which contained 30–60% of the original tritium residues in urine, were analysed on silica-gel TLC plates to quantitate T-2 metabolites. Ten radioactive peaks appeared on the radiochromatogram and were designated as shown in Fig. 5a. None of the metabolites designated as TC-1, TC-3, TC-5, TC-7 and TC-8 corresponded to known T-2 derivatives. TC-6 showed an $R_F$ value similar to that of T-2 tetraol in chloroform–methanol (9:1, v/v), but moved a little faster than T-2 tetraol in chloroform–methanol (5:1, v/v). TC-8 located at the origin of the TLC plate seemed to be composed of several metabolites.

As is shown in Table 1, major urinary metabolites were TC-1, TC-3 and TC-6, ranging in concentrations from 340 to 530 ppb T-2 equivalents at 12 and 24 hr after dosing, and from 18 to 66 ppb T-2 at 48 hr. These three metabolites accounted for about 40% of the total radioactive metabolites in the 12-hr sample of cow urine. The urinary level of unmetabolized T-2 toxin was 44 and 11 ppb at 12 and 24 hr, respectively, and only 3 ppb 48 hr after treatment. Concentrations of neosolaniol, HT-2 toxin and 4-deacetylneosolaniol, which were chromatographically identical with standard compounds, ranged from 30 to 67 ppb T-2 equivalents at 12 and 24 hr, and were below 10 ppb 48 hr after treatment.

*Faecal metabolites of T-2 toxin*

The efficiency of extraction with acetonitrile was about 90–95%; the unextractable tritium (5–10%) remained in the residue (Table 1). An insignificant amount (below 1%) of radioactivity was found in the acetonitrile distillate. After chromatography on XAD-2 and Florisil columns, 53 to 64% of the faecal tritium residue was concentrated in the Florisil–chloroform/methanol fraction, with concentrations of 173 ppb (12 hr), 3749 ppb (24 hr) and 1740 ppb (48 hr) equivalents of T-2 toxin. Appreciable amounts, ranging from 12 to 19% of the original faecal radioactivity, were eluted in the XAD-water fraction, probably as conjugates of T-2 metabolites. Radioactivity of the Florisil–methanol eluate located at the origin of the TLC plate (referred to as TC-8) was 3–8% of the original faecal radioactivity. Total recovery of tritium residues through Florisil chromatography was 85–91%.

At least eight radioactive components were present in the extract of the 24-hr faecal sample subjected to TLC. As is shown in Table 1, most of the faecal metabolites were more polar than neosolaniol. Neither T-2 toxin nor the unidentified metabolite TC-1 was found in the faeces. Major faecal metabolites were TC-3 and TC-6, present in concentrations of 820–1500 ppb 24 hr after dosing. A considerable amount of TC-4 (4-deacetylneosolaniol) was also noted (530 and 220 ppb at 24 and 48 hr, respectively).

*T-2 metabolites in milk*

The recovery of radioactivity in milk by acetone extraction was around 90–96% in 12-, 24- and 36-hr milk samples, and decreased with time after dosing (Table 2). Appreciable quantities (over 20% of the original activity) were collected in the distillate of the acetone extracts; the increase in activity with time after treatment was probably due to radioactive water. After chromatography on Amberlite XAD-2 and Florisil columns, the tritium residues in the concentrate of the acetone extract were efficiently concentrated in the Florisil-chloroform/methanol (3:1) eluate, the yields for the 12-, 24-, 36- and 48-hr

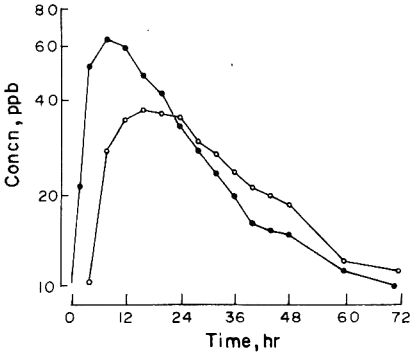


Fig. 4. Concentration of [$^3$H]T-2 equivalents in the milk (○) and plasma (●) of a lactating cow after administration of [$^3$H]T-2 toxin.

Table 1. *Urinary and faecal metabolites of T-2 toxin in a lactating cow given an oral dose of [$^3$H]T-2 toxin*

| Fraction/Metabolite | Concn/Recovery* | Metabolites in urine samples taken at† 12 | 24 | 48 | Metabolites in faecal samples taken at† 12 | 24 | 48 |
|---|---|---|---|---|---|---|---|
| **Solvent extraction and column chromatography** | | | | | | | |
| $CH_3CN$ residue | ppb | — | — | — | 21 | 709 | 144 |
| | % | | | | 7·65 | 10·01 | 5·26 |
| XAD-$H_2O$ eluate | ppb | 1027 | 2693 | 556 | 32 | 1035 | 506 |
| | % | 31·21 | 55·45 | 69·04 | 11·77 | 14·62 | 18·51 |
| XAD-$H_2O$ concentrate | ppb | 810 | 2153 | 435 | — | — | — |
| | % | 24·63 | 44·32 | 53·97 | | | |
| Florisil-$CHCl_3$/MeOH | ppb | 1981 | 1883 | 243 | 173 | 3749 | 1740 |
| eluate | % | 60·20 | 38·77 | 30·14 | 62·90 | 52·95 | 63·67 |
| **Separation by TLC** | | | | | | | |
| T-2 toxin | ppb | 44 | 11 | 3 | — | — | — |
| TC-1 | ppb | 515 | 366 | 18 | — | — | — |
| Neosolaniol | ppb | 49 | 35 | 4 | — | 30 | — |
| TC-2‡ | ppb | 67 | 66 | 9 | 10 | 99 | 28 |
| TC-3 | ppb | 474 | 494 | 46 | 56 | 822 | 217 |
| TC-4§ | ppb | 40 | 33 | 5 | 35 | 529 | 219 |
| TC-5 | ppb | 65 | 40 | 7 | — | 468 | 143 |
| TC-6 | ppb | 339 | 528 | 66 | 52 | 1500 | 1092 |
| TC-7 | ppb | 27 | 36 | 5 | 8 | 211 | 33 |
| TC-8‖ | ppb | 267 | 263 | 25 | 23 | 551 | 71 |

*Concn in ppb calculated in terms of T-2 toxin; recovery expressed as a percentage of the original radioactivity of the urines or faeces.
†Time (hr) after administration of [$^3$H]T-2 toxin.
‡HT-2 toxin.
§4-Deacetylneosolaniol.
‖Calculated by adding TC-8 to the Florisil-MeOH eluate.

samples being, respectively, 81, 67, 35 and 17% of the original radioactivity, and representing 29, 19, 7 and 3 ppb equivalents of T-2 toxin.

Figure 5b shows a representative thin-layer radiochromatogram of the milk extract of a 12-hr sample. Eight radioactive components were present in the extract. Three major components corresponded to TC-1, TC-3 and TC-6, as found in the urine. Table 2 provides a quantitative breakdown of the T-2 metabolites in the milk. The concentration of unmetabolized T-2 toxin in milk was about 2 ppb at 12 hr and below 0·1 ppb 36 hr after treatment. The major metabolites TC-1, TC-3 and TC-6 were present in concentrations ranging from 2·5 to 8·7 ppb in 12- and 24-hr samples. These three metabolites accounted for about 60–70% of the extractable metabolites in milk samples up to 36 hr after dosing. The relative amounts of these metabolites changed with time after dosing and the tendency was toward relatively greater quantities of more polar metabolites such as TC-3 and TC-6. After 36 hr, concentrations of individual metabolites except TC-6 were below 1 ppb, while TC-6 was still present at about 2 ppb equivalents of T-2 toxin 48 hr after dosing.

*T-2 metabolites in plasma*

After chromatography on XAD-2 columns, the tritium residues in plasma were efficiently concentrated in XAD-methanol eluates with recoveries of 69–93%, but appreciable quantities of radioactivity (4–13 ppb T-2 equivalents; 7·4–27·5% of the radioactivity in plasma) were eluted in the XAD-water fraction. The proportion in the latter fraction increased with time after dosing (Table 2), probably as a result of both radioactive water and polar conjugates of T-2 metabolites.

As is shown in Fig. 6, at least eight radioactive metabolites were present in the plasma. The major metabolites, TC-1, TC-3, TC-6 and TC-8 accounted for between 51 and 64% of the extracted tritium residue; the relative amounts changed with time after

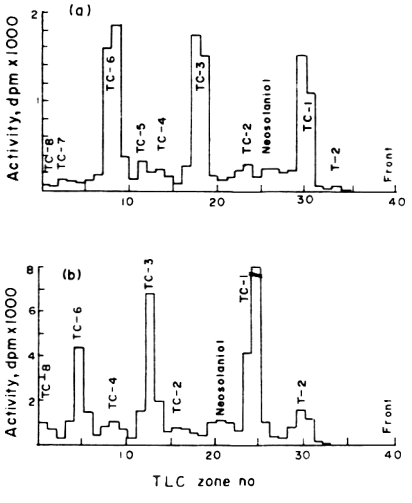


Fig. 5. Thin-layer radiochromatograms of Florisil-chloroform methanol eluates fractionated from (a) the urine and (b) the milk of a lactating cow intubated with [$^3$H]T-2 toxin, the urine sample being taken 24 hr and the milk sample 12 hr after treatment.

Table 2. *Metabolites of T-2 toxin in milk and plasma of a lactating cow given an oral dose of [$^3H$]T-2 toxin*

| Fraction/Metabolite | Concn/Recovery* | Metabolites in milk samples taken at† | | | | Metabolites in plasma from blood samples taken at† | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 12 | 24 | 36 | 48 | 4 | 8 | 16 | 20 |
| **Solvent extraction and column chromatography** | | | | | | | | | |
| Acetone residue | ppb | 1·4 | 2·5 | 2·1 | 3·0 | — | — | — | — |
| | % | 4·03 | 8·86 | 10·41 | 18·25 | | | | |
| Acetone distillate | ppb | 7·4 | 8·3 | 8·9 | 9·5 | — | — | — | — |
| | % | 20·91 | 29·45 | 43·95 | 57·81 | | | | |
| XAD-$H_2O$ eluate | ppb | 0·7 | 1·3 | 0·7 | 0·7 | 3·7 | 8·3 | 13·4 | 11·4 |
| | % | 2·10 | 4·53 | 3·26 | 4·26 | 7·36 | 13·18 | 26·12 | 27·53 |
| XAD-MeOH eluate | ppb | — | — | — | — | 47·0 | 56·5 | 37·2 | 28·7 |
| | % | | | | | 92·84 | 89·34 | 72·25 | 69·29 |
| Florisil-$CHCl_3$/MeOH | ppb | 28·5 | 18·82 | 7·06 | 2·87 | — | — | — | — |
| eluate | % | 80·91 | 66·86 | 34·78 | 17·48 | | | | |
| **Separation by TLC** | | | | | | | | | |
| T-2 toxin | ppb | 2·0 | 0·2 | <0·1 | <0·1 | 8·4 | 5·9 | 1·1 | 0·5 |
| TC-1 | ppb | 8·7 | 2·5 | 0·5 | 0·1 | 16·3 | 12·2 | 5·5 | 3·8 |
| Neosolaniol | ppb | 1·8 | 0·9 | 0·1 | <0·1 | 0·6 | 2·0 | 0·7 | 0·6 |
| TC-2‡ | ppb | 1·2 | 0·9 | 0·2 | 0·1 | 1·1 | 1·7 | 0·8 | 0·3 |
| TC-3 | ppb | 5·7 | 4·7 | 0·8 | 0·7 | 4·7 | 4·7 | 7·9 | 2·6 |
| TC-4§ | ppb | 1·5 | 1·3 | 0·5 | 0·2 | 0·5 | 1·2 | 0·6 | 0·8 |
| TC-6 | ppb | 3·9 | 6·8 | 3·5 | 1·9 | 4·1 | 7·1 | 6·9 | 7·5 |
| TC-8 | ppb | 1·9‖ | 1·7‖ | 1·0‖ | 0·6‖ | 3·3 | 12·1 | 8·4 | 9·1 |

*Concn in ppb calculated in terms of T-2 toxin; recovery expressed as a percentage of the original radioactivity of the milk or plasma.
†Time (hr) after administration of [$^3$H]T-2 toxin.
‡HT-2 toxin.
§4-Deacetylneosolaniol.
‖Calculated by adding TC-8 to the Florisil-MeOH eluate.

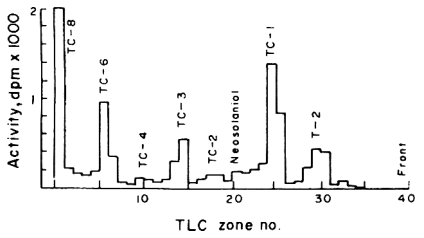


Fig. 6. Thin-layer radiochromatogram of XAD-methanol eluate from a plasma sample taken 8 hr after treatment from a lactating cow intubated with [$^3$H]T-2 toxin.

dosing (Table 2), with a tendency towards increasing quantities of the more polar metabolites such as TC-3, TC-6 and TC-8.

The plasma levels of T-2 toxin and TC-1 peaked at 8 ppb and 16 ppb, respectively, as early as 4 hr after dosing. With TC-3, the peak level (around 8 ppb T-2 equivalents) was maintained during the period 12–16 hr after treatment. The unidentified major metabolites, namely TC-1, TC-3, TC-6 and TC-8, were still present in appreciable concentrations (3·8, 2·6, 7·5 and 9·1 ppb T-2 equivalents, respectively) 20 hr after treatment. On the other hand, concentrations of T-2 toxin, HT-2 toxin (TC-2), neosolaniol and 4-deacetylneosolaniol were below 1 ppb 20 hr after treatment.

## DISCUSSION

As indicated by the presence of radioactivity in the blood system within 2 hr of intubation (Fig. 4), T-2 toxin intubated in crystalline form was absorbed from the gastro-intestinal tract. It became evenly distributed in many tissues and organs, but within 72 hr almost all of the toxin and its metabolites had been rapidly excreted in the urine and faeces in a ratio of 3:7 (Fig. 2). This figure was in close agreement with those found in rodents (Matsumoto *et al.* 1978), in which the toxin administered in olive oil was excreted in the urine and faeces in a ratio of 1:5 (rats) and 1:3 (mice). Thus faeces rather than urine represent a more important excretion route for T-2 metabolites.

The quick incorporation and higher rate of disappearance of [$^3$H]T-2 toxin was also reported in broiler chickens (Chi *et al.* 1978b) and swine (Robison *et al.* 1979b). The toxin did not accumulate in specific organs in the cow, but the tritium residues in the bile and liver were a little higher than the whole blood level. Retention of radioactivity by these organs was also observed in broiler chickens (Chi *et al.* 1978b) and in rats and mice (Matsumoto *et al.* 1978). This fact indicates that a large amount of the absorbed toxin and its metabolites was eliminated into the intestinal tract through the biliary excretion system.

The delayed elimination of a large amount of radioactivity in the cow faeces, compared with the urinary excretion (Fig. 3), suggests that T-2 toxin and its metabolites probably circulate in the enterohepatic system of the cow. Consequently, T-2 metabolites circulated in this system may cause several histopathological lesions in the intestinal tract of animals, such as have been found in rodents (Saito & Ohtsubo, 1974), the guinea-pig (DeNicola, Rebar, Carlton & Yagen. 1978), cattle (Hibbs *et al.* 1974) and swine (Weaver, Kurtz, Bates, Chi, Mirocha, Behrens & Robison, 1978). Further investigations on the relation between the level of T-2 metabolites in the intestines and the extent of the histopathological lesions found there could provide information on the mechanism by which T-2 toxin exerts its effects.

In view of the possible human consumption of edible tissues contaminated with T-2 toxin, the tritium residues noted in the meat, liver, heart and milk may be important. Table 3 shows the experimentally derived relationships between the tritium residues in cow tissues or plasma and [$^3$H]T-2 toxin levels in the feed, in comparison with data obtained for broiler chickens (Chi *et al.* 1978b) and swine (Robison *et al.* 1979b). The tissue/feed ratio of tritium in the edible tissues of the cow 72 hr after dosing ranged from 0·0003 to 0·0006. These figures were 5–10% of those in swine 18 hr after dosing. As for the chickens, the tissue/feed ratio was higher than those in the cow and pig, ranging from 0·010 to 0·014 for meat and heart, and from 0·021 to 0·027 for liver, regardless of T-2 dosage.

It is notable that the tissue/plasma ratios of T-2 metabolites, ranging from 0·8 to 1·0 in meat and heart and from 1·6 to 3·5 in liver, are independent of animal species, of T-2 dosage and of time after dosing (Table 3). This fact suggests that the residue of T-2 metabolites in edible tissues could be estimated diagnostically from the residues in plasma. As for the milk, the milk/plasma ratio of radioactivity in the cow treated with [$^3$H]T-2 toxin increased linearly up to 24 hr and thereafter ranged from 1·0 to 1·3 (data not shown).

Chromatographic analyses of the tritium residues in cow tissues and excreta revealed that the major metabolites of T-2 toxin were unidentified compounds designated TC-1, TC-3 and TC-6. Within the first 24 hr, these three metabolites accounted for 30–40% of the extractable radioactivity in urine, 60–70% in milk, and 50–60% in plasma (Tables 1 & 2).

Unmetabolized T-2 toxin in the plasma represented less than 20% of the extractable radioactivity shortly (4 hr) after dosing (Table 2). This indicated that T-2 toxin absorbed from the intestinal tract was rapidly metabolized to the major metabolites (TC-1, TC-3, TC-6), which then circulated in the blood to distribute evenly in many tissues and organs.

The unidentified metabolites TC-1 and TC-3 were found in bovine excreta. These correspond to TB-1 and TB-3, respectively, found in the excreta (Yoshizawa *et al.* 1980b) and edible tissue (T. Yoshizawa, S. P. Swanson & C. J. Mirocha, unpublished data, 1979) of broiler chickens given [$^3$H]T-2 toxin. Moreover, these compounds may correspond to one of the unknown metabolites found in rats given [$^3$H]T-2 toxin (Matsumoto *et al.*, 1978). This suggests that many animals are capable of metabolizing T-2 toxin in a pattern similar to that of cow, and that the metabolites TC-1, TC-3 and TC-6 are appropriate indexes for estimating intake of the toxin.

It has already been reported (Yoshizawa *et al.* 1980a) that in the liver homogenate and intestinal

Table 3. *The relationship between the level of [$^3$H]T-2 toxin in the feed or the tritium residues in plasma and tritium levels in the edible tissues of the cow, chick and pig*

| Tissue | Animal | Time after dosing* (hr) | Feed level† (ppm) | Tissue level‡ (ppb) | Tissue/feed ratio | Tissue/plasma§ ratio |
|---|---|---|---|---|---|---|
| Meat | Cow | 72 | 31·38 | 8·8 | 0·0003 | 0·863 |
| | Chick‖ | 24 | 1·26 | 17·3 | 0·0137 | 1·000 |
| | | | 5·0 | 59·2 | 0·0118 | 0·938 |
| | | | 18·95 | 228·6 | 0·0121 | 0·875 |
| | Swine¶ | 18 | 1·25 | 3·1 | 0·002 | 0·775 |
| Heart | Cow | 72 | 31·38 | 10·1 | 0·0003 | 0·991 |
| | Chick‖ | 24 | 1·26 | 13·7 | 0·011 | 0·792 |
| | | | 5·0 | 49·4 | 0·010 | 0·783 |
| | | | 18·95 | 207·7 | 0·011 | 0·795 |
| | Swine¶ | 18 | 1·25 | 3·9 | 0·003 | 0·975 |
| Liver | Cow | 72 | 31·38 | 18·5 | 0·0006 | 1·863 |
| | Chick‖ | 24 | 1·26 | 34·0 | 0·0270 | 1·965 |
| | | | 5·0 | 107·3 | 0·0215 | 1·700 |
| | | | 18·95 | 431·0 | 0·0227 | 1·649 |
| | Swine¶ | 18 | 1·25 | 13·8 | 0·011 | 3·450 |
| Milk | Cow | 72 | 31·38 | 11·4 | 0·0004 | 1·118 |

*Animals were intubated with a single dose of [$^3$H]T-2 toxin.

†Estimates of feed levels were based on the assumption that each animal would consume the following amount of feed daily: cow, 5 kg; chick, 100 g; swine, 600 g.

‡Values were calculated from residual tritium levels in the edible tissues of animals given [$^3$H]T-2 toxin and were expressed as equivalents of [$^3$H]T-2 toxin (ppb).

§The plasma levels were 10·2 ppb equivalents of T-2 toxin in the cow, and, in chicks, 17·3, 63·1 and 261·3 ppb equivalents of T-2 toxin at feed levels of 1·26, 5·0 and 18·95 ppm, respectively. The whole-blood level of residual tritium in swine was 4·0 ppb equivalents of T-2 toxin.

‖The values in chicks were calculated from the data reported by Chi *et al.* (1978b).

¶The values in swine were calculated from the data reported by Robison *et al.* (1979b).

strips of rats, T-2 toxin was deacylated stepwise at the C-4, C-8 and C-15 positions; the toxin was metabolized initially to HT-2 toxin, which was converted into T-2 tetraol *via* 4-deacetylneosolaniol. These compounds were minor metabolites in the tissues and excreta of the cow, as indicated in the present study. Although none of the unidentified metabolites (TC-1, TC-3, TC-6) were formed by *in vitro* metabolism of T-2 toxin in rat-liver homogenate, we anticipate the participation of a liver-enzyme system in the formation of TC-1, TC-3 and TC-6, having ruled out the possibility of the formation of these unidentified metabolites in the cow rumen; none was detected in the rumen contents of cows receiving unlabelled T-2 toxin daily for 4 consecutive days (unpublished data).

After XAD-2 chromatography of excreta, milk and plasma samples, appreciable amounts of radioactivity were found in the XAD-water eluates, which may contain very polar metabolites (Tables 1 & 2). Some portions of the tritium residue in this fraction were distilled, but the distillable radioactivity was estimated to be below 3% of the total dose and was probably due to radioactive water. The relative amount of the remaining polar metabolites (XAD-$H_2O$ concentrate) increased with time after dosing. We consider that conjugates of T-2 metabolites may be present in this fraction, since a significant amount of the less polar metabolites was liberated by enzymatic hydrolysis (data not shown).

Isolation and characterization of the major metabolites are in progress, but none of these metabolites corresponds to a partial deacetylation product of T-2 toxin. Metabolites TC-1 and TC-3 both showed a positive colour reaction with a 4-(*p*-nitrobenzyl)pyridine/tetraethylenepentamine reagent (Kato & Takitani, 1978), indicating the presence of an intact 12,13-epoxide ring.

On the basis of these data, it is concluded that T-2 toxin was rapidly metabolized, mainly to three unidentified metabolites in addition to very polar metabolite(s), and in these forms was distributed evenly in cow tissues and organs. Within 72 hr of dosing, almost all of the T-2 metabolites had been eliminated, mainly in the faeces and urine. The metabolites transmitted into the milk represented about 0·2% of the administered dose.

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# THREE-GENERATION REPRODUCTION STUDY OF RATS INGESTING 2,4,5-TRICHLOROPHENOXYACETIC ACID IN THE DIET

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**Abstract**—Male and female 4–6-wk-old Sprague–Dawley rats ($F_0$) were fed lab chow containing 2,4,5-T to provide dosage levels of 0, 3, 10 or 30 mg/kg/day for 90 days and were then bred. At day 21 of lactation, pups were randomly selected for the following generation ($F_1$) and the rest were autopsied. Subsequent matings were conducted to produce $F_2$, $F_{3a}$ and $F_{3b}$ litters, successive generations being fed from weaning on the appropriate test or control diet. Fertility was decreased in the matings for the $F_{3b}$ litters in the group on the 10-mg/kg/day dose level. Postnatal survival was significantly decreased in the $F_2$ litters of the 10-mg/kg group and the $F_1$, $F_2$ and $F_{3a}$ litters of the 30-mg/kg/day group. The relative liver weight of weanlings was significantly increased in the $F_2$, $F_{3a}$ and $F_{3b}$ litters of the 30-mg/kg/day group. A significant decrease in relative thymus weight was seen only in the $F_{3b}$ generation of this dosage group. Thus, dose levels of 2,4,5-T that were sufficiently high to cause signs of toxicity in neonates had no effect on the reproductive capacity of rats, except for a tendency toward a reduction in postnatal survival at a dose level of 30 mg/kg/day. Reproduction was not impaired at the lowest dose level (3 mg/kg/day).

## INTRODUCTION

Numerous studies have been conducted, using a variety of laboratory and non-laboratory animal species, to evaluate the teratogenic potential of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). Studies have been reported on mice, rats, hamsters, rabbits, sheep, rhesus monkeys and reindeer, using samples of 2,4,5-T of varying degrees of purity (Binns & Balls, 1971; Collins & Williams, 1971; Courtney, Gaylor, Hogan, Falk, Bates & Mitchell, 1970; Courtney & Moore, 1971; Emerson, Thompson, Strebing, Gerbig & Robinson, 1971; Erne & Nordkvist, 1972; Khera & McKinley, 1972; Neubert & Dillman, 1972; Roll, 1971; Sparschu, Dunn, Lisowe & Rowe, 1971; Wilson, 1971). The content of the contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) ranged from less than 0·01 ppm to approximately 30 ppm in the samples used. In some studies, 2,4,5-T and TCDD were administered simultaneously, using oral dose levels of 50–100 mg 2,4,5-T/kg/day in combination with 0·01–3 μg TCDD/kg/day.

Considered overall, these studies indicate that purified 2,4,5-T is teratogenic in mice when administered at high dose levels. Dose levels of 35 mg 2,4,5-T/kg/day or higher given orally were generally associated with cleft palate and/or kidney malformations in mice. High doses given to other mammalian species by various routes of administration resulted primarily in adverse effects on the development of skeletal structures, changes that reflected retarded development rather than malformation. These changes are very probably a reflection of the stress to the maternal organism resulting from large doses of 2,4,5-T rather than of the direct interaction of the agent with the developing foetus. The potential for 2,4,5-T to cause a teratogenic effect is dependent upon the TCDD content of the samples as well as on the dose administered.

In response to a lack of reproduction data in the literature, this three-generation reproduction study in rats was initiated. The objective of the study was to determine whether chronic ingestion of 2,4,5-T by rats has a detrimental effect on reproduction and on the development of the resultant offspring.

## EXPERIMENTAL

*Test material.* The purified 2,4,5-T (ref. AGR 133711) used for this study was prepared by the Dow Chemical Company, Midland, MI. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin was not present in the sample down to the detection limit of 0·03 ppb.

*Diet preparation.* A 1% premix was prepared by dissolving an appropriate amount of 2,4,5-T in acetone and mixing it thoroughly with ground laboratory chow. This premix, after drying, was mixed with laboratory chow to prepare diets supplying 3, 10 and 30 mg 2,4,5-T/kg body weight/day. The body weights and food consumption of 20 rats/sex/group were used as the basis for preparing these diets. Control rats were supplied with ground laboratory chow treated with an equivalent amount of acetone. Portions of each prepared diet from each dose level were retained during wk 1, 8, 13, 52 and 104, and samples were analysed for 2,4,5-T.

*Animals.* Male and female 4–6-wk-old Sprague–Dawley rats (Spartan substrain) obtained from Spartan Research Animals, Haslett, MI, were placed randomly in suspended wire-bottom cages (two per cage) and allowed laboratory chow (Ralston Purina Company, St. Louis, MO) and tap water *ad lib.*

Animals were housed in a room controlled for temperature, humidity and light cycle.

*Experimental design.* Randomized groups of male and female $F_0$ rats were fed a diet containing sufficient 2,4,5-T to provide dose levels of 0, 3, 10 or 30 mg/kg/day for 90 days, beginning at 4–6 wk of age. The $F_0$ rats in the control and high-dose groups consisted of 16 males and 32 females, while groups of ten males and 20 females were used for the low and intermediate dose levels. Mating of the $F_0$ treated animals to produce the $F_1$ litters began after 90 days on the test diets. The breeding period consisted of two 6-day mating periods, each with a different male, separated by a 6-day rest period. The $F_1$ and $F_2$ rats were mated at an average age of 130 days, and were fed the appropriate test or control diets from weaning.

After the second mating period, the $F_0$ females were placed in individual cages containing ground corncob litter for nesting. A screen was placed in the bottom of each cage at the end of wk 1 of lactation. After mating, the $F_1$ and $F_2$ females were placed in plastics shoe-box-type cages until their young were weaned or killed. The $F_2$ adults were mated a second time to produce the $F_{3b}$ litters because of a questionable effect on the survival of neonates in the $F_{3a}$ litters. Because the type of cage used to rear the $F_2$ and $F_3$ litters in this study differed from those used for the $F_1$ litters and for all the earlier reproduction studies in this laboratory, there were no historical control data which could be used for comparison.

*Observations.* For each generation, body weights of the male and female rats were recorded weekly during the period prior to mating. The amount of food consumed was recorded twice weekly prior to mating. The females were observed daily for signs of normal and abnormal parturition. The date of parturition was recorded, and the number of days from the first cohabitation to parturition was calculated. The numbers of live and dead newborn pups were recorded on the day of parturition (day 0) and the numbers of live pups were noted on days 1, 7, 14 and 21 after delivery. Each litter was weighed on days 1, 7 and 14 of lactation. On day 21, individual body weights of the weanlings were recorded. At each weighing period, individual pups were observed for external anomalies and overt signs of toxicity and their sex was verified.

*Mating.* To ensure a high overall pregnancy rate, each female was placed with a male for two 6-day matings, each with a different male, separated by a 6-day resting period. The pan beneath the cage was examined each morning for the presence of a vaginal plug. This design was used for all matings. During the mating period, both sexes were maintained on diets containing 2,4,5-T at the concentrations calculated for the females. After the mating period, males were returned to their original cages and given the 2,4,5-T-containing diets as prepared for the males. The females were placed in individual cages containing ground corn-cob litter for nesting. The females continued to receive the test diets throughout gestation and lactation.

*Gross autopsy and histopathology of weanlings.* A gross pathological examination was conducted on the weanlings that were not randomly selected as future parents. Internal soft tissue anomalies were recorded. The liver, kidneys and thymus from between four and six 21-day-old pups of each sex from the $F_1$, $F_2$, $F_{3a}$ and $F_{3b}$ litters of each dosage group were weighed and histological examination of these tissues was conducted on paraffin-embedded sections stained with haematoxylin and eosin.

*Gross autopsy of adults.* Rats dying during the course of the study were subjected to a gross pathological examination. At the termination of each generation, a gross autopsy was conducted on all surviving rats.

*Statistical evaluation.* The indices of reproduction, such as fertility and survival indices, were evaluated statistically by the Fisher's Exact Probability Test (Siegel, 1956). Analysis of the incidence of changes among the weanlings was made by the Wilcoxon test as modified by Haseman & Hoel (1974). Analysis of the body weights and of food consumption was made by a one-way analysis of variance and Dunnett's test (Steel & Torrie, 1960). The level of significance chosen in all cases was $P < 0.05$.

## RESULTS

### *Clinical observations*

No compound-related trends were seen in food consumption, body-weight change, demeanour or physical appearance. Among adult $F_0$ males on 10 mg 2,4,5-T/kg/day, the mean body weight was significantly decreased at day 148 of age. Mean body weights of $F_1$ adult females on 30 mg/kg/day were significantly decreased at age 48 and 64 days. Food consumption of $F_2$ female adults producing $F_{3a}$ litters and fed 3 or 10 mg/kg/day were significantly decreased at 83–94 days of age. At all other intervals, the body weights and food consumption were comparable between control and all treated groups.

During gestation of the $F_{3b}$ litters, some of the adult females in the control and treated groups suffered water deprivation at various periods as a result of malfunctioning of the automatic watering system. The rats affected were two controls, and two from the 3-mg/kg, six from the 10-mg/kg and one from the 30-mg/kg dosage groups. The affected rats showed dark and red-brown crusty material around the nostrils and eyes. This water deprivation occurred primarily in the latter third of gestation and after parturition. The litters most affected, as measured by the death of pups, were those deprived of water after parturition, except for one 30-mg/kg dam which had two periods of deprivation on about days 17 and 21 of gestation and which delivered its entire litter of 12 dead. The animals deprived of water after parturition included pups from one control (15/15 dead), from one adult on 3 mg/kg (8/11 dead), and from one on 10 mg/kg (10/11 dead). Body weight and eye–nose symptoms returned to normal within 24 hr of the provision of water. Because each dose group contained one dramatically affected litter, the inclusion or exclusion of these litters for statistical analysis would not have changed any conclusions on statistical significance. All indices for the $F_2$ adults and $F_{3b}$ litters include the litters from these water-deprived dams.

### *Fertility index*

The fertility index (the number of females delivering a litter expressed as a percentage of the total number

Table 1. *Fertility of rats given 2,4,5-T in the diet*

| 2,4,5-T dose (mg/kg/day) | Fertility Index† for generation: | | | |
|---|---|---|---|---|
| | | | $F_2$ | |
| | $F_0$ | $F_1$ | 1st mating | 2nd mating |
| 0 | 30/32 (94) | 30/32 (94) | 33/34 (97) | 29/32 (91) |
| 3 | 18/20 (90) | 21/22 (95) | 21/22 (95) | 17/22 (77) |
| 10 | 18/20 (90) | 20/20 (100) | 22/22 (100) | 15/22 (68*) |
| 30 | 32/32 (100) | 30/32 (94) | 32/34 (94) | 25/33 (76) |

†FI = no. of females delivering a litter/no. of females placed with a male (expressed as a percentage in brackets).
The value marked with an asterisk differs significantly from the control value: *$P < 0{\cdot}05$ by Fisher's exact probability test.

placed with a male) did not differ significantly from that of the controls for the $F_0$, $F_1$ and $F_2$ (first mating) adults of any group given 2,4,5-T in the diet (Table 1). The fertility index for the $F_2$ second mating was significantly lower than that of the controls at the 10-mg/kg/day dose level. All fertility indices for the $F_2$ second mating were below those of earlier generations, with the 3-, 10- and 30-mg/kg/day dosage groups showing a greater decline than the controls in this respect.

*Litter size at birth*

There was no significant reduction in litter size at birth at any dose level in any generation. The mean number of pups per litter at birth was significantly greater in the $F_2$ generation of the 3-mg/kg/day group than in the controls (Table 2). The average number of days from the first cohabitation to the day of parturition was not significantly altered at any dose level in any generation.

*Survival*

The gestation survival index (the percentage of pups alive at birth) was significantly less for the $F_1$ litters of the groups fed 3 or 30 mg/kg/day than for control pups (Table 3), but was significantly greater than the controls for the $F_{3a}$ litters of the 10-mg/kg/day group.

The postnatal survival index (the percentage of live-born pups surviving to 21 days of age) was significantly lower than the control figure in the pups of the $F_2$ litters of the 10-mg/kg group and the $F_1$, $F_2$ and $F_{3a}$ litters of the 30-mg/kg/day group (Table 3). However, when the $F_2$ adults of the 30-mg/kg/day dosage group were re-mated to obtain the $F_{3b}$ litters, survival at 21 days was significantly greater than that in the controls. The survival of the 3-mg/kg/day litters was significantly greater than that of the controls in the $F_1$ generation and did not differ from the control value in the following three generations of litters.

*Postnatal body weights and sex ratios*

The average body weight of 21-day-old rats from dams given 30 mg 2,4,5-T/kg/day was significantly lower than those of the $F_2$ male and $F_{3b}$ male and female control litters. The average body weight of 21-day-old rats in the 3-mg/kg/day $F_2$ litters was significantly lower than the weight of the corresponding controls. This significant decrease is attributable to a significantly larger litter size rather than to a compound-related effect.

The sex ratio (M:F) of the $F_1$ 21-day-old pups at the 3-mg/kg/day dose level differed significantly from the control ratio in favour of females (40:60) but no other significant differences were recorded in this parameter.

*Autopsy findings*

Anomalies found included dilated renal pelvis, subcapsular haemorrhage of the kidney, small thymus and small testes, but no generation at any dose level differed significantly from the controls in the incidence of any finding.

No alterations in the weight of liver, kidney or thymus were seen in the $F_1$ weanlings. Liver weight (absolute but not relative) in the male $F_2$ weanlings of

Table 2. *Numbers of pups per litter at birth among rats given 2,4,5-T in the diet*

| 2,4,5-T dose (mg/kg/day) | Mean no. of pups/litter at birth in generation: | | | |
|---|---|---|---|---|
| | $F_1$ | $F_2$ | $F_{3a}$ | $F_{3b}$ |
| 0 | 12 ± 2 | 10 ± 4 | 12 ± 2 | 9 ± 3 |
| 3 | 10 ± 3 | 13 ± 2* | 10 ± 4 | 11 ± 3 |
| 10 | 11 ± 3 | 11 ± 3 | 11 ± 3 | 7 ± 4 |
| 30 | 10 ± 4 | 10 ± 4 | 11 ± 4 | 9 ± 4 |

Values are means of the nos of pups (alive or dead)/litter ± 1 SD, for the numbers of litters indicated in Table 1. That marked with an asterisk differs significantly from the control value: *$P < 0{\cdot}05$ by Dunnett's test.

Table 3. *Gestation survival and postnatal survival among litters of rats given 2,4,5-T in the diet*

| 2,4,5-T dose (mg/kg/day) | Survival index in generation: $F_1$ | $F_2$ | $F_{3a}$ | $F_{3b}$ |
|---|---|---|---|---|
| | **Gestation Survival Index†** | | | |
| 0 | 341/346 (99) | 310/316 (98) | 361/367 (98) | 254/268 (95) |
| 3 | 177/187 (95*) | 271/279 (97) | 205/207 (99) | 186/193 (96) |
| 10 | 189/190 (99) | 246/253 (97) | 240/242 (99) | 105/114 (92) |
| 30 | 324/343 (94*) | 347/364 (95*) | 346/359 (96) | 234/253 (92) |
| | **Postnatal Survival Index‡** | | | |
| 0 | 317/364 (87) | 302/310 (97) | 358/372 (96) | 197/254 (78) |
| 3 | 177/190 (93*) | 263/271 (97) | 191/205 (93)†† | 141/186 (76) |
| 10 | 145/176 (82)§ | 232/255 (91*¶¶ | 225/240 (94) | 90/111 (81) |
| 30 | 227/315 (72*)‖ | 336/360 (93*) | 327/353 (93*) | 198/234 (85*) |

†GSI = no. of pups alive at birth/total no. of pups born (expressed as a percentage in brackets).

‡PSI = no. of liveborn pups surviving for 21 days/total no. of liveborn pups (expressed as a percentage in brackets). Litter data from dams observed in the initial stages of delivery in which no reasonable estimate of litter size on day 0 could be made were excluded from the calculation of GSI. These data were included for calculation of PSI, thus in some cases resulting in a denominator for post-natal survival that is greater than the corresponding numerator for gestation survival.

§No data were recorded for dam no. 8312 on day 21 of gestation; her litter of 13 was excluded for the calculation of PSI.

‖No data were recorded for dam no. 8342 on day 21 of gestation; her litter of 9 was excluded for the calculation of PSI.

¶The remaining two of the four pups of litter 855 were killed in error on day 18; this litter was not included in the PSI calculation.

††One dam (no. 2150) lost pups because of lack of food; her litter was excluded from the PSI calculations.

Values marked with an asterisk differ significantly from the control value: $*P < 0.05$ by Fisher's exact probability test.

the 3-mg/kg/day group was significantly lower than that of the controls. This was probably a manifestation of the effect of the significantly larger litter size rather than a compound-related effect. Relative liver weights (g/100 g body weight) in the $F_2$ males and females of the 30-mg/kg/day group were significantly higher than the control values, as were the relative liver weights in the $F_{3a}$ males and $F_{3b}$ males and females in this dosage group. Significantly decreased relative thymus weights (g/100 g body weight) were seen only in male and female $F_{3b}$ weanlings of the 30-mg/kg/day group. The absolute thymus weight was significantly decreased in the $F_{3b}$ males at 30 mg/kg/day. No significant difference in kidney weights was observed between control and experimental groups.

Light microscopic examination of the liver, kidney and thymus from each generation of weanlings revealed no morphological lesions that were attributed to 2,4,5-T at any dose level.

## DISCUSSION

This study evaluated the potential of 2,4,5-T to affect the reproductive capacity of rats given the compound in their diet throughout three generations.

No evidence of toxicity was noted among the $F_0$, $F_1$ or $F_2$ (parental) rats prior to mating. A decrease in the fertility index was seen only at the 10-mg/kg/day dose level in the $F_2$ dams producing the $F_{3b}$ generation. No decrease was seen at the higher or lower dose level. Since a decrease in fertility was not seen at 10 mg/kg/day in any other matings or at 30 mg/kg/day in any matings, the decrease seen in the second mating of the $F_2$ dams was more likely to have been due to chance than to ingestion of 2,4,5-T in the diet. The fertility index for all groups, including the control, was decreased in the $F_{3b}$ generation. This may have been a manifestation of the $F_2$ adults having their second litter. No effect on litter size attributable to the compound occurred in any generation.

There was no significant effect on 21-day survival at the 3-mg/kg/day dose level. A significant decrease in 21-day survival was seen at the 30-mg/kg day dose level in the $F_1$, $F_2$ and $F_{3a}$ generations, but in the two latter generations the percentage survival was still 90% or more. Normally a comparison with historical controls from previous studies would be utilized in interpreting whether the control values for 21-day survival in these two generations (97 and 96%, respectively) were unusually high. However, after the $F_1$ generation was weaned, subsequent generations were housed in new plastics cages. For previous studies in this laboratory and for the $F_1$ generation of this study, stainless-steel reproduction cages with steel screens in them were used after day 7 of gestation. As there could have been added stress due to an increased loss of body heat by the pups in the steel cages, the only controls we could justify using were the $F_1$ and $F_2$ generations of this study. It was apparent that the percentage survival at 21 days was higher

in these controls than in the historical controls of previous studies reared in steel cages in previous studies (97 and 96% *v.* 80 and 84%, respectively). Our experience in past reproduction studies has been that a deviation of less than 10% from the controls is equivocal or not a difference from control. In this study, survival at the high dose level was 15% below controls in the $F_1$ generation.

No morphological anomalies occurred in significantly higher incidence in the test groups than in the controls at any dose level. Dilated renal pelvis or hydronephrosis was observed in controls of the $F_1$ generation at an incidence above that of historical controls from other studies, but the reason for this is unknown. All were males and in right kidneys, and they were distributed in different litters. The only dilated renal pelvis seen in subsequent generations was one in a control pup in the $F_2$ generation. None were observed in the $F_{3a}$ or $F_{3b}$ generations in the controls or any dose group. The reason for this decreasing incidence of dilated renal pelvis in succeeding generations is not apparent.

The $F_1$ generation postnatal survival results (Table 3) indicate an apparent dose-response relationship, with the percentage survival decreasing with increasing dose. This relationship was not seen in any of the subsequent generations.

In conclusion, administration of 2,4,5-T to rats for three generations resulted in a consistent tendency toward a reduction in neonatal survival at a dose level of 30 mg/kg/day. No other effects on reproductive capacity were seen. No adverse effect on reproduction was seen among rats ingesting 3 mg 2,4,5-T/kg/day.

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Fd Cosmet. Toxicol. Vol. 19, pp 47 to 52

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# INDUCTION PAR LE LINDANE DES MONOXYGENASES MICROSOMALES DU FOIE CHEZ LE RAT: EFFETS D'UN JEÛNE DE 72 HEURES

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**Résumé**—On répartit des jeunes rats mâles en deux groupes: témoin et traité au lindane. Le traitement consiste en l'addition de 60 ppm dans le régime pendant 4 semaines. On tue ensuite tous les animaux, dans chaque groupe la première moitié après un jeûne de 18 heures et l'autre moitié après un jeûne prolongé de 72 heures. L'ingestion de lindane accélère la biotransformation de l'aminopyrine et de l'aniline dans les microsomes hépatiques et elle augmente la teneur en *P*-450 ainsi que la proportion de l'acide linoléique de la phosphatidylcholine. Le jeûne prolongé élève l'hydroxylation de l'aniline et abaisse la déméthylation de l'aminopyrine. Chez le rat traité au lindane, le jeûne efface en partie l'induction de la *N*-déméthylase de l'aminopyrine et celle du *P*-450 et il abolit complètement l'effet propre du pesticide sur la teneur en acide linoléique du phosphoglycéride. On discute l'éventualité de l'induction, sous l'effet du lindane, d'une forme particulière de *P*-450 sensible au jeûne, liée dans la membrane à une phosphatidylcholine riche en acide linoléique.

**Abstract**—Young male rats were divided into a control group and a group treated with lindane by addition of 60 ppm to the semi-synthetic diet for 4 wk. All the animals were then killed, half of each group after an 18-hr fast and the rest after a prolonged 72-hr fast. Ingestion of lindane accelerated the biotransformation of aminopyrine and of aniline by the liver microsomes and increased the *P*-450 content and the proportion of linoleic acid in the phosphatidylcholine. Prolonged fasting increased aniline hydroxylation and depressed aminopyrine demethylation. In lindane-treated rats, the fasting partly counteracted the induction of aminopyrine *N*-demethylase and of *P*-450 and completely suppressed the characteristic effect of the pesticide on the linoleic acid content of the phosphoglyceride. It is suggested that lindane treatment leads to the induction of a particular form of *P*-450 sensitive to starvation and bound in the membrane to a phosphatidylcholine rich in linoleic acid.

## INTRODUCTION

Le risque d'intoxication par les pesticides augmente généralement avec le jeûne ou la sous-nutrition (Dale, Gaines & Hayes, 1962; Davison, Sell & Rose, 1971). Lambert & Brodeur (1976) rappellent que le jeûne mobilise les résidus de pesticide, à partir des graisses corporelles, et les répartit dans d'autres tissus (sang, cerveau, foie...). Des quantités importantes de pesticide dans le milieu circulant peuvent donc provoquer une accumulation toxique dans les organes- ou tissus-cibles. D'autre part, on sait que le jeûne modifie le métabolisme, et donc les effets biologiques, des médicaments. Kato (1977) souligne que ce type d'interaction, dont le mécanisme demeure obscur, a une grande importance en pratique médicale puisque la mise au jeûne des sujets est nécessaire dans certaines chimiothérapies et qu'une restriction alimentaire accompagne fréquemment les états pathologiques.

L'insecticide lindane (isomère gamma de l'hexachlorocyclohexane) accélère la biotransformation des xénobiotiques (Fouts, 1970): il induit les oxygénases microsomales du foie (Pélissier & Albrecht, 1976). Que devient l'induction de ces enzymes chez l'animal soumis à un jeûne prolongé?

Dans la présente expérience, nous avons étudié, dans les microsomes hépatiques du jeune rat mâle, l'interaction des effets du lindane et d'un jeûne de 72 heures sur les activités de *N*-déméthylation de l'aminopyrine, d'hydroxylation de l'aniline, sur la teneur en *P*-450 ainsi que sur la composition en acides gras de la phosphatidylcholine.

## METHODES EXPERIMENTALES

*Animaux et traitement.* Les animaux sont des rats mâles Wistar U, exempts d'organismes pathogènes spécifiques (SPF). A l'âge de 6 semaines (poids moyen, environ 150 g), nous les répartissons en deux groupes. Le premier (témoin) reçoit un régime semi-synthétique préparé au laboratoire, sans autre addition. Le second (traité) reçoit ce même régime auquel nous

ajoutons 60 ppm de lindane. Le traitement dure 4 semaines. Au sacrifice, les rats sont à jeûn depuis 18 heures ou 72 heures (jeûne prolongé). Le foie est rapidement prélevé, lavé et pesé. Nous préparons un homogénat total du foie dans 3 vols de saccharose 0,25 M refroidi dans lequel nous mesurons la quantité de lindane par chromatographie en phase gazeuse (Lovelock & Lipsky, 1960). Nous préparons ensuite les microsomes (250 mg foie/ml) selon le procédé de Kamath & Narayan (1972) tel que nous l'avons décrit (Albrecht, Pélissier, Manchon & Rospars, 1973). Nous déterminons dans les microsomes la composition en acides gras de la phosphatidylcholine (Metcalfe, Schmitz & Pelka, 1966), la teneur en protéines (Lowry, Rosebrough, Farr & Randall, 1951), la *N*-déméthylation de l'aminopyrine, l'hydroxylation de l'aniline ainsi que la quantité de *P*-450 (Pélissier, Faudemay, Manchon & Albrecht, 1978).

*Analyse statistique.* Les résultats sont établis par analyse de variance (Schwartz, 1966) après vérification de sa légitimité par l'épreuve de Bartlett (Snedecor & Cochran, 1967). Notre plan expérimental permet d'appliquer la méthode des blocs complets (Lellouch & Lazar, 1974). L'expérience est bifactorielle 2 × 2: nous étudions les effets des deux facteurs (lindane, jeûne), chacun étant à deux niveaux. Nous testons (1) l'absence d'interaction entre les deux facteurs; (2) l'effet du jeûne prolongé; (3) l'effet du pesticide. S'il n'y a pas d'interaction significative ($P > 0,05$) entre les deux facteurs, pour apprécier l'effet de l'un, nous comparons la moyenne de deux groupes à la moyenne des deux autres groupes; par exemple, pour tester l'effet du lindane: (témoins, jeûne court) et (témoins, jeûne 72 heures) *contre* (lindane, jeûne court) et (lindane, jeûne 72 heures).

## RESULTATS

Au moment du sacrifice, les rats pèsent environ 250 g. Nous ne notons pas d'effet significatif dû à l'ingestion de lindane sur la croissance corporelle. De même, la chute de poids consécutive au jeûne n'est pas sensiblement différente chez les rats traités au pesticide et chez les témoins.

Nous indiquons sur le Tableau 1 le poids du foie et sa teneur en lindane. La diminution du poids relatif due au jeûne prolongé est plus importante ($P < 0,05$) pour les rats traités: $-24\%$ ($P < 0,05$) au lieu de $-13\%$ ($P > 0,05$) chez l'animal témoin. Le jeûne de 72 heures réduit très sensiblement ($-66\%$, $P < 0,01$) la concentration de lindane dans le foie du rat traité.

### *Activité des microsomes hépatiques*

*Effet du jeûne prolongé.* Chez tous les animaux, le jeûne de 72 heures ralentit ($P < 0,01$) le métabolisme du substrat de type I, aminopyrine, en moyenne de $-23\%$ et $-33\%$ respectivement par rapport aux protéines et à l'unité de *P*-450 (Tableau 2). Chez le rat témoin, le jeûne prolongé réduit ($P < 0,01$) de $-33\%$ l'activité exprimée par foie entier. En revanche, le jeûne accélère ($P < 0,01$) de $+19\%$ le métabolisme du substrat de type II, aniline, quand l'activité est rapportée aux protéines. Nos résultats sont conformes aux observations de Kato & Gillette (1965).

Chez tous les animaux, la teneur en *P*-450 rapportée aux protéines est augmentée, en moyenne de $+12\%$ ($P < 0,05$), sous l'effet du jeûne prolongé. Nous confirmons là-aussi les données de la littérature (Gram, Guarino, Schroeder, Davis, Reagan & Gillette, 1970; Greim, 1971).

Le jeûne de 72 heures ne modifie pas significativement la proportion des acides gras de la phosphatidylcholine (Tableau 3).

*Effets du lindane.* L'ingestion de lindane accroît ($P < 0,01$) la biotransformation de l'aminopyrine: en moyenne de $+59\%$ et $+28\%$, respectivement par rapport aux protéines et au *P*-450 (Tableau 2). Chez le rat non soumis au jeûne prolongé l'activité par foie entier est augmentée ($P < 0,01$) de $+93\%$. L'hydroxylation de l'aniline est aussi accélérée ($P < 0,01$) sous l'effet du lindane, de $+22\%$ par rapport aux protéines et de $+35\%$ par foie; remarquons à nouveau que pour ce substrat le traitement n'a pas d'effet significatif sur l'activité moléculaire exprimée par unité de *P*-450 (Pélissier & Albrecht, 1976).

Tableau 1. *Poids du foie et teneur en lindane*

| Traitement | Poids relatif du foie† (g/100 g rat) | Teneur en lindane† (μg/g foie) |
|---|---|---|
| Jeûne de 18 hr | | |
| Témoin | 3.89 ± 0.149 | 0.063 ± 0.0101 |
| Lindane | 4.76 ± 0.147 | 3.34 ± 0.260 |
| Jeûne de 72 hr | | |
| Témoin | 3.38 ± 0.120 | 0.0518 ± 0.0430 |
| Lindane | 3.64 ± 0.097 | 1.15 ± 0.140 |
| | **Comparaisons‡** | |
| Effet lindane (a) | NS | ** |
| (b) | NS | ** |
| Effet jeûne (c) | NS | NS |
| (d) | * | ** |
| Interaction | * | ** |

†Moyenne, écart-type sur la moyenne (16 rats/groupe).
‡Comparaisons par rapport à (a) jeûne court, (b) jeûne prolongé, (c) rats témoins et (d) rats traités au lindane: $*P \leqslant 0.05$; $**P \leqslant 0.01$; NS = $P > 0.05$.

Tableau 2. *Activités enzymatiques des microsomes du foie*

| Paramètre | Activité enzymatique† | | | | Comparaisons‡ | | |
|---|---|---|---|---|---|---|---|
| | Jeûne de 18 hr | | Jeûne de 72 hr | | | | |
| | Témoin | Lindane | Témoin | Lindane | Effet lindane | Effet jeûne | Interaction |
| *N*-Deméthylation de l'aminopyrine: | | | | | | | |
| nmol aminoantipyrine/min/100 mg protéines | 10,4 ± 1,35 | 18,0 ± 2,25 | 9,0 ± 1,41 | 12,8 ± 1,57 | ** | ** | NS |
| nmol aminoantipyrine/min/foie | 34,9 ± 2,88 | 67,3 ± 5,15 | 23,4 ± 2,61 | 35,9 ± 2,49 | (a)** (b)** | (c)** (d)** | ** |
| nmol aminoantipyrine/min/nmol *P*-450 | 0,153 ± 0,0179 | 0,203 ± 0,0213 | 0,108 ± 0,0127 | 0,130 ± 0,0101 | ** | ** | NS |
| Hydroxylation de l'aniline: | | | | | | | |
| nmol *p*-aminophénol/min/100 mg protéines | 27,2 ± 1,43 | 31,4 ± 1,86 | 30,5 ± 1,74 | 39,0 ± 2,08 | ** | ** | NS |
| nmol *p*-aminophénol/min/foie | 99,9 ± 8,68 | 134 ± 12,9 | 86,0 ± 7,06 | 117,4 ± 6,62 | ** | NS | NS |
| nmol *p*-aminophénol/min/nmol *P*-450 | 0,412 ± 0,0239 | 0,372 ± 0,0268 | 0,401 ± 0,0298 | 0,480 ± 0,0173 | NS | NS | NS |
| Hémoprotéine *P*-450: | | | | | | | |
| nmol/100 mg protéines | 67,2 ± 3,32 | 88,1 ± 3,81 | 80,0 ± 4,18 | 94,7 ± 5,68 | ** | * | NS |
| nmol/foie | 240 ± 12,9 | 367 ± 21,1 | 223 ± 13,9 | 285 ± 13,5 | (a)** (b)** | (c)NS (d)** | * |

†Moyenne, écart-type sur la moyenne (16 rats/groupe).
‡Comparaisons par rapport à (a) jeûne court, (b) jeûne prolongé, (c) rats témoins et (d) rats traités au lindane: $*P \leqslant 0{,}05$; $**P \leqslant 0{,}01$; NS = $P > 0{,}05$.

Tableau 3. *Teneur en acides gras de la phosphatidylcholine des microsomes hépatiques*

| Acide gras | Acides gras individuels (mg/100 mg acides gras totaux)† | | | | Comparaisons‡ | | |
|---|---|---|---|---|---|---|---|
| | Jeûne de 18 hr | | Jeûne de 72 hr | | | | |
| | Témoin | Lindane | Témoin | Lindane | Effet lindane | Effet jeûne | Interaction |
| C16:0 | 36,5 ± 1,41 | 35,9 ± 1,30 | 37,3 ± 1,18 | 38,2 ± 1,31 | NS | NS | NS |
| C18:0 | 36,8 ± 1,58 | 33,5 ± 1,12 | 33,57 ± 0,501 | 33,55 ± 0,648 | NS | NS | NS |
| C18:1 | 11,68 ± 0,545 | 10,60 ± 0,503 | 11,79 ± 0,200 | 11,70 ± 0,290 | NS | NS | NS |
| C18:2 | 6,71 ± 0,351 | 8,85 ± 0,571 | 8,11 ± 0,507 | 7,86 ± 0,401 | (a)** (b) NS | (c) NS (d) NS | * |
| C20:3 | 2,90 ± 0,450 | 2,61 ± 0,291 | 3,20 ± 0,302 | 3,14 ± 0,444 | NS | NS | NS |
| C20:4 | 5,5 ± 1,02 | 7,07 ± 0,774 | 7,26 ± 0,939 | 5,59 ± 0,757 | NS | NS | NS |

†Moyenne, écart-type sur la moyenne (huit rats/groupe).
‡Comparaisons par rapport à (a) jeûne court, (b) jeûne prolongé, (c) rats témoins et (d) rats traités au lindane: $*P \leqslant 0{,}05$; $**P \leqslant 0{,}01$; NS = $P > 0{,}05$.

Nous confirmons que le lindane élève ($P < 0{,}01$) la biosynthèse nette du *P*-450. Chez tous les animaux, la teneur en hémoprotéine est en moyenne augmentée de $+24\%$ par rapport aux protéines et chez le rat non soumis au jeûne prolongé la quantité totale par foie est accrue de $+53\%$.

Chez l'animal non soumis au jeûne de 72 heures, le lindane élève de 32% ($P < 0{,}01$) la proportion de l'acide linoléique de la phosphatidylcholine (Tableau 3).

*Interaction des effets du lindane et du jeûne*

N-*Déméthylation de l'aminopyrine.* Nous observons une interaction ($P < 0{,}01$) entre les effets du pesticide et du jeûne de 72 heures sur l'activité enzymatique exprimée par foie: la diminution d'activité par le jeûne est plus importante chez les rats traités, $-47\%$ au lieu de $-33\%$ pour les témoins; l'augmentation de l'activité, sous l'effet du lindane, est moindre chez les animaux soumis au jeûne de 72 heures, $+53\%$ au lieu de $+93\%$ pour les rats n'ayant pas subi de jeûne prolongé. Le jeûne efface donc en partie l'induction par le lindane de la *N*-déméthylation de l'aminopyrine.

*Hémoprotéine P-450.* Nous remarquons une interaction entre les deux facteurs sur la quantité totale de *P*-450 par foie: le jeûne réduit de $22\%$ ($P < 0{,}01$) la teneur en *P*-450 du foie des rats traités alors qu'il est sans effet significatif chez les témoins; le lindane augmente toujours la quantité de *P*-450 mais l'effet est moindre chez les animaux au jeûne de 72 heures, $+28\%$ au lieu de $+53\%$ pour les rats non soumis au jeûne prolongé. Le jeûne de 72 heures supprime donc en partie l'augmentation de la quantité de *P*-450 par foie entier.

Pour ces deux paramètres, l'analyse statistique ne montre d'interaction significative entre les effets du lindane et du jeûne que pour les valeurs exprimées par foie entier mais un examen attentif de nos résultats suggère qu'il en serait de même pour les valeurs rapportées aux protéines: le jeûne prolongé réduit de $41\%$ l'induction spécifique par le lindane de la *N*-déméthylation de l'aminopyrine et celle du *P*-450.

*Acide linoléique de la phosphatidylcholine.* Nous notons une interaction ($P < 0{,}05$) entre les effets du lindane et du jeûne sur la proportion de l'acide linoléique: le lindane accroît de $32\%$ ($P < 0{,}01$) la teneur en acide linoléique chez les rats non soumis au jeûne prolongé alors qu'il n'a pas d'effet significatif lorsque le jeûne est de 72 heures. Le jeûne supprime donc l'augmentation de la proportion de l'acide linoléique due au lindane.

## DISCUSSION

Antérieurement, nous avons établi que l'induction par le lindane des oxygénases hépatiques s'accompagne d'une augmentation de la teneur en phosphatidylcholine des microsomes (Pélissier *et al.* 1978). De plus, nous avons constaté que le pesticide modifie la composition en acides gras du phosphoglycéride: il élève la proportion de l'acide linoléique (Albrecht, Dooh-Priso, Faudemay, Pélissier & Carreau, 1979). Par la présente expérience, nous remarquons que le jeûne de 72 heures supprime complètement cet effet propre du pesticide. D'autre part, le jeûne efface en partie l'induction de la déméthylation de l'aminopyrine et celle du *P*-450 tandis qu'il n'a pas d'effet, ou même renforce, l'induction de l'hydroxylation de l'aniline.

L'ensemble de ces résultats nous amène à proposer une hypothèse, proche des conceptions de Hayes, Mgbodile & Campbell (1973), rendant compte des variations des effets du jeûne suivant le type de substrat.

Chez le rat mâle, il existerait *au moins* deux espèces différentes de *P*-450 dans les microsomes hépatiques. Le traitement par le lindane accroît la quantité totale d'hémoprotéine, mais l'augmentation porterait essentiellement sur l'une des deux espèces. Cette dernière serait liée à la phosphatidylcholine synthétisée *de novo*, sous l'effet du pesticide, caractérisée par sa richesse en acide linoléique. Nous l'appelons *P*-450-PC (18:2) pour la distinguer de l'autre espèce, le *P*-450 'natif'. La déméthylation de l'aminopyrine serait plus rapide en présence de *P*-450-PC (18:2) que de *P*-450 'natif'; ce qui ne serait pas le cas pour l'hydroxylation de l'aniline. Le jeûne prolongé fait disparaître en partie l'hémoprotéine induite par le lindane. Mais cet effet porterait essentiellement, sinon totalement, sur le *P*-450-PC (18:2) puisque nous avons vu

que le jeûne abolit complètement l'augmentation par le pesticide de la teneur en acide linoléique de la phosphatidylcholine. D'où le sensible abaissement par le jeûne de la déméthylation de l'aminopyrine 'induite'.

Nous admettons que les microsomes du foie contiennent plusieurs formes de *P*-450. Il s'agit là d'un domaine de recherches très étudié depuis quelques années. On s'accorde pour considérer qu'effectivement, aussi bien chez l'animal témoin que chez le traité par un inducteur, des espèces différentes d'hémoprotéines existent et qu'elles présentent des affinités variables pour les substrats (Lu & West, 1978; Nebert, 1979). D'autre part, nous considérons aussi que le métabolisme de l'aminopyrine, substrat de type I, met en jeu deux systèmes enzymatiques (dans notre hypothèse, deux formes de *P*-450) alors qu'un seul est impliqué pour l'hydroxylation de l'aniline, substrat de type II. Les travaux de Burke & Bridges (1972) nous semblent donner des résultats conformes à notre hypothèse. Ces chercheurs montrent que le biphényl (type I) présente deux affinités pour le *P*-450 tandis que l'aniline en a une seule. De plus, le stockage des microsomes à 2–20°C supprime en 48 heures la forte affinité du biphényl pour le *P*-450 mais sa faible affinité ne décroît que lentement et progressivement; la 'liaison' de l'aniline au *P*-450 et la vitesse de son métabolisme évoluent beaucoup moins que pour le biphényl au cours du stockage des microsomes. Les deux affinités du biphényl pour le *P*-450 dans l'expérience de Burke & Bridges (1972) ne résultent-elles pas de la mise en jeu de deux formes différentes de *P*-450 dont l'une très 'active' (forte affinité) serait liée à la phosphatidylcholine et qui serait plus fragile *in vivo* chez l'animal soumis au jeûne (notre expérience) et *in vitro* sous l'effet du stockage à une température supérieure à 0°C (expérience de Burke & Bridges)?

Nous admettons aussi que chez le rat mâle, le métabolisme des substrat de type I dépend plus que pour celui de type II de la quantité et de la qualité des lipides membranaires liés au *P*-450. Ceci semble bien établi: le traitement des microsomes avec de la phospholipase C ou de l'isooctane détruit en partie la 'liaison' de l'éthylmorphine et de l'hexobarbital à l'hémoprotéine tandis qu'il augmente la 'liaison' de l'aniline (Chaplin & Mannering, 1970; Leibman & Estabrook, 1970); la phospholipase C abaisse la *N*-déméthylation de la benzphétamine mais elle n'a pas d'effet sur l'hydroxylation de l'aniline (Eling & DiAugustine, 1971).

Nous venons de présenter quelques données de la littérature qui nous paraissent en accord avec notre hypothèse rendant compte des variations, suivant le type de substrat, de l'effet du jeûne prolongé sur l'induction par le lindane des oxygénases. Néanmoins, il nous faut reconnaître que la même argumentation peut s'appliquer pour les effets du jeûne sur l'induction due au phenobarbital. Or, le jeûne atténue l'induction par le pesticide (substrats de type I) tandis qu'il accentue celle que produit le barbiturique (Kato, 1967; Lambert & Brodeur, 1976; Marselos & Laitinen, 1975). Nous admettrons que la forme de *P*-450 'induite' par le phénobarbital est differente de celle que fait apparaître le lindane. Toutefois des recherches ultérieures s'avèrent nécessaires pour préciser ce point, d'autant que même chez le rat témoin, malgré de très nombreux travaux (cf. Kato, 1977), on n'a pas encore donné d'explication totalement claire des variations des effets du jeûne selon le type de substrat et selon le sexe.

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# RELATIONSHIP BETWEEN THE CONSUMPTION OF TOXIC RICE OIL AND THE LONG-TERM CONCENTRATION OF POLYCHLORINATED BIPHENYLS IN THE BLOOD OF YUSHO PATIENTS

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**Abstract**—The relationship between the consumption of the toxic rice oil and the concentration of polychlorinated biphenyls (PCBs) in the blood five or more years after the poisoning was examined in Yusho patients. In each year from 1973 to 1976, significant positive correlations were observed between the blood PCB concentration and the total amount of the rice oil consumed but not between the blood PCB concentration and the amount of the rice oil consumed per kg per day.

## INTRODUCTION

A mass outbreak of poisoning (Yusho) due to the ingestion of a rice oil contaminated with polychlorinated biphenyls (PCBs) and their related compounds occurred in western Japan in 1968 (Kuratsune, Yoshimura, Matsuzaka & Yamaguchi, 1972). In order to prove the causal relationship, a crude estimate of the quantity of the rice oil consumed by each patient and members of his or her family was made without regard to their age, sex or food intake or to the possible loss of the oil during and after cooking. This demonstrated that both the incidence and the clinical severity of the poisoning correlated positively with the consumption of the rice oil (Kuratsune *et al.* 1972; Yoshimura, 1971). Recently, our original data were reanalysed taking the age, sex and food intake of the patients into consideration, and the distributions of individual oil consumptions were estimated (Hayabuchi, Yoshimura & Kuratsune, 1979). PCB levels in the blood of Yusho patients have been determined since 1972. On the basis of the PCB levels in the blood of the patients and of the individual oil consumptions, we have examined whether the PCB levels in the blood during the five or more years following ingestion of the toxic oil are correlated with the initial (re-estimated) oil consumptions.

## EXPERIMENTAL

*Individual oil consumption.* Patients whose consumptions of the toxic canned rice oil produced or shipped by one company on 5/6 February 1968 had been estimated and whose blood PCB concentrations had been determined were included in the present study. Their personal total consumption of the oil and their daily consumption of the oil per kg body weight were as estimated in the previous study (Hayabuchi *et al.* 1979).

*Blood PCBs.* An annual physical check-up of Yusho patients has been made by the Health Examination Team for Yusho organized by the government in Fukuoka Ken since 1969. The blood levels of PCBs in patients attending the check-up have been determined. In this study, most of the data concerns PCB levels in blood samples collected at annual check-ups from 1973 to 1976. Chemical analysis of PCBs in the blood of Yusho patients was started in four laboratories in collaboration with the Study Group for Therapy of Yusho in 1972. In three of these laboratories, PCBs in whole blood were analysed by the government standard analytical method (Masuda, Kagawa, Shimamura, Takada & Kuratsune, 1974), while in the fourth, PCBs in plasma were analysed using Jensen's method (Takamatsu, Inoue & Abe, 1974). The PCB levels determined by the latter method were therefore divided by 1·6 in order to make them comparable with those in whole blood determined by the former method. Blood PCB concentrations determined in these four laboratories were thought to be comparable, because preliminary analyses of the same five blood samples in each laboratory gave very similar values, namely, 7, 7, 7 and 5 ppb for sample A, 4, 4, 3 and 5 ppb for sample B, 4, 4, 2 and 5 ppb for sample C, 7, 8, 7 and 7 ppb for sample D, and 4, 5, 5 and 6 ppb for sample E. Analysis of variance showed no significant difference between the results obtained in the different laboratories.

Table 1. *Mean and range of age, total amount of oil consumed, the amount of oil consumed per kg per day, and the blood PCB concentration of Yusho patients in 1973–1976*

| Year | Sex | No. of patients | Age (yr) | | Oil consumption | | | | Blood PCB (ppb) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Total (ml) | | Daily (μg/kg/day) | | | |
| | | | Mean | Range | Mean* | Range | Mean* | Range | Mean* | Range |
| 1973 | M | 21 | 35·7 | 6–74 | 700 | 230–2813 | 207 | 75–608 | 6·9 | 2–29 |
| | F | 27 | 29·3 | 8–60 | 784 | 195–3375 | 244 | 68–861 | 7·2 | 1–39 |
| | M + F | 48 | 32·1 | 6–74 | 746 | 195–3375 | 227 | 68–861 | 7·1 | 1–39 |
| 1974 | M | 14 | 38·9 | 8–68 | 748 | 220–2813 | 160 | 32–608 | 8·6 | 5–19 |
| | F | 24 | 33·4 | 9–58 | 842 | 212–3375 | 224 | 55–861 | 9·1 | 2–31 |
| | M + F | 38 | 35·4 | 8–68 | 806 | 212–3375 | 198 | 32–861 | 8·9 | 2–31 |
| 1975 | M | 13 | 44·0 | 10–76 | 828 | 216–2813 | 207 | 49–351 | 8·5 | 3–19 |
| | F | 19 | 32·7 | 10–59 | 856 | 288–3375 | 236 | 55–861 | 8·2 | 2–37 |
| | M + F | 32 | 37·3 | 10–76 | 845 | 216–3375 | 224 | 49–861 | 8·3 | 2–37 |
| 1976 | M | 9 | 50·4 | 23–77 | 623 | 288–1934 | 196 | 75–608 | 9·9 | 6–14 |
| | F | 10 | 42·2 | 11–60 | 888 | 288–3375 | 218 | 55–861 | 11·5 | 4–32 |
| | M + F | 19 | 46·1 | 11–77 | 751 | 288–3375 | 207 | 55–861 | 10·5 | 4–32 |

*Geometric mean.

*Statistical analysis.* Significant differences between means were determined by *t*-tests. Regression coefficients and correlation coefficients between the oil consumptions and the blood PCB concentrations were calculated after first transforming the data using each value transformed to natural logarithms, because the distributions of both the individual oil consumptions and the blood PCB concentrations approximated to log normal (Hayabuchi *et al.* 1979). The significance of regression coefficients was determined by *t*-tests. In order to eliminate the possible effect of age, partial correlation coefficients were also calculated (Snedecor & Cochran, 1974).

## RESULTS

The numbers of patients included in the present analysis were 48, 38, 32 and 19 for 1973, 1974, 1975 and 1976, respectively. Both the amounts of oil consumed and the blood PCB concentrations ranged widely and took a skew distribution with geometric means as shown in Table 1. No significant sex difference was noted in the distributions of the total amounts of oil consumed, the amounts of oil consumed per kg per day or the blood PCB concentrations in any one year, although the means of these values for females were generally larger than the corresponding ones for males in all the years.

The correlations between the total amounts of oil consumed and the blood PCB concentrations by year are shown in Table 2. Fairly large correlation coefficients were observed for males, and all but the 1976 value were statistically significant. For females, similar but more definite positive correlations were observed in all of the years. Partial correlation coefficients calculated by eliminating the effect of age were close to the simple correlation coefficients, indicating that the correlations between the total amounts of oil

Table 2. *Correlation between the total amount of oil consumed† and the blood PCB concentration† in Yusho patients in 1973–1976*

| Year | Sex | Regression equation ($y=$) | Correlation coefficient | Partial correlation coefficient‡ |
|---|---|---|---|---|
| 1973 | M | $0{\cdot}475X - 0{\cdot}515$ | 0·529* | 0·529* |
| | F | $0{\cdot}792X - 1{\cdot}430$ | 0·787*** | 0·784*** |
| | M + F | $0{\cdot}652X - 1{\cdot}024$ | 0·681*** | 0·680*** |
| 1974 | M | $0{\cdot}326X - 0{\cdot}004$ | 0·771** | 0·770** |
| | F | $0{\cdot}764X - 1{\cdot}277$ | 0·834*** | 0·849*** |
| | M + F | $0{\cdot}535X - 0{\cdot}606$ | 0·762*** | 0·769*** |
| 1975 | M | $0{\cdot}548X - 0{\cdot}671$ | 0·697** | 0·702** |
| | F | $0{\cdot}915X - 1{\cdot}768$ | 0·797*** | 0·804*** |
| | M + F | $0{\cdot}744X - 1{\cdot}258$ | 0·746*** | 0·751*** |
| 1976 | M | $0{\cdot}325X - 0{\cdot}068$ | 0·586 | 0·760* |
| | F | $0{\cdot}675X - 0{\cdot}930$ | 0·858** | 0·866** |
| | M + F | $0{\cdot}550X - 0{\cdot}559$ | 0·783*** | 0·827*** |

†Log-transformed values were used.
‡Calculated by controlling age.
Values marked with asterisks are significant at $*P < 0{\cdot}05$, $**P < 0{\cdot}01$ or $***P < 0{\cdot}001$.

Table 3. *Correlation between the amount of oil consumed per kg per day† and the blood PCB concentration† in Yusho patients in 1973–1976*

| Year | Sex | Correlation coefficient | Partial correlation coefficient‡ |
|---|---|---|---|
| 1973 | M | 0·250 | 0·265 |
| | F | 0·693*** | 0·690*** |
| | M + F | 0·515*** | 0·519*** |
| 1974 | M | 0·456 | 0·452 |
| | F | 0·223 | 0·115 |
| | M + F | 0·286 | 0·278 |
| 1975 | M | 0·057 | 0·023 |
| | F | 0·343 | 0·251 |
| | M + F | 0·265 | 0·198 |
| 1976 | M | 0·447 | 0·546 |
| | F | 0·573 | 0·682 |
| | M + F | 0·533* | 0·627** |

†Log-transformed values were used.
‡Calculated by controlling age.
Values marked with asterisks are significant at $*P < 0·05$, $**P < 0·01$ or $***P < 0·001$.

consumed and the blood PCB concentrations were independent of age. The regression equations for the relationship between the total amount of oil consumed and blood PCB levels are also shown in Table 2. The regression coefficients for the females were larger than those for the males in all years, but these sex differences in the coefficients were not significant except in 1974.

As shown in Table 3, positive correlation coefficients were also observed between the amounts of oil consumed per kg per day and the blood PCB concentrations for both males and females and for males and females combined, but they were much smaller than the corresponding coefficients between the blood PCB concentrations and the total amounts of oil consumed, estimated disregarding the body weight and the duration of oil consumption (Table 2). The partial correlation coefficients also showed a similar trend. The regression equations for the relationship between the amounts of oil consumed per kg per day and the blood PCB levels are not shown in Table 3, since in most cases the regression coefficients were not significant.

## DISCUSSION

As expected, the clinical severity of the response in Yusho patients examined in 1969 was closely correlated with their individual consumptions of the toxic rice oil (Hayabuchi *et al.* 1979). However, it seemed unlikely that blood levels of PCBs determined five or more years after the initiation of poisoning, would be correlated with the initial consumption of the oil. The basis of this assumption was that after the long lapse of time, the blood levels of PCBs would approach the normal range which is low and relatively narrow and that these almost normal values might not reflect the original exposure. However, the present study clearly demonstrates that the decreased PCB levels closely correlated with the initial intake of the oil or of PCBs. This is probably due to the fact that the PCBs ingested by the patients contained PCB congeners. These are present in relatively small amounts, but if they are absorbed they are hardly excreted from the body. A large intake of the toxic rice oil will result in a large intake of such congeners and lead to an elevated level of PCBs in the blood for a very long time after the initial ingestion. Thus it seems that in the case of Yusho poisoning the PCB level in the blood is a good indicator of previous exposure to PCBs.

One of the most interesting findings in our study is that the PCB levels in blood did not correlate with the daily intake of the toxic rice oil per kg of body weight but with the total consumption of the oil. This finding is in accord with our previous observation that the clinical severity of the response in Yusho patients was not correlated with the oil consumption per kg body weight per day but was closely correlated with the total oil consumption (Hayabuchi *et al.* 1979). The levels of PCBs remaining in the blood even several years after exposure may be determined primarily by the total amount of PCB congeners that are ingested, since the level of excretion of these congeners is very low. Thus, our observation seems to be explained by this peculiar feature of PCBs.

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# CHRONIC TOXICITY OF 2,3,7,8-TETRACHLORODIBENZOFURAN FOR RHESUS MACAQUES

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**Abstract**—2,3,7,8-Tetrachlorodibenzofuran (TCDF) at levels of 50 and 5 μg/kg (ppb) in food caused sickness and some deaths in groups of three rhesus macaques fed for 2 months and 6 months, respectively. The principal pathological changes were atrophy or squamous metaplasia of the sebaceous glands, mucous metaplasia and hyperplasia of the gastric mucosa; involution of the thymus and hypoplasia of the bone marrow. In animals that did not die during the experimental feeding, recovery was complete after 3 months of a TCDF-free diet.

## INTRODUCTION

Polychlorinated dibenzofurans (PCDFs) are structurally similar to polychlorinated dibenzo-*p*-dioxins (Fig. 1), at least one of which, the 2,3,7,8- homologue, is among the most toxic low-molecular-weight organic chemicals known. PCDFs are unwanted contaminants in the wood preservative pentachlorophenol (Firestone, Ress, Brown, Barron & Damico, 1972) and in polychlorinated biphenyls (PCBs; Bowes, Mulvihill, Simoneit, Burlingame & Risebrough, 1975; Roach & Pomerantz, 1974). Their significance as environmental risks is unknown, though it has been speculated that the toxicity of PCBs may be largely due to contaminating PCDFs (Vos, Koeman, van der Maas, ten Noever de Brauw & de Vos, 1970). High levels of PCDF were in fact found in Kanemi rice oil, the PCB-contaminated rice oil that caused Yusho, an epidemic of human poisoning in Japan (Buser, Rappe & Garå, 1978), and PCDFs were found in the tissues of Yusho victims (Nagayama, Masuda & Kuratsune, 1977). They have also been detected in samples of gelatin from supermarket shelves (Firestone, 1977). The presumed source was pentachlorophenol contamination of the animal hides used in the manufacture of the gelatin.

Single doses of 2,3,7,8-tetrachlorodibenzofuran (TCDF), or mixtures of PCDFs, have been found to be toxic for rats (Oishi, Morita & Fukuda, 1978), mice (Nishizumi, 1978), chicks (McKinney, Chae, Gupta, Moore & Goldstein, 1976), guinea-pigs, and monkeys (Moore, McConnell, Dalgard & Harris, 1979).

We report here on the chronic toxicity of low levels of TCDF incorporated into the diet of young male rhesus macaques.

## EXPERIMENTAL

*Diet preparation.* The TCDF, 98% pure, was part of a lot synthesized by Gray, Dipinto & Solomon (1976) under contract to the Food and Drug Administration (FDA). It dissolved readily in acetone (5·258 mg in 50 ml), and this stock solution was stored in the dark at room temperature for over a year. Diet cakes were prepared by homogenization of ground Purina Monkey Chow, bananas, water, vitamin and mineral supplements, and a 1:9 acetone:corn oil mixture (4·2 ml/kg of cake), which in the experimental cakes contained enough TCDF to bring the final levels to 5 or 50 μg/kg (ppb).

*Analytical determination of TCDF in diet batches.* The level of TCDF was determined in several samples of 50 ppb and 5 ppb cakes, prepared at the beginning and the end of the experiment (about a year apart) from the same stock solution described above.

A 20-g sample of pulverized diet cake was comminuted with 50 ml of acetone and allowed to settle for about 30 min; the extract was filtered through a plug of glass wool into a 400-ml beaker. Extraction was repeated three more times, and the filter was washed with an additional 50 ml of acetone. The combined extracts were evaporated on a steam bath; caution was taken to avoid heating the residue beyond incipient dryness. After remaining traces of acetone had evaporated at room temperature, the acetone-free residue was mixed with about 2 g of silica gel and transferred with several small portions of petroleum ether to a 22 × 250 mm chromatography column containing 10 g of silica gel (J. T. Baker Co., Cat. No. 3405, 60 to 200 mesh, activated overnight at 130°C in a vacuum oven and slowly added while hot to the column half filled with petroleum ether). The TCDF was eluted from the silica gel with 200 ml of petroleum ether directly onto a Florisil chromatography column (Horwitz, 1975). The Florisil column was eluted with 200 ml of 6% ethyl ether in petroleum ether to remove possible pesticide or PCB interferences. The TCDF was eluted from the Florisil with 200 ml of $CH_3CN$–petroleum ether–methylene chlor-

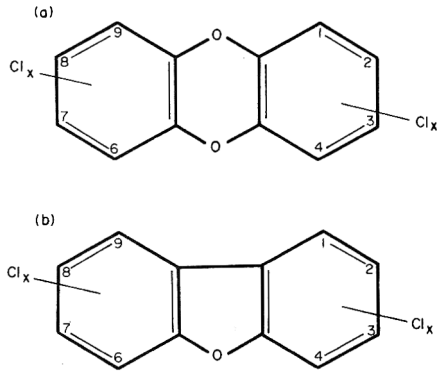


Fig. 1. (a) Polychlorinated dibenzo-*p*-dioxin; (b) polychlorinated dibenzofuran.

ide (1·5:48·5:50, by vol.) eluting solvent directly into a Kuderna-Danish concentrator equipped with a 10-ml graduated receiver. The eluate was evaporated, and the final volume was adjusted to 1 or 10 ml with isooctane.

A Varian 3700 gas chromatograph equipped with a constant current $^{63}Ni$ electron capture detector (ECD), glass capillary column inlet system, and capillary column was used for the quantification of TCDF. Several different columns were used, and each was found to be satisfactory. Typical operating conditions for a 20 m × 0·25 mm ID column coated with $C_{87}H_{176}$ hydrocarbon liquid phase (Boksanyi & Kovats, 1976; Riedo, Fritz, Tarjan & Kovats, 1976) were as follows: temperature (°C)—column, 200, inlet, 300, detector, 320; average linear velocity of hydrogen carrier gas, 66 cm/sec (1·9 ml/min); splitter flow rate, 50 ml/min (split ratio, 25:1); column make-up nitrogen gas flow, 15 ml/min; ECD controller attenuation, 1 × 32; Hewlett-Packard 7130A strip chart recorder, 1 millivolt span, 0·25 in./min.

Under these conditions, 400 pg of injected TCDF produced an ECD response of about 70% full-scale deflection at a retention time of about 12 min (Fig. 2a). Injection volumes of cleaned-up extracts were equivalent to 8 mg of the 50 ppb cake and 80 mg of the 5 ppb cake. The equivalent amounts of extracts from control cakes were injected in each case.

No TCDF was detected in two different lots of the control cakes (Fig. 2b) with a detection limit estimated to be about 0·5 ppb (7% full-scale deflection).

Cake prepared at 50 ppb was analysed to assure the accuracy and homogeneity of the preparation. Several subdivisions and a composite were all found to contain 49 to 55 ppb or 98 to 110% of the expected value. Several subdivisions of 5 ppb cake were found to contain about 3·2 ppb, or 64% of the expected value (Fig. 2c). Several attempts to improve the extraction failed to raise these values to the expected level of 5 ppb. It is believed that the extraction did not recover all of the TCDF from the cake, and the loss was significant only at the lower level.

Fortification experiments with TCDF added to the control cakes to test the extraction procedure gave recovery values of 93% at the 50 ppb level and 85% at the 5 ppb level (Fig. 3).

*Analytical determinations of other compounds.* The cakes were analysed by the FDA multi-residue method for chlorinated hydrocarbon pesticides and PCBs as described in the Pesticide Analytical Manual (1973). No pesticides or PCBs were detected (limit of detection was approximately 0·2 ppm Aroclor 1254).

*Animal feeding and maintenance.* Young male rhesus macaques (*Macaca mulatta*), 1 to 2 years old, were individually caged in a single room in which no toxic compounds other than TCDF were or had been used. They were fed *ad lib.* with cubes of diet cake twice daily. Three monkeys were tested at each dietary level and the experimentally fed monkeys served as their own controls. In addition, during both tests, which were done at different times, a fourth monkey fed TCDF-free cakes was housed in an adjacent cage and subjected to blood and biopsy examinations to monitor possible cross-contamination of food and faeces.

The animals were observed daily and weighed weekly; blood samples were taken every 2 wk (50 ppb diet) or monthly (5 ppb diet) for haemogram and automated serum chemical analyses (sodium, potassium, chloride, bicarbonate, calcium, phosphorus, glu-

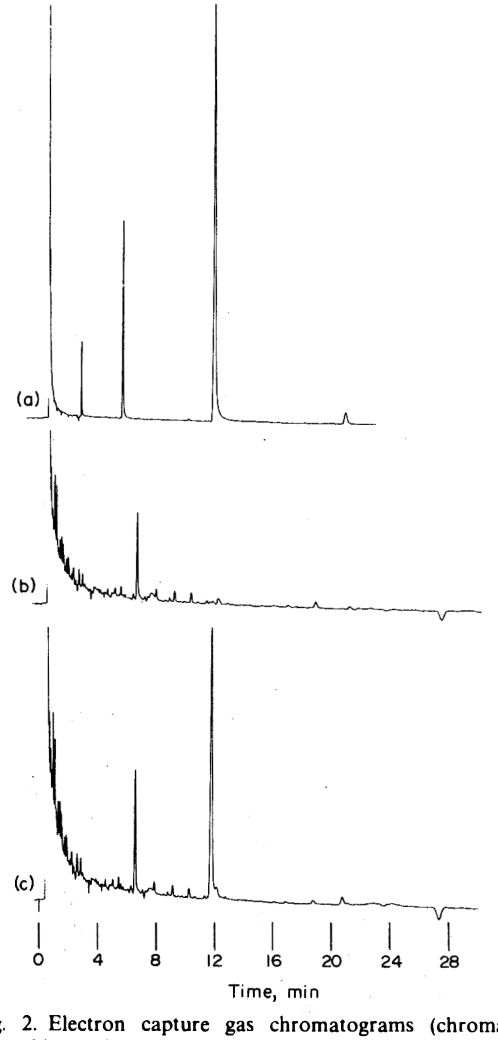


Fig. 2. Electron capture gas chromatograms (chromatographic conditions are described in the text.). (a) A mixture of polychlorinated dibenzofuran standards: 2,8-di- (420 pg), 2,3,8-tri- (444 pg), and 2,3,7,8-tetrachlorinated dibenzofuran (400 pg) in that order; (b) control diet; (c) diet prepared with 5 ppb added 2,3,7,8-tetrachlorodibenzofuran.

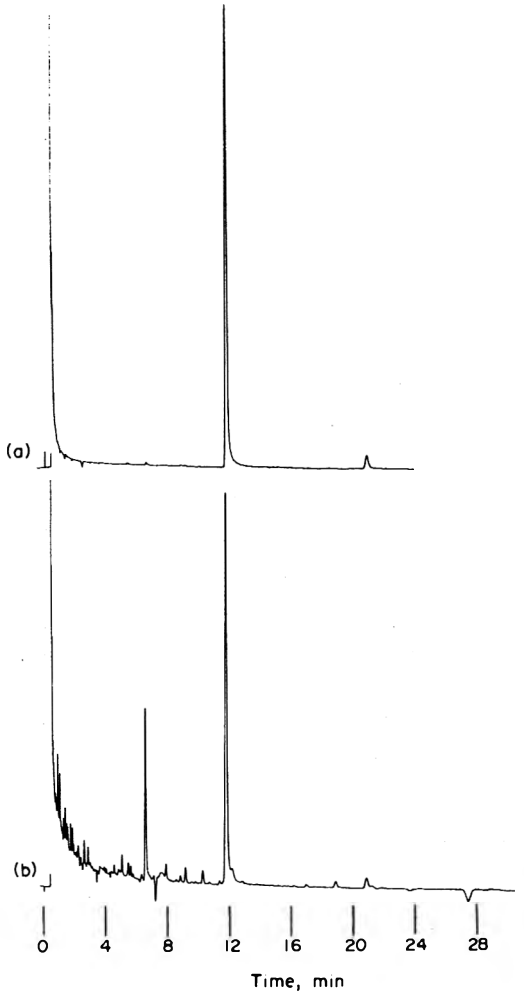


Fig. 3. Electron capture gas chromatograms (chromatographic conditions are described in the text) (a) 400 pg of TCDF standard (small peak at about 21 min is an unidentified pentachlorodibenzofuran isomer impurity in the TCDF reference material) (b) control diet fortified with 5 ppb TCDF; there was an 85% recovery.

cose, urea nitrogen, total protein, albumin, bilirubin, creatinine, uric acid, cholesterol, triglycerides, alkaline phosphatase, glutamic-oxaloacetic transaminase, and lactic dehydrogenase). Biopsy samples of cheek skin were taken under light ketamine (Ketalar, Parke-Davis) anaesthesia, before exposure to TCDF and at 1-month intervals during exposure and recovery. Moribund monkeys were killed and examined by conventional autopsy procedures. All biopsy and autopsy tissue samples were fixed in formaldehyde–glutaraldehyde; embedded in glycol methacrylate; and stained with haematoxylin, methylene blue, and basic fuchsin.

*Precautions.* Stock solutions of TCDF were prepared with gloved hands over disposable bench covers under a hood. The monkeys were housed in an isolated building entirely lined with stainless steel. The excreta and spilled food were collected in sheet plastic. Disposable coveralls, plastic booties, masks, and gloves were worn by personnel who fed the monkeys and collected the excreta. All waste was burned in a detached, gas-fired incinerator with a high-temperature (800°C) afterburner.

## RESULTS

### *Animals exposed at the 50 ppb level*

After 1 month all three monkeys showed periorbital oedema, reddening and thickening of the eyelids, enlargement of facial hair follicles, and partial loss of tactile hairs from the cheeks, lips, and chin. The normally abundant sebaceous glands in the skin of the cheek were reduced in size and number. At this time, one of the three sick monkeys was returned to the control diet.

After 2 months the physical activity of the two remaining exposed animals was markedly reduced and skin changes were severe (Fig. 4). The meibomian glands were enlarged and beaded. Body hair was easily pulled out in tufts, and the skin of the trunk was dry and scaly. The fingernails and toenails were elevated by subungual thickening or were lost altogether. Sebaceous glands were totally absent, and hair follicles were reduced to narrow squamous pegs in the cheek skin. At this time, one of the two monkeys died, and the other was returned to the normal diet.

Recovery was rapid in the two monkeys that had been fed TCDF for 1 and 2 months, respectively, and then given uncontaminated cakes. By the end of a week they were more active. By the end of the first month the facial changes had largely receded, the nails were regrowing, and sebaceous glands were reappearing in the cheek skin. Within 3 months, the behaviour, clinical appearance, and histological structure of the skin were normal. There were no significant changes in haematology or serum-chemical values during TCDF feeding or during recovery.

The abnormal findings at autopsy in the monkey that died after consuming TCDF at 50 ppb for 2 months were limited to the skin, thymus and stomach. The body weight at death was 23% less than at the start of feeding, and the body hair was almost gone. Sebaceous glands in lip, cheek, scalp, scrotum and external auditory canal were replaced by small squamous nubbles or occasionally by squamous cysts. The meibomian glands were converted to tubular squamous cysts filled with keratin (Fig. 5). The nails were lost (although the root remained), and the nail bed was thickly keratinized. The thymic lobules, which are normally large and cellular at this age, were small and without cortices (Fig. 6). In the mucosa of the body of the stomach, parietal cells were absent. The normal pattern of pit, neck, and tortuous glands was replaced by an array of straight, sometimes dilated glands lined only by tall mucous cells, save for a few zymogenic cells at the very bases of the glands. Mucous glands occasionally extended into the submucosa to form irregular cysts (Fig. 7).

The liver was grossly normal in colour and size. The only light-microscopic abnormality was an increase in the height and number of goblet cells in the epithelium of intrahepatic bile ducts. The overall cellularity of the bone marrow was decreased (this was not reflected in peripheral blood counts before death).

### *Animals exposed at the 5 ppb level*

One of the three monkeys showed periorbital oedema and a 20% weight loss after 6 wk. During wk 7, a cough and diarrhoea developed, and the animal was killed when moribund.

The other two monkeys first showed periorbital oedema and enlargement of the meibomian glands at 3 months, and their appearance remained the same for the next 3 months. There was no apparent loss of hair. Just before the end of month 6, one monkey abruptly stopped eating, lost weight, and passed frequent watery stools in which there were many strongyloides. After a week of acute illness, during which the animal was treated with thiabendazole, paromomycin sulphate, and parenteral fluids, this monkey died overnight.

The third monkey was returned to the TCDF-free diet at the end of month 6. The only physical findings at that time were swollen eyelids and hyperkeratotic nail beds.

Normal growth was maintained in the treated animals, except for a brief preterminal period of rapid weight loss in the two monkeys that died. The values for haemoglobin, haematocrit, serum albumin, serum cholesterol, and serum calcium fell slightly in the two animals that lived for 6 months, but these values remained within normal limits.

As was the case at the higher dietary level, recovery was rapid in the single surviving monkey that had been fed TCDF at 5 ppb for 6 months. Within 1 month, physical activity was normal and the face and nails were unremarkable. Biopsy samples of the cheek showed a partial return of sebaceous glands after 1 month and complete restoration at 2 months.

The abnormal post-mortem findings in the monkey that was killed when moribund at 7 wk were similar to those found in the monkey given a higher dose, though less severe. The thymus was highly involuted and the gastric mucosa altered, but atrophy and squamous metaplasia of the sebaceous glands were only partial.

The monkey that died at 6 months showed atrophy and squamous metaplasia of the sebaceous glands, hyperkeratotic nail beds, involution of the thymus, and mucous metaplasia of the gastric mucosa, as well as focal acute and organizing haemorrhagic pneumonitis and acute focal ulcerative colitis, both due to strongyloides. The vertebral bone marrow was hypocellular (Fig. 8), although peripheral erythrocyte and leukocyte counts had been within normal limits.

## DISCUSSION

Chronic oral intake by monkeys of small amounts of TCDF caused a disease clinically and pathologically similar to that following a single large dose of TCDF (Moore *et al.* 1979), acute or chronic ingestion of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; Allen, Barsotti, Van Miller, Abrahamson & Lalich 1977; McConnell, Moore & Dalgard, 1978; McNulty, 1977), or chronic intake of PCBs (Allen, 1975). The chief histopathological effects in all cases were seen in the thymus, sebaceous glands, nail beds, mucosa of the stomach and bile ducts, and bone marrow. The mechanism of these diverse effects is not known.

The reason for death in the TCDF-poisoned monkeys was obscure. The thymic and epithelial changes hardly seemed lethal, and there was no histopathological, haematological, or serum chemical evidence for significant disease of the liver, or indeed of any other organ. Death was preceded by weight loss, anorexia, and depression—nonspecific signs for which there was no apparent explanation. Only in one of these deaths (the later at 5 ppb) was an intercurrent disease identified—severe strongyloidiasis—which may have caused or at least hastened death by dehydration and electrolyte imbalance.

The toxic potency of TCDF, when chronically ingested by rhesus macaques, was approximately equivalent to that of TCDD, which caused sickness in adult rhesus females after months of exposure at dietary concentrations of 0·05 and 0·5 ppb (Allen *et al.* 1977; Schantz, Barsotti & Allen, 1979). Although the levels of TCDD used in these studies were lower than the levels of TCDF reported here, a quantitative distinction between potencies cannot be made because the ages and sexes were different and the numbers of animals were small.

However, TCDF is much more toxic for monkeys than the commercial PCB mixtures Aroclor 1248 (Allen, 1975) and Aroclor 1242 (Becker, McNulty & Bell, 1979), both of which caused sickness and some deaths in rhesus macaques in a few months at dietary levels of 2·5 to 25 ppm. We therefore estimate that TCDF is 1000 times more potent. This estimate can only be provisional because chronic dose-response relationships have not been established either for TCDF or for the Aroclors.

Commercial PCB mixtures have been reported to be contaminated with PCDFs at up to 33 ppm (Bowes *et al.* 1975; Miyata, Nakamura & Kashimoto, 1976; Morita, Nakagawa, Akiyama, Mimura & Isono, 1977; Nagayama, Kuratsune & Masuda, 1976; Roach & Pomerantz, 1974). In each report, tetrachlorodibenzofurans as a class were identified as components of the contaminating PCDFs, but in only one case was the 2,3,7,8- isomer tentatively identified (Bowes, Mulvihill, DeCamp & Kende, 1975); the concentration of this isomer was less than 1 ppm in American and Japanese PCB mixtures. Therefore, since the toxic ratio of TCDF to PCB mixtures is of the order of 1000, TCDF alone cannot account for the toxicity of PCB mixtures.

In the absence of any information on the toxicities of other individual PCDFs, except for one report on 2,3,4,7,8-pentachlorodibenzofuran (Moore *et al.* 1979), it is possible that these other congeners, sometimes present in the aggregate at levels above 1 ppm, contribute to the action of the mixture, even though their specific toxicities may be less than that of TCDF. Furthermore, the toxicities of contaminating PCDFs are likely to be additive one with another and with those of the PCBs themselves, if uncontaminated PCBs are toxic. These uncertainties can in principle be resolved only by toxicological tests with pure PCDFs, very pure single PCBs, and PCB mixtures free from all contaminants (if such mixtures can be prepared without alteration of the spectrum of the individual PCBs present).

The toxicity of PCDFs is much more significant with respect to Yusho oil, in which PCDFs were concentrated 250 to 500 times and enriched in the more chlorinated congeners, relative to the PCB mixture—Kanechlor 400—with which it was contaminated (Miyata, Kashimoto & Kunita, 1977; Morita *et al.* 1977; Nagayama *et al.* 1976). Buser *et al.* (1978) identified over 40 individual PCDFs, among which the two

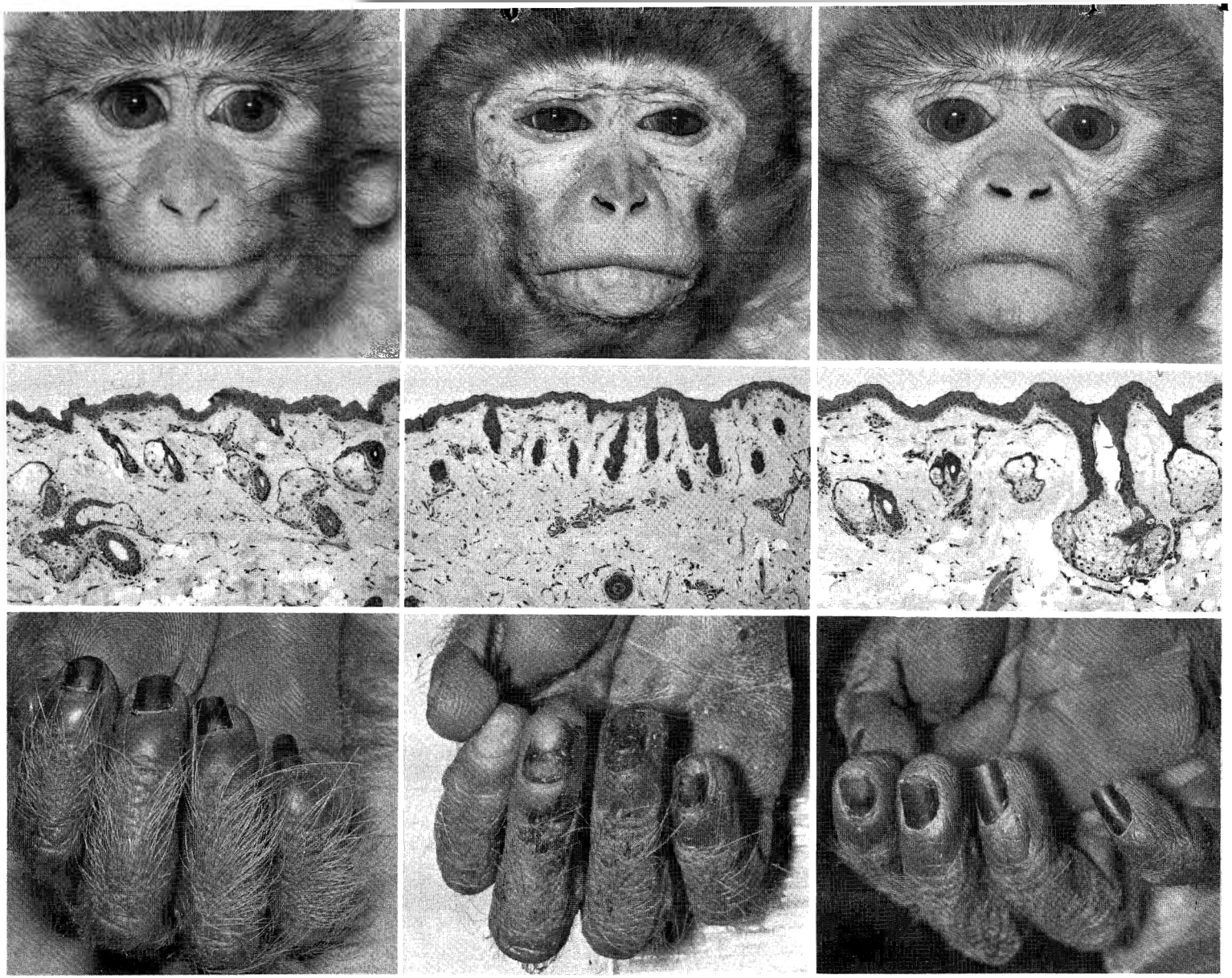

Fig. 4. Face, micrograph of skin of cheek ( $\times 82$), and fingernails of young rhesus male before exposure (left), after 2 months of consumption of cakes containing 50 ppb TCDF (centre), and after 3 more months of a control diet (right). At the end of the exposure, the eyelids were thickened and the facial skin was shiny, granular, and hairless; sebaceous glands had disappeared and hair follicles were small epithelial pegs. The fingernails were shortened and elevated or lost. Recovery was complete after 3 months of a TCDF-free diet.

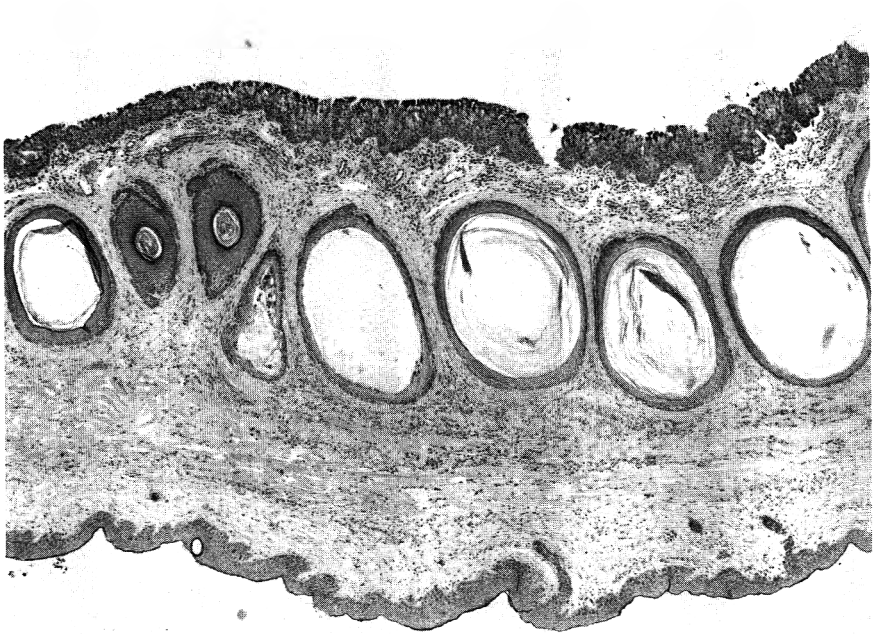

Fig. 5. Upper eyelid of a monkey that died after ingesting cakes containing 50 ppb TCDF for 2 months. Conjunctival surface at the top. Meibomian glands were converted to simple, distended squamous cysts. × 35.

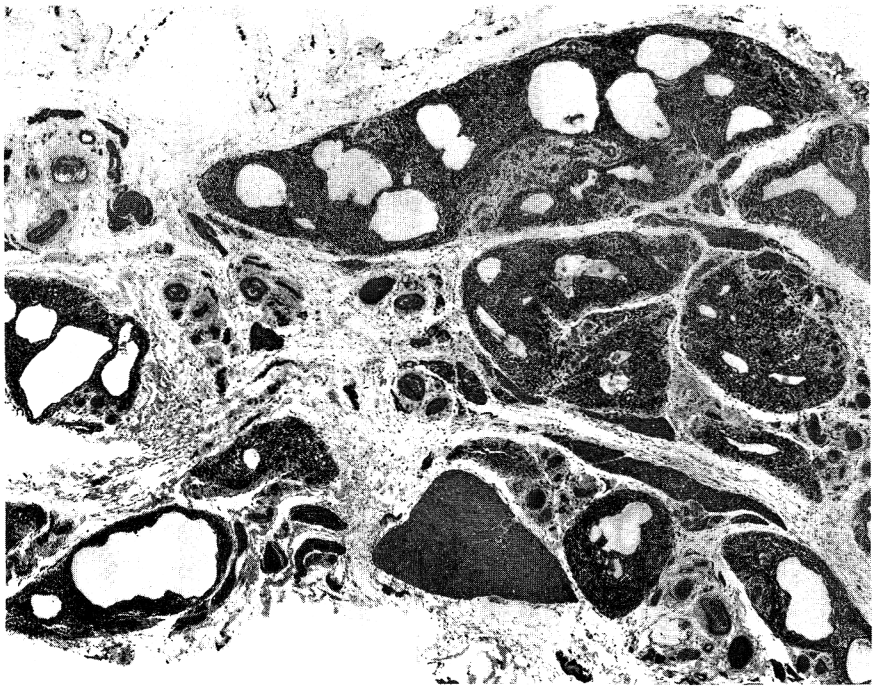

Fig. 6. Thymus of a monkey that died after ingesting cakes containing 50 ppb TCDF for 2 months. The lobules shrank and had no cortices; the corpuscles were cystic. × 35.

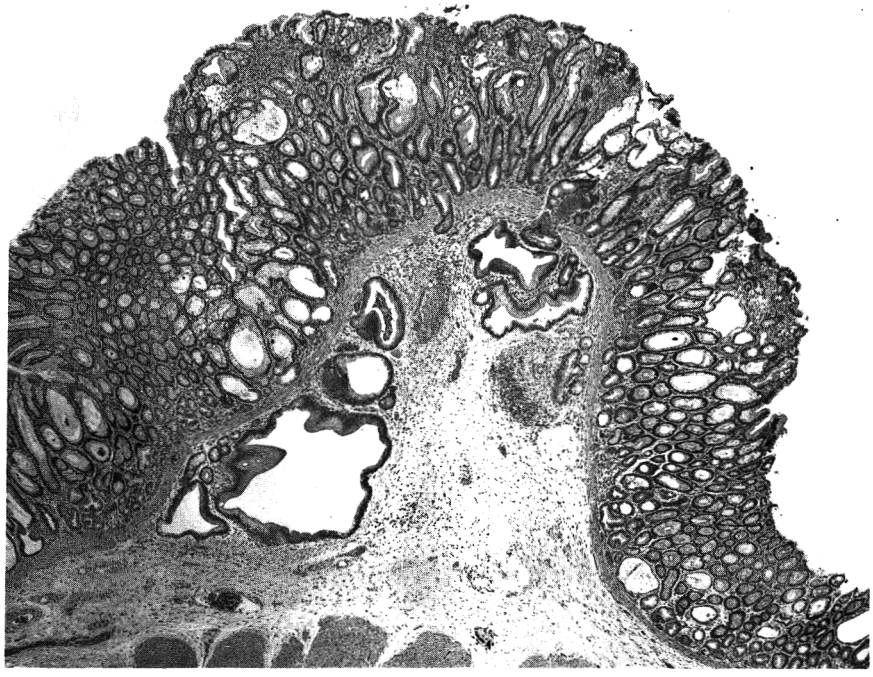

Fig. 7. Mucosa of body of stomach of a monkey that died after ingesting cakes containing 50 ppb TCDF for 2 months. Mucous cells entirely replaced parietal and zymogenic cells. There were numerous small intramucosal cysts and cystic growth of mucous glands in the submucosa. × 20.

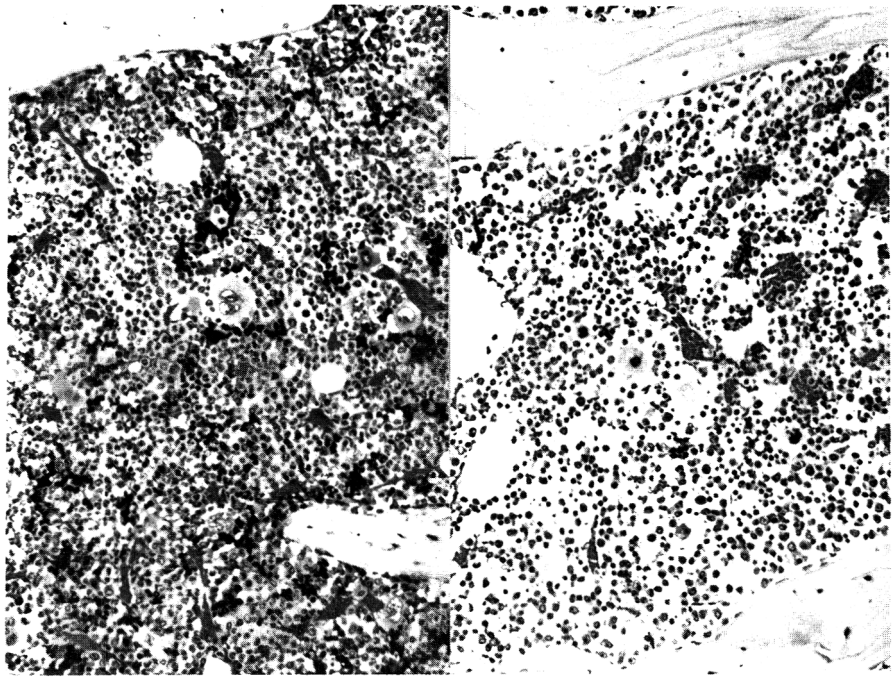

Fig. 8. Vertebral bone marrow (left) of a normal juvenile rhesus male and (right) of a monkey that died after ingesting cakes containing 5 ppb TCDF for 6 months. Cellularity was reduced in the treated monkey. × 130.

known to be toxic, TCDF and 2,3,4,7,8-pentaCDF, were major components, in Yusho oil and in 'used' Kanechlor 400 (Kanechlor 400 that has been subjected to prolonged heating).

The action of TCDF appears to be cumulative with chronic exposure. Young male monkeys consume about 100 g of diet cake/kg body weight/day, and the intake of TCDF for the 50 ppb diet was therefore about 5 μg/kg/day, or 300 μg/kg in 2 months. This figure compares with an estimate of the acute $LD_{50}$ for rhesus macaques of 1000 μg/kg (Moore *et al.* 1979). The effects of a given amount of TCDF seem to be similar, whether administered orally all at once or ingested gradually over a period of weeks or months.

On the other hand, cumulative action is inconsistent with the rapid recovery of the poisoned monkeys that were returned to a TCDF-free diet. This quick return to health contrasted with the course of TCDD poisoning in monkeys, in which illness progressed to death or recovery which was much delayed even after chronic exposure had ended (Allen *et al.* 1977).

Almost nothing is known of the pharmacokinetics of TCDF and TCDD in monkeys. Probably TCDD is slowly excreted; less than 5% of a single dose was found in urine and faeces during the week after dosing (Van Miller, Marlar & Allen, 1976). No such data exist for TCDF.

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# RAT TERATOLOGY STUDY OF ORALLY ADMINISTERED TRIS-(2,3-DIBROMOPROPYL) PHOSPHATE*†

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**Abstract**—A study was undertaken to determine the teratogenic potential of tris-(2,3-dibromopropyl) phosphate (TRIS), the flame retardant formerly used in clothing. TRIS, in undiluted propylene glycol, was orally intubated into pregnant Sprague–Dawley rats on days 6–15 of gestation at a level of 0, 5, 25 or 125 mg/kg/day. A group of untreated females served as a negative control group. Weight gain during gestation was significantly decreased in the animals treated with 125 mg/kg/day, but no other compound-related toxic or teratogenic effects were observed.

## INTRODUCTION

The US Flammable Fabrics Act of 1953, which was intended to decrease the risk of death or injury due to fires, regulated the manufacture of highly flammable clothing and was amended in 1954 to include minimum flammability standards for general clothing. The standards were further extended to carpets and rugs in 1971, to mattresses in 1973 and to children's sleepwear in 1973 and 1975. All these flammability standards were codified in 1975 (*Federal Register* 1975, **40**, 59889).

In order to provide flame-retardant chemicals to meet the new standards, chemical manufacturers increased their production markedly. For example, the estimated 150 million pounds produced in 1971 was increased to over 300 million pounds in 1977 (Sanders, 1978). In 1976, tris-(2,3-dibromopropyl) phosphate (TRIS) was the most extensively used flame retardant for textiles. The following year it was prohibited in the USA for use in children's clothing on the basis of its mutagenic and carcinogenic potential (*Federal Register* 1977, **42**, 18850, 28060, 61593 & 61621).

Several subchronic oral studies of TRIS have been performed. Rats given TRIS in corn oil by gavage at dose levels of 10, 50 or 100 mg/kg for 4 wk (Brieger, Gabriel & Rieders, 1968) had increased blood-bromide concentrations. Dogs given TRIS in the diet at dose levels of 50 or 100 mg/kg had decreased body weights and elevated blood-bromide concentrations (Ulsamer, Osterberg & McLaughlin, 1980). In a 2-wk oral toxicity study, dogs and rats fed 15% shredded TRIS-treated acetate fibres three times per week showed no changes in blood-bromide concentrations and no adverse effects were observed (Ulsamer *et al.* 1980). Similarly, no adverse effects were observed in dogs fed shredded TRIS-treated rayon and acetate fibres for 3 wk (Ulsamer *et al.* 1980).

Decreased body weights and increased bromide concentrations in muscle, liver and fat were found in rats fed 100 or 1000 ppm TRIS for 28 days (Kerst, 1974). TRIS in propylene glycol intubated into rats for 5 days/wk for 13 wk at 25, 100 or 250 mg/kg produced renal tubular nephritis (Osterberg, Bierbower, Ulsamer, Porter & Jones, 1978).

To date, no teratology study of TRIS has been published, but a teratology study was conducted on a similar flame retardant, tris-(1,3-dichloroisopropyl) phosphate (Fyrol FR-2). This compound differs from TRIS in that it contains chlorine instead of bromine and has branched organic groups. When Fyrol FR-2 was intubated into pregnant rats on days 6–15 of gestation at dose levels of 25, 100 or 400 mg/kg, no foetal malformations were observed, but maternal and foetal toxicity occurred at the highest dose level (Stauffer Chemical Co. report, cited by Ulsamer *et al.* 1980). The present report is an attempt to furnish information about the teratogenic potential of TRIS.

## EXPERIMENTAL

*Materials and animals.* TRIS was purchased from Great Lakes Chemical Co., West Lafayette, IN (Lot

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no. NY-7431). By solid-probe mass-spectrometric and gas-chromatographic analyses, the sample was determined to contain 99·7% TRIS, 0·14% 1,2,3-tribromopropane and 0·17% 2,3-dibromopropyl alcohol. TRIS is a slightly yellow, viscous liquid that is practically insoluble in water; at 25°C it has a density of 2·27 g/ml and a viscosity of 9200 cP. Appropriate volumes of TRIS were mixed on a weight/volume basis with undiluted laboratory-grade propylene glycol obtained from Fisher Scientific Co., Pittsburgh, PA. Sexually mature, timed-pregnant Sprague–Dawley rats were obtained from Charles River Breeding Laboratories, Wilmington, MA, and were shipped to our laboratories on either day 1 or day 2 of pregnancy. Their body weight on day 2 of pregnancy was 192 ± 13·8 g (mean ± 1 SD). Each experimental or control group consisted of at least 30 animals. All of the animals were fed Purina Lab Chow (Ralston Purina Co. Inc., St. Louis, MO) and given tap water *ad lib*. The animals were housed in stainless-steel hanging cages and the light cycle provided 12 hr darkness and 12 hr light.

*Procedure.* The timed-pregnant rats were randomly allocated to an experimental or control group. The day on which sperm was found in the vagina was designated day 0 of gestation. On days 6–15 of gestation, concurrent groups were intubated with TRIS in undiluted propylene glycol at a level of 0, 5, 25 or 125 mg/kg/day; a group of untreated animals served as negative controls.

The animals were intubated at the same time each day by the same personnel (whenever possible), and a 15-gauge needle with a smooth bead soldered onto the end was used to administer 0·25 ml/100 g body weight. On day 20 of gestation, the animals were killed by $CO_2$ asphyxiation. The uteri were opened and examined *in situ* for the presence and location of resorption sites and of live and dead foetuses. Deciduomas, brownish implantation sites without placentas, were called early deaths, and implantation sites with placentas and with complete but non-viable foetuses that were of subnormal size, showed retarded development or were in a macerated condition were classed as late deaths, according to the terminology of Bateman & Epstein (1971) and the MARTA Committee on Terminology (1969). A runt was considered to be any foetus that weighed at least 30% less than the average weight of the concurrent negative controls (Leuschner & Czok, 1973). Each live foetus was weighed, sexed and examined for gross external malformations under magnification, and the crown–rump length was measured. Corpora lutea were counted under magnification. Approximately half of the foetuses from each litter were fixed in alcohol, stained with Alizarin Red S (Dawson, 1926) and examined for skeletal variations. The remaining foetuses were fixed in Bouin's solution, razor-blade sectioned (Wilson, 1965) and examined for internal variations of the soft tissues. The investigators performing the examinations had no knowledge of which treatment group the litters belonged to.

*Statistical analysis.* Data on the numbers of corpora lutea per dam, implantations per dam, number of viable implants per dam, foetal body weights, crown–rump lengths and food consumption were submitted to an analysis of variance followed by a least signifi-

Table 1. *Maternal and reproductive data for rats given tris-(2,3-dibromopropyl) phosphate (TRIS) during gestation*

| Compound | Dose level (mg/kg) | No. of pregnant females examined | Autopsy findings (mean/dam) | | | | | | Females with resorptions (%) | | Mean food consumption (g/day) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Maternal body-weight gain (g)* | Corpora lutea* | Implantations* | Resorptions | | Viable foetuses* | Total resorptions (%) | Preimplantation loss (%) | One or more | Two or more | |
| | | | | | | Early | Late | | | | | | |
| None | — | 30 | 157·3 ± 4·5 | 13·7 ± 0·7 | 10·3 ± 0·6 | 0·5 | 0 | 9·9 ± 0·6 | 4·5 | 24·4 | 33·3 | 10·0 | 23·4 |
| Propylene glycol (2·5 ml/kg) | | 32 | 147·6 ± 5·1 | 13·8 ± 0·6 | 10·2 ± 0·5 | 0·7 | 0 | 9·5 ± 0·6 | 6·4 | 26·4 | 31·2 | 15·6 | 21·7† |
| TRIS | 5 | 24 | 151·2 ± 5·2 | 13·0 ± 0·8 | 10·1 ± 0·6 | 0·3 | 0 | 9·8 ± 0·6 | 3·3 | 22·7 | 29·2 | 4·2 | 22·7 |
| | 25 | 28 | 150·7 ± 6·9 | 13·5 ± 0·6 | 11·1 ± 0·6 | 0·5 | 0 | 10·6 ± 0·6 | 4·8 | 17·7 | 25·0 | 7·1 | 22·7 |
| | 125 | 31 | 130·3 ± 5·3‡ | 14·7 ± 0·7 | 10·1 ± 0·6 | 0·5 | 0·03 | 9·6 ± 0·7 | 5·4 | 31·5 | 32·3 | 6·4 | 20·8 |

*Values are means ± SEM.
†Significantly different ($P < 0·01$) from the corresponding value for the untreated (negative control) group.
‡Significantly different ($P < 0·05$) from the corresponding value for the solvent (propylene glycol) control group.

cant difference (LSD) test. The numbers of resorptions per litter were transformed by use of the Freeman–Tukey arc-sine transformation for binomial proportions (Mosteller & Youtz, 1961) followed by an analysis of variance and an LSD test. Preimplantation loss data were transformed using the Freeman–Tukey arc-sine transformation followed by an analysis of variance and an LSD test. The numbers of litters with one or more resorptions, one or more skeletal or soft-tissue variations and specific external, soft-tissue and skeletal variations were analysed by the Fisher exact one-tailed test (Siegel, 1956). An analysis of variance was used to test maternal weight gain. An Armitage test for linearity of proportions (Armitage, 1973) was also used to detect trends.

## RESULTS AND DISCUSSION

Preliminary single-dose studies on rats in our laboratory gave $LD_{50}$ values of 1·88 g/kg for 10% (w/v) TRIS in propylene glycol and 20 g/kg for undiluted propylene glycol.

Prior to the present experiment, a pilot study was conducted in which groups of ten pregnant rats were orally intubated with TRIS at 0, 250 or 1000 mg/kg/day on days 6–15 of gestation. The resulting mortality was 0, 10 and 100%, respectively. The rats given 1000 mg/kg/day died on days 9–11 of gestation. In another pilot study, groups of ten non-pregnant rats were orally intubated with TRIS for 10 days at dose levels of 100, 150, 500 or 1000 mg/kg/day; mortality was 0, 0, 70 and 100%, respectively.

In the present study, no adverse effects were observed except for a significantly decreased body-weight gain during gestation in the animals treated with 125 mg TRIS/kg compared with the value for the solvent control group (Table 1). The average amount of food consumed by the solvent control group was significantly decreased ($P < 0·01$) when compared

Table 2. *Foetal data for rats given tris-(2,3-dibromopropyl) phosphate (TRIS) during gestation*

| Compound | Dose level (mg/kg) | Foetal body weight (g)* | | Crown–rump length (cm)* | | Sex distribution (% of live foetuses) | |
|---|---|---|---|---|---|---|---|
| | | M | F | M | F | M | F |
| None | — | 4·30 ± 0·07 | 4·06 ± 0·06 | 4·0 ± 0·03 | 3·9 ± 0·02 | 53·7 | 46·3 |
| Propylene glycol (2·5 ml/kg) | — | 4·36 ± 0·08 | 4·10 ± 0·06 | 4·0 ± 0·03 | 3·9 ± 0·02 | 48·5 | 51·5 |
| TRIS | 5 | 4·19 ± 0·05 | 4·02 ± 0·06 | 4·0 ± 0·01 | 3·9 ± 0·03 | 50·0 | 50·0 |
| | 25 | 4·26 ± 0·04 | 4·01 ± 0·06 | 4·0 ± 0·02 | 4·0 ± 0·03 | 49·3 | 50·7 |
| | 125 | 4·28 ± 0·06 | 4·05 ± 0·04 | 4·0 ± 0·03 | 3·9 ± 0·02 | 48·3 | 51·7 |

*Values are means ± SEM.

Table 3. *Incidence of specific external and soft-tissue variations in foetuses of rats given tris-(2,3-dibromopropyl) phosphate (TRIS) during gestation*

| Variation | Incidence* in foetuses and litters of rats given | | | | |
|---|---|---|---|---|---|
| | No treatment | Propylene glycol (2·5 ml/kg) | TRIS (mg/kg) 5 | 25 | 125 |
| **External variations** | | | | | |
| No. of foetuses examined | 296 (30) | 305 (32) | 234 (24) | 296 (28) | 296 (31) |
| Haemorrhage | 15 (9) | 8 (6) | 6 (5) | 7 (7) | 10 (8) |
| Runt | 3 (3) | | 3 (3) | 1 (1) | 2 (1) |
| Deformed head and nose | 1 (1) | | | | |
| **Soft-tissue variations** | | | | | |
| No. of foetuses examined | 151 (30) | 143 (32) | 113 (24) | 143 (27) | 146 (30) |
| Hydrocephalus | 1 (1) | | | | |
| Vessel inside vena cava | | | | 1 (1) | |
| Reversed dorsal aorta | | | | 1 (1) | |
| Diaphragmatic hernia | | | | | 1 (1) |
| Discoloured kidney | 25 (10) | 35 (14) | 25 (13) | 37 (13) | 39 (15) |
| Ectopic kidney | 2 (2) | 5 (3) | | 7 (5) | 1 (1) |
| Enlarged renal pelvis: severe | 3 (2) | 2 (2) | 6 (5) | 7 (6) | 10 (6) |
| moderate | 8 (7) | 10 (6) | 11 (8) | 6 (5) | 8 (7) |
| Hydroureter | 48 (18) | 41 (21) | 36 (16) | 59 (21) | 37 (20) |
| Bladder missing | | | | | 1 (1) |
| Discoloured adrenal | 7 (4) | 15 (9) | 14 (7) | 17 (9) | 7 (4) |
| Ectopic ovary | | 1 (1) | | | |
| Ectopic testes | 3 (1) | | | | |
| Testes incompletely descended | 1 (1) | | 1 (1) | | |
| Haemorrhage | 3 (3) | 3 (2) | 4 (4) | 7 (5) | 4 (4) |

*No. of foetuses affected and, in parentheses, no. of litters.

Table 4. *Incidence of soft-tissue variations in foetuses of rats given tris-(2,3-dibromopropyl) phosphate (TRIS) during gestation*

| Compound | Dose level (mg/kg) | Soft-tissue variations: No. | Soft-tissue variations: Mean/ litter | Foetuses with one or more soft-tissue variations: No. | Foetuses with one or more soft-tissue variations: Mean/ litter | Foetuses with one or more soft-tissue variations: % of total foetuses | Litters with one or more foetuses with variations: No. | Litters with one or more foetuses with variations: % of total litters |
|---|---|---|---|---|---|---|---|---|
| None | — | 101 | 3·4 | 66 | 2·2 | 43·7 | 21 | 70·0 |
| Propylene glycol (2·5 ml/kg) | — | 112 | 3·5 | 73 | 2·3 | 51·0 | 27 | 84·4 |
| TRIS | 5 | 97 | 4·0 | 64 | 2·7 | 56·6 | 22 | 91·7 |
| | 25 | 142 | 5·3 | 94 | 3·5* | 65·7 | 25 | 92·6 |
| | 125 | 108 | 3·6 | 80 | 2·7 | 54·8 | 27 | 90·0 |

*Significantly different ($P < 0·05$) from corresponding value for solvent (propylene glycol) control group.

with the negative control group. However, there were no significant differences ($P > 0·10$) in the amounts consumed by any of the treated groups when compared to the solvent controls. At autopsy, no effects of treatment on the numbers of corpora lutea, implantations, early deaths or late deaths were apparent (Table 1). Neither did the percentage of females with resorptions, the number of viable foetuses, the percentage of resorptions or the percentage of preimplantation loss show any changes due to compound dosage. Approximately equal numbers of males and females were produced in the control groups and at each dose level (Table 2). Neither foetal body weight nor crown–rump length was affected by administration of TRIS (Table 2).

Runts were found in all of the groups except the propylene glycol control group, and one foetus in the negative control group had an externally deformed head and nose (Table 3). Of the foetal soft-tissue variations found, the incidence of severely enlarged renal pelvises appeared to be increased with TRIS dosage, but the increases were not dose-related and were not statistically significant ($0·080 < P < 0·111$) compared with the value for the solvent controls. The number of hydroureters was increased at the 25-mg/kg level but not at the 100 mg/kg level. No dose-response was evi-

Table 5. *Incidence of specific skeletal variations in foetuses of rats given tris-(2,3-dibromopropyl) phosphate (TRIS) during gestation*

| Variation | Incidence* in foetuses and litters of rats given: No treatment | Propylene glycol (2·5 ml/kg) | TRIS (mg/kg): 5 | 25 | 125 |
|---|---|---|---|---|---|
| No. of foetuses examined | 145 (28) | 159 (31) | 117 (23) | 151 (28) | 148 (30) |
| Sternebrae: RO | 22 (13) | 8 (7) | 18 (8) | 23 (16) | 15 (6) |
| bipartite | | 4 (4) | 1 (1) | 2 (2) | |
| missing | 18 (12) | 8 (6) | 14 (10) | 11 (5) | 9 (6) |
| malaligned | 3 (3) | 7 (7) | 5 (5) | 7 (6) | 6 (6) |
| Vertebrae: RO | | | | | 1 (1) |
| Centrum: bipartite | | 1 (1) | 1 (1) | 3 (2) | 1 (1) |
| Fourteenth rib bud | 39 (19) | 34 (15) | 43 (16) | 48 (18) | 37 (17) |
| Fourteenth rib | 2 (2) | 2 (2) | 2 (2) | 3 (3) | |
| Fifteen ribs | | 1 (1) | | | |
| Ribs: RO | | | | | 1 (1) |
| missing | | | | | 1 (1) |
| wavy | 4 (1) | 4 (4) | 6 (4) | 9 (6) | 11 (5) |
| Hyoid bone: RO | 1 (1) | 1 (1) | 1 (1) | 3 (3) | |
| missing | | | 1 (1) | | 1 (1) |
| Pubis: missing | | | | | 1 (1) |
| Metacarpals: RO | | | | | 1 (1) |
| Phalanges: RO | | | | | 1 (1) |
| Interparietal bone: RO | 17 (10) | 13 (9) | 23 (12) | 22 (15) | 18 (12) |
| Parietal bone: RO | 1 (1) | 1 (1) | 6 (2) | 1 (1) | 1 (1) |
| Supraoccipital bone: bipartite | | | | | 1 (1) |

RO = Reduced ossification

*No. of foetuses affected and, in parentheses, no. of litters.

Table 6. *Incidence of sternebral variations in foetuses of rats given tris-(2,3-dibromopropyl) phosphate (TRIS) during gestation*

| Compound | Dose level (mg/kg) | Sternebral variations: Total | Sternebral variations: Mean/ litter | Foetuses with one or more sternebral variations: No. | Foetuses with one or more sternebral variations: Mean/ litter | Foetuses with one or more sternebral variations: % of total foetuses | Litters with one or more foetuses with variations: No. | Litters with one or more foetuses with variations: % of total litters |
|---|---|---|---|---|---|---|---|---|
| None | — | 43 | 1·5 | 34 | 1·2 | 23·4 | 17 | 60·7 |
| Propylene glycol (2·5 ml/kg) | — | 27 | 0·9 | 23 | 0·7 | 14·5 | 17 | 54·8 |
| TRIS | 5 | 38 | 1·6 | 33 | 1·4 | 28·2 | 15 | 65·2 |
| | 25 | 43 | 1·5 | 40 | 1·4* | 26·5 | 21 | 75·0 |
| | 125 | 30 | 1·0 | 27 | 0·9 | 18·2 | 12 | 40·0 |

*Significantly different ($P < 0·05$) from the corresponding value for the solvent (propylene glycol) control group.

dent in the incidence of foetal soft-tissue variations (Table 4). No increase in the incidence of such variations occurred at the highest dose level, but at 25 mg/kg, the total number of soft-tissue variations was increased and the number of foetuses with one or more variations was increased significantly ($P < 0·05$). However, the percentage of litters affected showed no corresponding increase.

Evaluation of skeletal variations also showed no evidence of a dose-response (Table 5). There were apparent increases in the incidences of malaligned sternebrae and of wavy ribs in the foetuses of animals given propylene glycol compared with the untreated controls, but these differences were neither significant nor dose related. Among the foetuses of the 125-mg/kg group, a single poorly developed foetus had multiple defects: missing hyoid bone, sternebrae and pubis, bipartite supraoccipital bone, and incompletely ossified interparietal bone, vertebrae, metacarpals and phalanges.

The incidence of sternebral variations showed no compound-related effect of propylene glycol or TRIS (Table 6). The single significant increase ($P < 0·05$) in the number of foetuses with one or more sternebral variations occurred at 25 mg/kg. At the lowest TRIS level, the average number of skeletal variations (excluding sternebral variations) was increased (Table 7) and the number of foetuses with one or more variations was increased significantly ($P < 0·05$) compared with the solvent control value, but there was no corresponding increase in the number of litters affected.

Because of the lack of previous TRIS teratology studies, the results of this study can only be compared with those from the study of a similar flame retardant, Fyrol FR-2 (Stauffer Chemical Co. report, cited by Ulsamer *et al.* 1980). Fyrol FR-2 given by oral intubation to pregnant rats at 25, 100 or 400 mg/kg/day on days 6–15 of gestation caused no foetal malformations at any dose level, but there was evidence of foetal and maternal toxicity at 400 mg/kg. The TRIS study reported here showed a similar lack of teratogenicity and toxicity at the levels tested. On the basis of results of the preliminary pilot studies with TRIS, it seems likely that increasing the dose to 400 mg/kg/day would also have caused toxicity.

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Table 7. *Incidence of skeletal variations (excluding sternebral variations) in foetuses of rats given tris-(2,3-dibromopropyl) phosphate (TRIS) during gestation*

| Compound | Dose level (mg/kg) | Skeletal variations: Total | Skeletal variations: Mean/ litter | Foetuses with one or more skeletal variations: No. | Foetuses with one or more skeletal variations: Mean/ litter | Foetuses with one or more skeletal variations: % of total foetuses | Litters with one or more foetuses with variations: No. | Litters with one or more foetuses with variations: % of total litters |
|---|---|---|---|---|---|---|---|---|
| None | — | 64 | 2·3 | 55 | 2·0 | 37·4 | 24 | 85·7 |
| Propylene glycol (2·5 ml/kg) | — | 57 | 1·8 | 47 | 1·5 | 29·6 | 21 | 67·7 |
| TRIS | 5 | 83 | 3·6 | 63 | 2·7* | 53·8 | 20 | 87·0 |
| | 25 | 89 | 3·2 | 66 | 2·4 | 43·7 | 22 | 78·6 |
| | 125 | 76 | 2·5 | 57 | 1·9 | 38·5 | 22 | 73·3 |

*Significantly different ($P < 0·05$) from the corresponding value for the solvent (propylene glycol) control group.

trometric and gas–liquid-chromatographic analyses of TRIS.

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# THE DOSE-DEPENDENT METABOLISM OF [$^{14}$C]METHYLENE CHLORIDE FOLLOWING ORAL ADMINISTRATION TO RATS

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**Abstract**—[$^{14}$C]Methylene chloride ($^{14}CH_2Cl_2$) was administered to Sprague–Dawley rats in a single oral dose of 1 or 50 mg/kg. The disposition of radioactivity was followed for 48 hr after dosing. The major metabolites of $CH_2Cl_2$ were CO and $CO_2$, both of which were found in expired air. The fate of $^{14}CH_2Cl_2$ was dependent upon the administered dose. Rats given 1 mg $^{14}CH_2Cl_2$/kg metabolized approximately 88% of the dose, whereas those given 50 mg/kg metabolized only about 28%. Although all tissues examined 48 hr after dosing contained $^{14}$C activity, the overall tissue retention of $^{14}$C was low (< 10%) after either dose of $^{14}CH_2Cl_2$. The highest concentrations of $^{14}$C activity were found in the liver and the lowest in the fat. The data indicate that the observed dose-dependency was due to saturation of metabolic pathways for $CH_2Cl_2$ by the 50-mg/kg dose.

## INTRODUCTION

Methylene chloride ($CH_2Cl_2$) is widely used in a variety of medical, industrial and commercial applications and has become the solvent of choice for several new consumer products as well as for some food processing applications, particularly the decaffeination of coffee.

The fate of $^{14}CH_2Cl_2$ following ip injection in rats was reported by DiVincenzo & Hamilton (1975). Although these authors established that the major metabolites of $^{14}CH_2Cl_2$ were $^{14}CO$ and $^{14}CO_2$, the high doses used in these studies (412–930 mg/kg) preclude the application of these data to the consideration of low-level $CH_2Cl_2$ exposure. More recently, Yesair, Jaques, Schepis & Liss (1977) reported that the fate of $^{14}CH_2Cl_2$ given orally to mice was dose-dependent; high doses were not metabolized as extensively as low doses.

Several laboratories have initiated programmes for the safety evaluation of $CH_2Cl_2$. The studies reported here represent a portion of our investigations into the pharmacokinetics and metabolism of $^{14}CH_2Cl_2$ with the object of providing a data base sufficient to aid in the design and interpretation of laboratory investigations related to the potential hazards of exposure to the solvent.

## EXPERIMENTAL

*Animals.* Male Sprague–Dawley rats (Spartan Research Animals, Inc., Haslett, MI) weighing approximately 250 g were used. The rats were acclimatized to the experimental environment (Roth cages, Wyse Glass Specialities, Freeland, MI) for a minimum of 48 hr before treatment. Food (Purina Rat Chow; Ralston Purina Co. Inc., St. Louis, MO) and water were provided *ad lib.*

*Methylene chloride.* [$^{14}$C]Methylene chloride ($^{14}CH_2Cl_2$; specific activity 2·25 mCi/mmol) was obtained from New England Nuclear (Boston, MA; lot number 816-297). The minimum radiochemical purity of this material was 96·4% when assayed by liquid scintillation counting of the trapped effluent from a gas-chromatographic column (see below). The $^{14}CH_2Cl_2$ was diluted to the desired specific activity with cold, carrier technical-grade $CH_2Cl_2$ (minimum purity, 99%; Dow Chemical Co., Plaquemine, LA).

*Experimental procedure.* $^{14}CH_2Cl_2$ in distilled water was administered by gavage to groups of three rats at doses of 1 or 50 mg/kg. The final concentrations of $^{14}CH_2Cl_2$ in the dose solutions were assayed by gas chromatography and liquid scintillation spectrometry. The specific activities of $^{14}CH_2Cl_2$ in the dose solutions were 4·29 and 0·13 $\mu$Ci/mg $CH_2Cl_2$ for the 1- and 50-mg/kg dose levels respectively.

Immediately after administration of the single dose of $^{14}CH_2Cl_2$, the rats were placed in individual Roth-type metabolism cages, and urine, faeces and expired air were collected over the next 48 hr. Expired $^{14}CO$ and $^{14}CO_2$, and urine and faeces were collected at 12-hr intervals. The urine receptacles were kept at solid-$CO_2$ temperature to minimize the possible loss of volatile urinary excretion products. Expired $^{14}CH_2Cl_2$ was collected at 0·5-hr intervals for the first 5 hr after dosing. Subsequently a single sample was collected for the ensuing 8-hr period to ensure complete collection of all expired $^{14}CH_2Cl_2$.

The rats were killed by exsanguination 48 hr after treatment. The carcasses were skinned, the tissues were excised and all of the samples were stored frozen.

*Breath analysis.* The collection of radiolabelled volatiles in expired air was carried out by drawing room air (500 ml/min) through the glass metabolism cages and subsequently through a series of traps for the absorption of $^{14}CH_2Cl_2$, $^{14}CO_2$ and $^{14}CO$. The air leaving each metabolism cage was passed through the following: (a) a 1-cm diameter glass tube containing 6 g activated charcoal (grade 252; Witco Chemical Co., New York, NY) to trap expired $^{14}CH_2Cl_2$; (b) an

impinger containing 120 ml 5 M-ethanolamine in 2-methoxyethanol to trap expired $^{14}CO_2$; (c) a 50-ml impinger containing 45 g Hopcalite catalyst (Mine Safety Appliances, Pittsburgh, PA) to convert $^{14}CO$ to $^{14}CO_2$; (d) an additional 5 M-ethanolamine trap to collect $^{14}CO$ oxidized to $^{14}CO_2$. A Drierite cartridge (W. A. Hammond Drierite Co., Xenia, OH) was placed before the Hopcalite catalyst to absorb moisture which might otherwise have adversely affected its catalytic activity.

*Measurement of radioactivity.* A Searle Mark II or Mark III liquid scintillation spectrometer was used for all radioactivity measurements. External standard-channel ratios were used to determine counting efficiency. Known volumes of the $^{14}CO_2$ traps were counted in a toluene-based scintillation cocktail. The charcoal adsorbent for $^{14}CH_2Cl_2$ was divided into four equal aliquots and desorbed for 24 hr in the toluene-based scintillation fluid before counting. Samples of excreta and tissues were prepared for combustion in a Beckman Biological Materials Oxidizer (Beckman Instruments, Palo Alto, CA) and/or liquid scintillation counting as described previously (McKenna, Zempel, Madrid, Braun & Gehring, 1978).

*Gas chromatography.* The determination of the radiochemical purity of $^{14}CH_2Cl_2$ was performed using two different columns in a Hewlett Packard Model 5750 gas chromatograph equipped with a thermal conductivity detector. The conditions for these analyses were as follows: *System 1*—6-ft long and 1/8-in. OD stainless-steel column packed with 50/100 mesh Poropak QS (The Annspec Co., Ann Arbor, MI); injector and detector temperature, 200°C; column temperature, 80°C; carrier gas, helium at 40 ml/min; *System 2*—6-ft long and 1/8-in. OD stainless-steel column packed with 100/200 mesh Chromosorb 102 (The Annspec Co.); injector and detector temperature 230°C; column temperature 210°C; carrier gas, helium at 30 ml/min. The carrier gas effluent from the detector was bubbled through traps of toluene-based scintillation cocktail which were changed at regular intervals. $^{14}C$ activity was assayed by liquid-scintillation counting. The radiochemical purity of $^{14}CH_2Cl_2$ was expressed as the percentage of the total $^{14}C$ activity injected onto the column that was associated with the methylene chloride peak.

$^{14}CH_2Cl_2$ in the dose solutions was assayed on a Varian Model 2440 gas chromatograph using an $H_2$ flame-ionization detector. Column and instrument conditions for this analysis were essentially the same as those described for System 1 above.

*Pharmacokinetic analysis.* Concentration–time data obtained for the excretion of $^{14}C$ activity in the expired air or urine of each animal were characterized by a two-compartment open model. The best pharmacokinetic parameter estimates were obtained by a least squares linear regression curve stripping programme on a Hewlett Packard Model 9820A calculator. The curves shown in the figures represent mathematical reconstructions of the elimination curves using the mean rate constants and intercepts determined from the data for the individual animals.

## RESULTS

During the 48 hr following oral administration, $^{14}CH_2Cl_2$ *per se* was found only in the expired air (Table 1). The major metabolites were $^{14}CO$ and $^{14}CO_2$, which were also present in expired air. Urine, faeces and tissues were devoid of volatile radioactivity. Thus the $^{14}C$ activity in these samples was attributed to biotransformation products of $^{14}CH_2Cl_2$.

Rats given the 1-mg/kg dose metabolized a greater percentage of the administered dose than those given 50 mg $^{14}CH_2Cl_2$/kg. Increased percentages of the lower dose were recovered as $^{14}CO_2$, $^{14}CO$ and urinary $^{14}C$ activity, and as $^{14}C$-labelled metabolites retained in the carcass. In contrast, rats given 50 mg $^{14}CH_2Cl_2$/kg exhaled approximately 72% of the dose unchanged, with proportionally less metabolism of the dose.

Figure 1 shows the time course for the pulmonary elimination of $^{14}CH_2Cl_2$ during the first 5 hr after intubation. Elimination of $^{14}CH_2Cl_2$ in the expired air of rats receiving the 1-mg/kg dose was characterized by a biexponential decay curve which was resolved into two first-order processes. The mean ($\pm 1$ SD, n = 3) rate constants of these first-order processes were $3{\cdot}34 \pm 0{\cdot}47\ hr^{-1}$ ($t_{1/2}$, 0·21 hr) and $0{\cdot}93 \pm 0{\cdot}17\ hr^{-1}$ ($t_{1/2}$, 0·76 hr) for the initial and terminal portions of the curve, respectively.

The pulmonary excretion of $^{14}CH_2Cl_2$ by rats given the 50-mg/kg dose approximated zero-order kinetics for the first hour after administration of $CH_2Cl_2$. However as the level of $^{14}CH_2Cl_2$ in expired air declined, the elimination of the unchanged solvent could be described by the same kinetic parameters that characterized pulmonary elimination of $^{14}CH_2Cl_2$ following the 1-mg/kg dose.

The pulmonary excretion of $^{14}CO$ by rats given 1 or 50 mg $^{14}CH_2Cl_2$/kg orally is shown in Fig. 2. When $^{14}CO$ excretion is expressed as a percentage of the administered dose against time, the data clearly indicate that rats given 1 mg $^{14}CH_2Cl_2$/kg metabolized a significantly greater fraction of the dose to $^{14}CO$ than did those given 50 mg/kg. The time course for the $^{14}CO$ in expired air following administration of 1 mg $^{14}CH_2Cl_2$/kg was described by a biphasic exponential decay curve which was resolved into two first-order processes. The apparent first-order rate constants were $0{\cdot}38 \pm 0{\cdot}06\ hr^{-1}$ and $0{\cdot}08 \pm 0{\cdot}01\ hr^{-1}$ (mean $\pm$ 1 SD, n = 3) for the initial and terminal portions of the curve, respectively. These rate constants corresponded to mean half-lives of 1·8 and 8·7 hr, respectively. Similar kinetic analysis of the data obtained following administration of the 50-mg $^{14}CH_2Cl_2$/kg dose was hampered by the incomplete collection of $^{14}CO$ exhaled during the 24–36 hr period after exposure. When this data point was ignored, the terminal phase of the excretion curve was adequately characterized by the same kinetic parameters obtained from the 1-mg/kg experiment. The rate constant for the rapid phase of the excretion process was $0{\cdot}26 \pm 0{\cdot}02\ hr^{-1}$, with a half-life of approximately 2·7 hr, similar to that obtained for the initial portion of the $^{14}CO$ excretion curve from the rats given 1 mg $^{14}CH_2Cl_2$/kg.

Figure 3 shows the time course for the elimination of $^{14}CO_2$ in expired air. Once again, the rats given 1 mg $^{14}CH_2Cl_2$/kg metabolized a greater percentage of the administered dose to $^{14}CO_2$ throughout the entire 48-hr collection period. The biphasic elimin-

Table 1. *Fate of [$^{14}$C]methylene chloride in rats 48 hr after a single dose*

| $^{14}CH_2Cl_2$ dose (mg/kg) | Percentage of dose recovered in | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Expired air as | | | Urine | Faeces | Carcass | Skin | Cage wash | Total percentage recovery |
| | $CH_2Cl_2$ | $CO_2$ | CO | | | | | | |
| 1 | 12·33 ± 1·43 | 35·01 ± 0·85 | 30·92 ± 1·67 | 4·52 ± 0·05 | 0·93 ± 0·02 | 5·84 ± 0·24 | 1·56 ± 0·05 | 0·53 ± 0·04 | 91·64 ± 2·07 |
| 50 | 72·09 ± 0·07 | 6·33 ± 0·39 | 11·87 ± 0·073 | 1·96 ± 0·05 | 0·25 ± 0·02 | 2·40 ± 0·24 | 1·15 ± 0·06 | 0·08 ± 0·01 | 96·13 ± 1·02 |

Values are means ± SEM for groups of three rats.

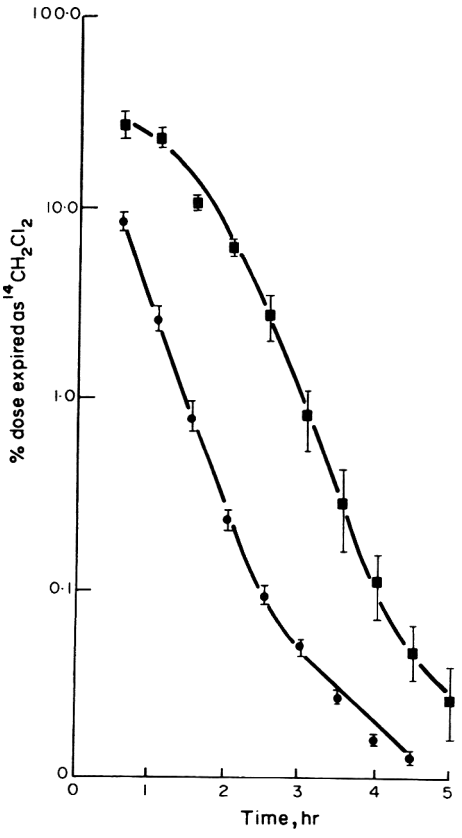


Fig. 1. Pulmonary excretion of $^{14}CH_2Cl_2$ following oral administration to rats of a single dose of either 1 (●) or 50 (■) mg $^{14}CH_2Cl_2$/kg. Data are presented as a percentage of the administered dose exhaled as $^{14}CH_2Cl_2$ during each 0·5-hr collection period. Each point represents the mean ± SEM for groups of three rats.

ation curves for $^{14}CO_2$ excretion at the two doses shown in Fig. 3 were essentially parallel. The data from the 1-mg/kg group gave mean (±1 SD, n = 3) first-order rate constants of 0·27 ± 0·02 $hr^{-1}$($t_{1/2}$, 2·6 hr) and 0·04 ± 0·010 $hr^{-1}$($t_{1/2}$, 17·3 hr) for the initial and terminal phases of the curves, respectively. For the 50-mg/kg group these values were 0·49 ± 0·18 $hr^{-1}$($t_{1/2}$, 1·4 hr) and 0·045 ± 0·004 $hr^{-1}$($t_{1/2}$, 15·4 hr).

The time course for urinary excretion of $^{14}C$ activity following $^{14}CH_2Cl_2$ administration is shown in Fig. 4. Rats given 1 mg/kg excreted a greater percentage of the administered dose of $^{14}CH_2Cl_2$ in the urine. The rates of $^{14}C$-elimination in the urine following either dose of $^{14}CH_2Cl_2$ were not significantly different. The biphasic elimination of urinary $^{14}C$ activity after 1 mg/kg was resolved into two first-order processes with mean (±1 SD, n = 3) rate constants of 0·21 ± 0·03 $hr^{-1}$($t_{1/2}$, 3·3 hr) and 0·03 ± 0·01 $hr^{-1}$ ($t_{1/2}$, 23·1 hr) for the initial and terminal portions of the curves, respectively. For the 50-mg/kg dose group these values were 0·14 ± 0·01 $hr^{-1}$($t_{1/2}$, 5 hr) and 0·03 ± 0·01 $hr^{-1}$($t_{1/2}$, 23·1 hr).

The tissue distribution of $^{14}C$ activity during the 48 hr following administration of $^{14}CH_2Cl_2$ is shown in Table 2. The highest concentrations of $^{14}C$ activity were found in the liver, kidney and lung. All other

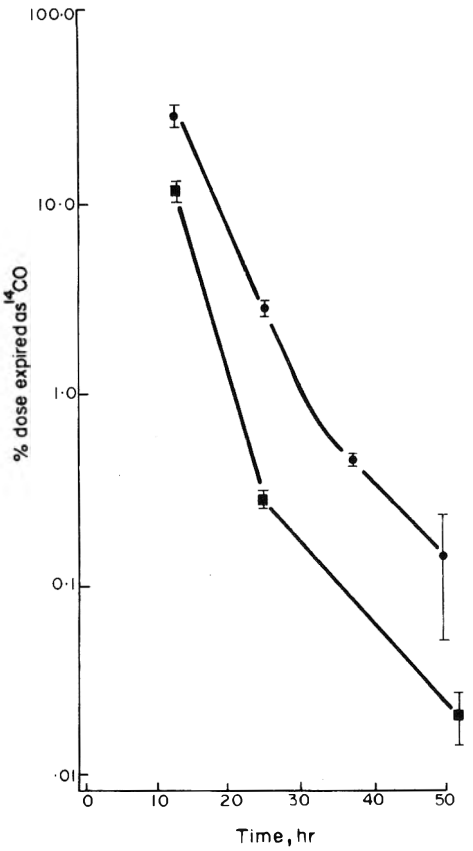


Fig. 2. Excretion of $^{14}CO$ in expired air following administration to rats of a single oral dose of 1 (●) or 50 (■) mg $CH_2Cl_2$/kg. Each point represents the mean ± SEM for groups of three rats. In drawing the curve for the 50-mg/kg data the value for the 24–26 hr collection period has been ignored; there was incomplete collection of $^{14}CO$ expired during this time.

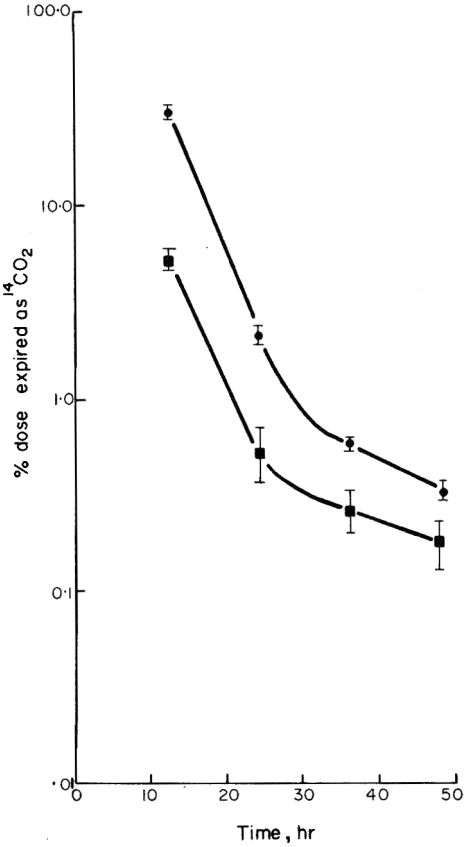


Fig. 3. Excretion of $^{14}CO_2$ in expired air following administration to rats of a single oral dose of 1 (●) or 50 (■) mg $^{14}CH_2Cl_2$/kg. Each point represents the mean ± SEM for groups of three rats.

tissues contained less $^{14}C$ activity per gram than the concentration observed in either blood or the remaining carcass, with the exception of skeletal muscle in the 1-mg/kg dose group. The lowest concentration of $^{14}C$ activity was observed in the fat.

## DISCUSSION

The results of this study indicate that the fate of $CH_2Cl_2$ in rats is dose-dependent. The metabolic and excretory pathways for $CH_2Cl_2$ were qualitatively similar after administration of either the 1- or 50-mg/kg dose of the solvent. Moreover, the rates of elimination of $^{14}C$ activity as metabolites of $^{14}CH_2Cl_2$ were essentially unaffected by dose despite a significant difference in the amounts of $^{14}CH_2Cl_2$ metabolites excreted and in the fraction of the dose metabolized. Therefore the dose-dependent fate of $^{14}CH_2Cl_2$ appears to be due to the saturation of one or more metabolic pathways for the solvent.

Dose-dependent metabolism of $CH_2Cl_2$ is also apparent from a comparison of the relative percentage recoveries of the major metabolites, $^{14}CO_2$ and $^{14}CO$. After the 1-mg/kg dose, the ratio of $^{14}CO_2$ to $^{14}CO$ recovered was 1·13. However, after the 50-mg/kg dose, this ratio decreased to 0·5, indicating

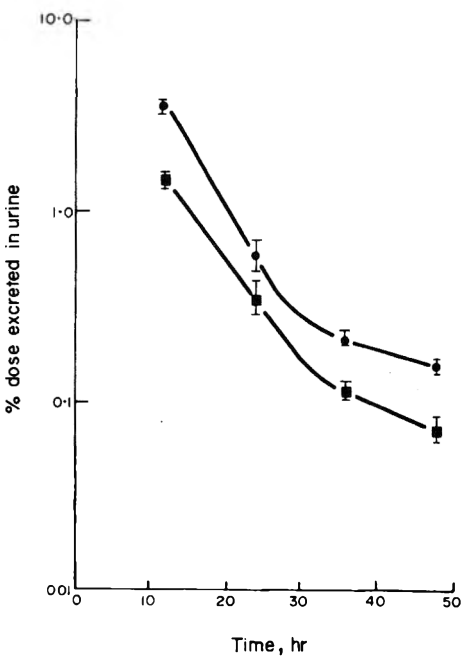


Fig. 4. Excretion of $^{14}C$ activity in the urine following administration to rats of 1 (●) or 50 (■) mg $^{14}CH_2Cl_2$/kg. Each point represents the mean ± SEM for groups of three rats.

Table 2. *Tissue distribution of $^{14}C$ activity in rats 48 hr after oral administration of a single dose of [$^{14}C$]methylene chloride*

| $^{14}CH_2Cl_2$ dose (mg/kg) | $^{14}C$ activity (μg equivalents $^{14}CH_2Cl_2$/g tissue) in | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Liver | Kidney | Lung | Brain | Epididymal fat | Skeletal muscle | Testes | Whole blood | Remaining carcass |
| 1 | 0·40 ± 0·04 | 0·15 ± 0·01 | 0·09 ± 0·01 | 0·041 ± 0·06 | 0·022 ± 0·002 | 0·076 ± 0·02 | 0·040 ± 0·002 | 0·057 ± 0·003 | 0·051 ± 0·002 |
| 50 | 6·67 ± 0·69 | 2·82 ± 0·21 | 1·67 ± 0·16 | 0·63 ± 0·10 | 0·33 ± 0·006 | 0·86 ± 0·04 | 0·92 ± 0·10 | 1·41 ± 0·11 | 0·99 ± 0·60 |

Values are means ± SEM for groups of three rats.

that the production of these metabolites is probably governed by two different metabolic pathways. Since the percentage of the dose recovered as either $^{14}CO_2$ or $^{14}CO$ decreased when the dose of $^{14}CH_2Cl_2$ was increased to 50 mg/kg, both metabolic pathways exhibited dose-dependency. This may have been due to the saturation of the pathway or to limited access of the $CH_2Cl_2$ to the actual site of metabolism following oral administration. Although the available data are inadequate to describe the exact mechanism by which metabolism of $^{14}CH_2Cl_2$ is limited in this situation, the net result of either of the above alternatives would be the same: a decrease in the percentage of the dose metabolized with increasing doses of $CH_2Cl_2$.

The observed differences in pulmonary excretion of $^{14}CH_2Cl_2$ after administration of the 50-mg/kg dose may also be accounted for in terms of the saturation of metabolism of $CH_2Cl_2$. The apparent zero-order pulmonary elimination of $^{14}CH_2Cl_2$ during the first hour after dosing may have been due to greater concentrations of the solvent being available for pulmonary clearance as a result of limited biotransformation. This interpretation of the data is consistent with the finding that following the initial zero-order excretion phase, the rate constants for pulmonary elimination were virtually identical to those observed after the 1-mg/kg dose.

The metabolism of $^{14}CH_2Cl_2$ to $^{14}CO$ and $^{14}CO_2$ has been described previously (DiVincenco & Hamilton, 1975). Further studies (Anders, Kubic & Ahmed, 1977), on the enzyme systems involved in the biotransformation of $CH_2Cl_2$ demonstrated that metabolism to CO is localized in hepatic microsomal fractions and requires both NADPH and molecular oxygen for optimum activity. Additional evidence for the dose-dependent metabolism of $CH_2Cl_2$ to CO *in vivo* has been reported (Kubic & Anders, 1975; Kubic, Anders, Engel, Barlow & Caughey, 1974; Hogan, Smith & Cornish, 1976). In these studies, saturation of the CO pathway was indicated by the lack of a dose-related increase in blood carboxyhaemoglobin levels with increasing doses of $CH_2Cl_2$.

Heppel & Porterfield (1948) reported that $CH_2Cl_2$ was dehalogenated in rat tissue slices to formaldehyde ($CH_2O$), and hydrogen and chloride ions. This pathway has been investigated in detail by Ahmed & Anders (1976). $CH_2O$ was not found to be a major metabolite of $^{14}CH_2Cl_2$ in this study or in that of DiVincenzo & Hamilton (1975). However, Neely (1964) reported that $^{14}CH_2O$ was primarily metabolized in the rat to $^{14}CO_2$. Thus it is likely that the production of $^{14}CO_2$ observed in this study resulted from further oxidation of the $^{14}CH_2O$ produced from $^{14}CH_2Cl_2$.

In view of the nature of the major biotransformation reactions for $CH_2Cl_2$ discussed above, it is evident that appreciable potential exists for the incorporation of $CH_2Cl_2$-derived one-carbon fragments into a variety of endogenous substrates involved in normal synthetic and metabolic pathways. Thus, it would be expected that much of the residual $^{14}C$ observed in tissues 48 hr after administration of $^{14}CH_2Cl_2$ would be derived from such reactions. In addition, studies by Anders *et al.* (1977) on the mechanisms of the conversion of $^{14}CH_2Cl_2$ to $^{14}CH_2O$ and $^{14}CO_2$ have suggested that the biotransformation pathways yield

intermediates that are capable of alkylating various tissue nucleophiles. Covalent binding of $^{14}CH_2Cl_2$ metabolites to liver microsomal lipid and protein has been demonstrated *in vitro* (Anders *et al.* 1977). However, experiments to evaluate this possibility in rats exposed to $^{14}CH_2Cl_2$ by inhalation failed to reveal any measurable alkylation of subcellular macromolecules (M. J. McKenna & J. A. Zempel, unpublished data, 1979).

The relationship of $CH_2Cl_2$ metabolism to the toxicity of the solvent is not fully understood at this time. Although definitive evidence for the dose-dependency of the primary metabolic pathways for $CH_2Cl_2$ has been presented, the low order of toxicity of this material makes it difficult to judge the significance of these findings in terms of the toxicity or detoxification of $CH_2Cl_2$. Indeed the identification of critical biochemical or metabolic events in the development of $CH_2Cl_2$-induced toxicity is further confounded by observations such as those of Rampy, Nitschke, Bell, Burek & Gehring (1979), who found that the onset of minimal hepatocellular alterations in rats became evident only after 1 yr of inhalation exposure to 3500 ppm $CH_2Cl_2$. It is conceivable that age and environmental factors not usually evaluated in typical acute or short-term metabolic investigations may also be involved in the changes.

From a practical viewpoint, those planning laboratory investigations of orally administered volatile chemicals such as $CH_2Cl_2$ must recognize the influence of dose-dependent pharmacokinetics on experimental design and interpretation. The rapid elimination of $CH_2Cl_2$ following oral administration increases disproportionately with increasing oral doses of the solvent. Thus that fraction of the administered dose that remains in the animal for longer periods of time largely represents biotransformation products of the parent molecule. Although no evidence exists at present to associate the metabolism of $CH_2Cl_2$ with toxicity, evaluation of the dose-response relationships for many volatile chemicals suggests that the fraction of the dose retained as metabolites more closely represents the 'effective dose' of the administered material. This effective dose can be quantitatively related to the response of interest. In such studies it becomes desirable to distinguish between the administered dose of a volatile chemical such as $CH_2Cl_2$ and the effective dose (metabolized $CH_2Cl_2$) which may be more directly associated with toxicity.

The results of this study indicate that because of the dose-dependent or saturable character of $CH_2Cl_2$ metabolism, the discrepancy between the administered dose and the effective dose of the solvent becomes greater with increasing doses. Furthermore, as saturation of metabolism of $CH_2Cl_2$ is approached, the change in the magnitude of the effective dose becomes insignificant despite large increases in the administered dose of the solvent. Therefore the use of pharmacokinetic data is critical in the selection of appropriate doses of $CH_2Cl_2$ for toxicity studies if good dose-response information is to be obtained. Finally, the observation of dose-dependent pharmacokinetics for $CH_2Cl_2$ precludes the linear extrapolation of toxicity data from high doses to predict the hazard of exposure to low doses of the solvent following oral administration.

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# MUTAGENICITY OF COMMERCIAL *p*-PHENYLENEDIAMINE AND OF AN OXIDATION MIXTURE OF *p*-PHENYLENEDIAMINE AND RESORCINOL IN *SALMONELLA TYPHIMURIUM* TA98

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**Abstract**—One chemically purified sample of *p*-phenylenediamine and two commercial analytical-grade samples, as well as an oxidation mixture prepared by reaction of *p*-phenylenediamine and resorcinol with $H_2O_2$, were tested for their ability to revert the frameshift strain TA98 in the *Salmonella typhimurium*/microsomal assay. While the purified product was non-mutagenic in this system, the two commercial samples of *p*-phenylenediamine and the oxidation mixture were mutagenic in the presence of S-9 mix. Separation and purification of four major fractions of the oxidation mixture indicated that only one oxidation product of *p*-phenylenediamine and resorcinol was responsible for the mutagenic activity observed. When rats were treated topically with the oxidation mixture, their urine became mutagenic. This activity was apparently attributable to the previously isolated compound, which was found in the urine in acetylated form.

## INTRODUCTION

*p*-Phenylenediamine (*p*-PD) is the most common primary agent used in the preparation of the oxidative-type hair dyes. These preparations consist of mixtures of aromatic amines, aromatic nitro derivatives and/or phenols which are mixed with hydrogen peroxide just before use. The mutagenicity of *p*-PD and other hair-dye components was first evaluated by Ames, Kammen & Yamasaki (1975) using the Salmonella/microsome test. In this study, purified *p*-PD was not mutagenic in *Salmonella typhimurium*, but after oxidation by $H_2O_2$ it showed strong activity in the frameshift-detecting strain TA1538 in the presence of a microsomal fraction. The mutagenic product was thoroughly investigated in a number of *in vitro* and *in vivo* test systems, but with varying results. It was always reported as mutagenic when assayed in the presence of $H_2O_2$ in *in vitro* microbial test systems (Garner & Nutman, 1977; Nishioka, 1976; Venitt & Searle, 1976; Yoshikawa, 1977), but when tested alone it was found negative by some authors (Nishioka, 1976; Venitt & Searle, 1976) and positive by others (Garner & Nutman, 1977; Yoshikawa, 1977). Furthermore in *in vivo* test systems it was found to be weakly active *per se* in the induction of sex-linked recessive lethals in Drosophila (Blijleven, 1977) but inactive in the micronucleus test (Hossak & Richardson, 1977) and dominant lethal test (Burnett, Loehr & Corbett, 1977).

On the other hand, no attention has been paid up to now to the assessment of the genetic potential of the conjugates produced during the $H_2O_2$ oxidation of *p*-PD and phenols. For this reason, and to try to clarify the conflicting data on the mutagenic properties of *p*-PD, we have studied in *S. typhimurium* strain TA98, the most sensitive frameshift-detecting Salmonella strain, the mutagenicity of both purified and commercial samples of *p*-PD and the oxidation product of *p*-PD and resorcinol.

Some effort was also made to evaluate the percutaneous absorption of these conjugates and to identify their chemical structure.

## EXPERIMENTAL

*Test materials.* Two commercial samples of *p*-PD were used; they were obtained from E. Merck, Darmstadt, FRG, and from Carlo Erba, S.p.A., Milan, and both were analytical grade. Purified *p*-PD was prepared by dissolving 33 g of the Carlo Erba sample in 100 ml boiling deionized water containing 1 g $Na_2S_2O_3$ and adding 2 g activated charcoal (Norite, E. Merck Labs, Elmsford, NY, USA) previously washed with hot water. The mixture was stirred rapidly, quickly filtered through a hot funnel and then cooled at −10°C. The crystals of *p*-PD were collected, washed with 10 ml cold water and dried in the dark over $P_2O_5$.

The oxidation mixture was prepared by dissolving 10 g purified *p*-PD and 30 g resorcinol (Merck) in 20 ml 5% $NH_4OH$ and adding to this solution 1 ml 36% $H_2O_2$ (electronic grade from BDH Ltd., Poole, England) at room temperature in the dark. After 1 hr, during which it was stirred occasionally, the mixture was lyophilized and solutions were centrifuged at 20,000 **g** for 30 min.

Acetylation of the oxidation mixture was performed

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at 0°C and pH 8·5 (in an *N*-ethylmorpholine–acetic acid medium) for 60 min, 10 μl acetic anhydride (Merck) being added for each ml of solution containing 10–30 mg/ml. The acetylated compounds were recovered by lyophilization.

The two compounds used as positive controls in the mutagenicity studies were analytical-grade 4-nitro-*o*-phenylenediamine and 2-aminoanthracene, obtained from Prof. V. Quercia (Istituto Superiore di Sanità, Rome) and John Ashby (ICI Ltd., Macclesfield, UK), respectively.

*Chromatography.* Silica-gel thin-layer chromatography (TLC) was performed at room temperature using the solvent system dichloroethane–heptane–ethyl acetate–methanol (2:2:3:3, by vol.). Column chromatography was carried out at 4°C on silica gel (Biosil 27–44 μ from Bio-Rad Laboratories, SRL, Milan) using a distilled water–15% ethanol gradient (Ultragrad LKB instrument). The chromatogram was recorded (using an Ulvicord III and Recorder 2066 LKB) at two wavelengths (278 and 408 nm). Fractions collected on a TC 80 (Gilson Medical Electronics, Villiers-le-Bel, France) were frozen and lyophilized.

*Skin absorption studies.* Wistar rats (body weight *c.* 700 g) were treated as described by Ammenheuser & Warren (1979), except that dorsal shaving was omitted, and were kept in metabolism cages. Urine was collected, by means of the device shown in Fig. 1, usually for 24 hr, after which the XAD-2 resin (Serva Heidelberg, FRG) was washed in the cold with 100 vols water, to reduce the small amount of histidine present, as this can impair the mutagenicity tests. It was then eluted with 10 + 5 ml acetone (Merck) and the acetone eluate was concentrated under reduced pressure and lyophilized.

For quantitative studies the absorbance of the acetone eluate at 540 nm was determined and the absorbance of the control, processed in the same way, was subtracted. The data were related to the daily urinary volume, which was remarkably constant at about 15 ml. The dry weights of the isolated fractions were determined on a Cahn Gram Electrobalance.

*Mutagenicity assays. Salmonella typhimurium* strain TA98 was kindly supplied by Dr. B. N. Ames, University of California, Berkeley, CA, USA. The selection of induced *his*$^+$ revertants was performed in the plate incorporation assay according to the procedure described by Ames, McCann & Yamasaki (1975). Liver microsomal fractions were obtained from male Sprague–Dawley rats (body weight *c.* 250 g) pretreated with Aroclor 1254. The procedures followed for the induction in rats and the preparation of the S-9 mix were as described by Ames *et al.* (1975). Glusulase (a mixture of sulphatase and β-glucuronidase from Endo Laboratories, Garden City, NY, USA) was added for the assay of urine concentrates, in amounts to provide about 1000 U β-glucuronidase and 400 U sulphatase/plate. The microtitre fluctuation test with microsomal activation was performed as described by Gatehouse & Delow (1979). Positive controls were included to check both enzyme activity and strain sensitivity as well as routine controls of sample and S-9 mix sterility. For testing, *p*-PD and its conjugates were dissolved in distilled water, while 4-nitro-*o*-phenylenediamine and 2-aminoanthracene, the positive controls, were dissolved in DMSO. DMSO was also used to dissolve the acetone residues of the urines, 50 μl of solvent being added per ml of urine.

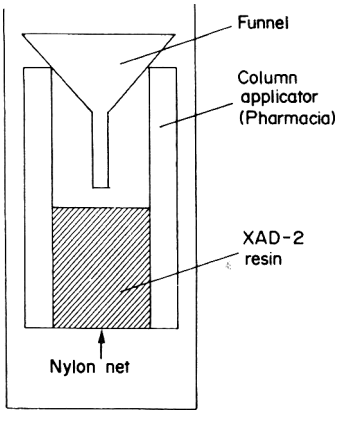


Fig. 1. Device for collecting aromatic urinary metabolites from rats.

## RESULTS

### *Chemical assay*

Chromatography of the centrifuged oxidation mixture is shown in Fig. 2A. The complex pattern was separated into four main peaks, which were tested for mutagenesis. More coloured bands were demonstrated by TLC (Fig. 3), indicating that the mixture contained many oxidation products. The three-ringed compound reported in the literature (Brody & Burns, 1968; Shah, 1977) is poorly soluble in water and was almost completely removed by centrifugation, the remaining traces being retained at the top of the column. Peak 4 was further purified by silica-gel chromatography. Acetylation of the mixture gave the chromatogram shown in Fig. 2B, peak 4 of which was discoloured.

Column chromatography of the urine concentrates and absorbance measurements on the eluates (Fig. 4) showed that extensive acetylation occurred *in vivo*, in accordance with the reported metabolism of the aromatic amines (Clayson, 1962). Two UV absorbing peaks, one coloured and composed of uroporphyrin and one colourless and probably composed of bile acids, were seen in the chromatograms of both control and test urines. In the latter, however, there were two small additional peaks, which corresponded to peaks 1 and 4 of the acetylated mixture.

Preliminary quantitative studies have shown that following skin application of 150 mg of the centrifuged oxidation product 0·4–0·6 mg is recovered in the urine, whereas with a dose of 300 mg, recovery rises to 2–3 mg.

### *Mutagenicity assays*

Each of the commercial samples of *p*-PD induced mutations in *S. typhimurium* strain TA98 when tested in the presence of rat-liver microsomal fractions. This activity was completely undetectable in the absence of S-9 mix (Table 1). The purified sample of *p*-PD produced no significant increase in the number of revertants when tested in the concentration range 0·25–2·0 mg/plate (Table 1). This negative result was

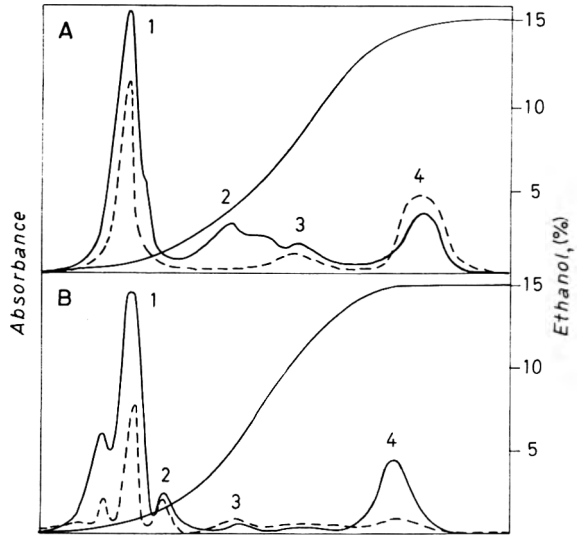


Fig. 2. Silica-gel column chromatography of (A) the oxidation mixture prepared from *p*-phenylenediamine and resorcinol and (B) the acetylated mixture. Absorbance was recorded at 278 nm (——) and at 408 nm (– – –). The smooth curve indicates the water–ethanol gradient.

confirmed by the highly sensitive microtitre fluctuation test performed with microsomal activation; in repeated experiments purified *p*-PD caused no significant increase in the number of positive wells when compared with the controls (Fig. 5).

A clearcut increase in the number of mutants was observed in assays of the centrifuged oxidation mixture of *p*-PD and resorcinol, but again only in the presence of S-9 mix. A significant increase was observed in both the plate incorporation assay (Table 1) and the microtitre fluctuation test (Fig. 5). No mutagenic activity was detected in either the purified *p*-PD or the resorcinol used for the oxidative reaction (Table 1).

The four major fractions of the oxidation mixture were tested separately with the aim of identifying the fraction(s) responsible for this mutagenic activity. The results reported in Table 2 indicate fraction 4 as the only active one. Comparison of the mutagenic potency of the whole mixture with that of fraction 4,

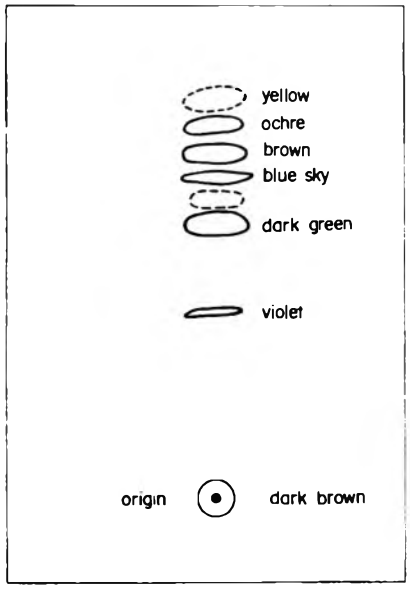


Fig. 3. TLC of the oxidation mixture, developed with dichloroethane–heptane–ethyl acetate–methanol (2:2:3:3, by vol.) over 30 min.

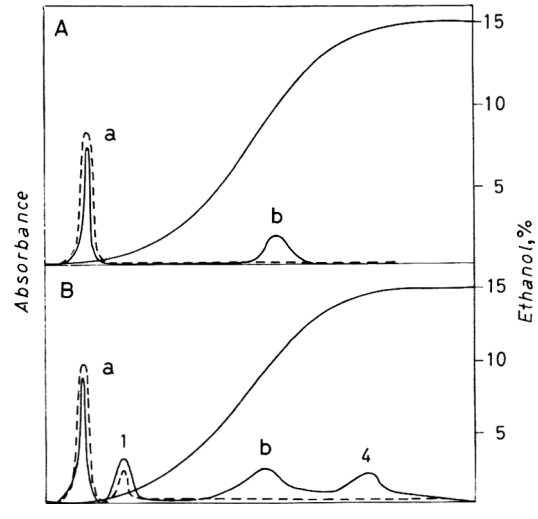


Fig. 4. Silica-gel column chromatography of urine concentrates (A) from control rats and (B) from rats treated with 300 mg of oxidation mixture. Absorbance was recorded at 278 nm (——) and at 408 nm (– – –). The smooth curve indicates the water–ethanol gradient.

Table 1. *Mutagenicity of commercial and purified* p-*PD, resorcinol and* p-*PD/resorcinol conjugates in plate incorporation assays using* Salmonella typhimurium *TA98*

| Test material | Concn (mg/plate) | No. of *his*$^+$ revertants/plate: Without S-9 mix | With S-9 mix |
|---|---|---|---|
| Commercial *p*-PD (Merck)* | 0 | 17 ± 2 | 46 ± 9 |
| | 0·25 | 13 ± 3 | 322 ± 62 |
| | 0·5 | 12 ± 2 | 270 ± 35 |
| | 1·0 | 10 ± 2 | 179 ± 41 |
| | 2·0 | (toxic) | 77 ± 2 |
| Commercial *p*-PD (Erba)* | 0 | 42 ± 10 | 46 ± 9 |
| | 0·25 | 36 ± 2 | 91 ± 2 |
| | 0·5 | 28 ± 3 | 99 ± 2 |
| | 1·0 | 26 ± 3 | 154 ± 20 |
| | 2·0 | 13 ± 1 | 223 ± 16 |
| Purified *p*-PD* | 0 | 23 ± 9 | 55 ± 3 |
| | 0·25 | 27 ± 5 | 81 ± 11 |
| | 0·5 | 22 ± 5 | 88 ± 7 |
| | 1·0 | 22 ± 1 | 89 ± 11 |
| | 2·0 | 21 ± 4 | 39 ± 5 |
| *p*-PD/resorcinol conjugates* | 0 | 39 ± 1 | 46 ± 8 |
| | 0·1 | 38 ± 1 | 61 ± 4 |
| | 0·25 | 42 ± 6 | 120 ± 19 |
| | 0·5 | 35 ± 6 | 123 ± 22 |
| | 1·0 | 51 ± 11 | 162 ± 4 |
| Resorcinol* | 0 | 39 ± 1 | 40 ± 5 |
| | 0·5 | 40 ± 7 | 59 ± 11 |
| | 1·0 | 28 ± 3 | 51 ± 8 |
| | 2·0 | 33 ± 5 | 53 ± 8 |
| 4-Nitro-*o*-phenylenediamine† | 0·01 | 1862 ± 526 | — |
| 2-Aminoanthracene† | 0·001 | — | 1009 ± 301 |

*p*-PD = *p*-Phenylenediamine

*Values are means ± 1 SD for three plates.

†Positive controls: values are means ± 1 SD derived from 36 observations made during the course of this work.

which represented about 15–20% of the mixture suggests that this fraction is responsible for all of the observed activity.

In further studies of the percutaneous absorption of the reaction mixture and its biotransformation, urine concentrates from rats treated topically with the *p*-PD/resorcinol conjugates were found to induce mutations in the TA98 strain when tested in the presence of S-9 mix but not in its absence (Table 3). Urine concentrates from untreated rats, however, induced

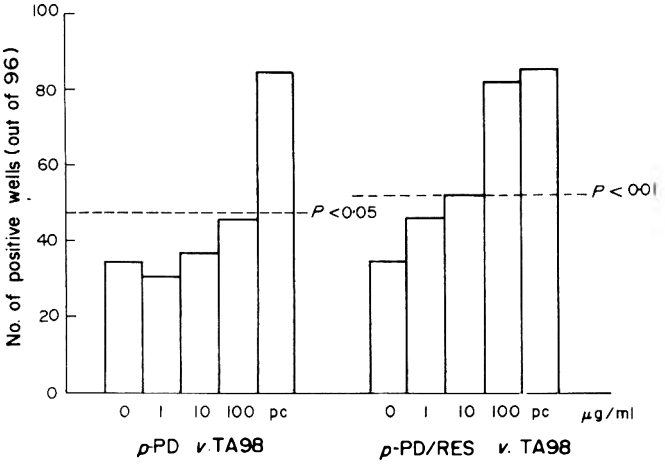


Fig. 5. Mutagenicity of purified *p*-phenylenediamine (*p*-PD) and *p*-PD/resorcinol conjugate (*p*-PD/RES) to *Salmonella typhimurium* TA98 as determined in the microtitre fluctuation test with microsomal activation. The positive control (pc) was 2-aminoanthracene at 1 µg/ml. *P* was determined by the chi-square test.

Table 2. *Mutagenicity of fractionated* p-*PD/resorcinol conjugates in plate incorporation assays using* Salmonella typhimurium, *with microsomal (S-9 mix) activation*

| Fraction no. | Concn (μg/plate) | No. of *his*$^+$ revertants/plate |
|---|---|---|
| 1 | 0 | 68 ± 7 |
| | 100 | 68 ± 10 |
| | 500 | 75 ± 10 |
| | 1000 | 77 ± 9 |
| 2 | 0 | 68 ± 7 |
| | 100 | 71 ± 13 |
| | 250 | 86 ± 13 |
| | 500 | 85 ± 6 |
| 3 | 0 | 65 ± 3 |
| | 50 | 84 ± 4 |
| | 100 | 80 ± 7 |
| | 200 | 92 ± 1 |
| 4 | 0 | 65 ± 3 |
| | 10 | 102 ± 0 |
| | 25 | 181 ± 32 |
| | 50 | 352 ± 19 |

*p*-PD = *p*-Phenylenediamine
Values are means ± 1 SD for three plates.

no appreciable increases in the numbers of mutants per plate (Table 3).

## DISCUSSION

The results of *in vitro* microbial mutagenicity studies on *p*-PD reported by Ames *et al.* (1975), Garner & Nutman (1977) and Yoshikawa (1977) contrasted with those of Nishioka (1976) and Venitt & Searle (1976). This divergence of results seemed to be related to the purity of the substances assayed. In our studies, mutagenic activity was detectable in commercial samples of analytical-grade *p*-PD but not in the freshly purified product. The mutagenic activity of the commercial samples may have been due to the presence of impurities and/or to a small amount of Bandrowski's base formed by spontaneous oxidation during storage.

The mutagenic product found in the oxidation mixture produced from *p*-PD and resorcinol, and identified as the green compound, fraction 4, seems to be an oxidized conjugation product of *p*-PD and resorcinol. In the oxidation mixture, the ratio in weight between *p*-PD and resorcinol (1:3) and the much higher affinity of iminoquinone for the latter (Brody & Burns, 1968) makes the formation of even small amounts of Bandrowski's base extremely improbable. Moreover, while the chemical nature of compound 4 remains unknown, it can be said that it is not the previously mentioned Bandrowski's base contaminant, which is poorly soluble in water and is easily removed by high-speed centrifugation. Under our experimental conditions, the specific mutagenic activity of fraction 4 produced about 4 revertants/μg, a degree of induction comparable to those of the carcinogens 2,4-diaminotoluene and ethyl methanesulphonate (McCann, Choi, Yamasaki & Ames, 1975).

The percutaneous absorption of the oxidation products and their urinary elimination were demonstrated by chromatography and by mutagenicity tests. The chromatogram of the urine eluates showed discoloration of peak 4, suggesting extensive acetylation *in vivo*. This acetylation has no effect on the biological activity of aromatic amines, as has been shown by *in vivo* carcinogenicity studies (Clayson, 1962). Quantitative data showed that at the lower topical dose (150 mg/rat) a large proportion of the absorbed oxidation mixture is retained in the body, probably on macromolecular targets. Doubling of the dose increased the urinary recovery of the oxidation products far beyond the expected amount, suggesting that the capacity of the tissue oxidation systems was being overloaded. On the other hand, acetylation was complete even with the higher doses, as was to be expected in view of the well-known efficiency of this metabolic pattern of detoxication.

Research is now in progress on commercial dye mixtures, in order to investigate the formation of mutagenic conjugates under actual conditions of use.

*Acknowledgement*—The authors are grateful to Mr. U. Cervelli for his skilled technical assistance.

Table 3. Salmonella typhimurium *TA98 plate incorporation assays of urine concentrate from a rat treated topically with 300 mg* p-*PD/resorcinol conjugates*

| Animal | Concn of urine concentrate* (μl/plate) | No. of *his*$^+$ revertants/plate: Without S-9 mix | With S-9 mix |
|---|---|---|---|
| Control | 0 | 32 ± 11 | 63 ± 3 |
| | 50 | 24 ± 4 | 69 ± 6 |
| | 100 | 16 ± 4 | 55 ± 6 |
| | 200 | 24 ± 6 | 63 ± 13 |
| Treated | 0 | 32 ± 11 | 63 ± 3 |
| | 50 | 32 ± 1 | 101 ± 20 |
| | 100 | 12 ± 2 | 197 ± 1 |
| | 200 | 21 ± 4 | 167 ± 19 |

*p*-PD = *p*-Phenylenediamine
*100 μl of concentrate = 2 ml urine.
Values are means ± 1 SD for three plates.

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Fd Cosmet. Toxicol. Vol. 19, pp. 85 to 88

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# SIZE-DISTRIBUTION ANALYSIS OF RESPIRABLE PARTICULATES IN COSMETIC AEROSOLS: A METHODOLOGICAL COMPARISON

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**Abstract**—The size spectra of respirable particulates from five cosmetic aerosol products were determined using the methods of microscopy, cascade impaction, and single particle aerodynamic relaxation-time (SPART) analysis. In order to facilitate methodological comparisons, the same sampling apparatus was used in all phases of the study. The results obtained using the three methods were similar in most cases. The pressurized aerosol products produced particulates with count median diameters of 0·6–1·5 μm and mass median diameters of 2·2–3·2 μm as measured by all methods. The pump spray also yielded particulates with median diameters in these ranges in the microscope and SPART analyses, but in the cascade impaction analysis, the mass median diameter was determined to be 12·8 μm.

## INTRODUCTION

Since the discovery of the propellant properties of various compounds, many cosmetic and household products have become available in aerosolized form. During the past 25 years concern has been growing about the inhalation toxicity of many of these aerosol products. Two major factors in the determination of the possible health hazard of these products are (1) the chemical composition of the products and (2) the amount and the site of deposition of the inhaled particulates (solid particles and/or liquid droplets) in the lungs during normal use. Predicting the amount of deposition requires a knowledge of the respiratory flow pattern and the aerodynamic behaviour of the inhaled particulates inside the lungs.

Methods of estimating probabilities and sites of deposition of inhaled aerosol are described in a report prepared by the Task Group on Lung Dynamics of the International Radiological Protection Commission (1966). The Task Group model was designed to predict deposition of polydispersed aerosols as a function of mass median aerodynamic diameter (Mercer, 1973). The aerodynamic diameter of a particulate is defined as the diameter of a unit density sphere having the same aerodynamic properties as the particulate. The significance of this size parameter is that it is a function of the size, shape and density of the particulate. According to the Task Group, the respirable fraction of aerosol consists of particulates smaller than 10 μm in aerodynamic diameter.

A number of techniques have been developed for size distribution analysis. Microscopy (Silverman, Billings & First, 1971) is the only technique that can be used to determine both the size and the shape of the aerosol particulates. The use of optical scattering devices (Silverman *et al.* 1971) is limited to the analysis of spherical particulates of known refractive index. One of the most widely used techniques is inertial impaction (Silverman *et al.* 1971; Mercer, 1973), which yields the aerodynamic diameter of the particulates. A recently developed method, relaxation-time analysis (Mazumder & Kirsch, 1977), utilizes a laser Doppler velocimeter (LDV) for measurement of particulate velocity in an acoustic field. A particulate entering the sensing volume of the LDV is excited by an acoustic wave. A microphone detects the acoustic signal and the LDV detects the oscillations of the particulate. The phase lag between the two signals is a measure of the aerodynamic diameter of the particulate. An interesting older method combines acoustic excitation with microscopy (Cassel & Schultz, 1952). Particulates in a microscope cell are excited acoustically and sinusoidal tracks appear on the photomicrographs; the amplitudes of the tracks depend on the size of the particulates.

Several investigators have reported size information for various cosmetic aerosols. The most frequently studied product is hair spray. Draize, Nelson, Newberger & Kelley (1959) reported that hair spray particulates ranged from less than 1 μm to 30 μm in diameter and had a mass median diameter of 8·5 μm; the method of analysis was not reported. Brunner, Giovacchini, Wyatt, Dunlap & Colandra (1963) reported a mass median diameter of 35 μm with no more than 0·5% of the particulate mass consisting of particulates less than 10 μm in diameter. Again, the method of analysis was not reported. McLaughlin, Bidstrup & Konstam (1963) took thermal precipitator and konimeter samples in hair-dressing establishments and found that the majority of particulates were less than 1 μm in diameter.

Hathaway (1973) used laser holographic microscopy to determine the count and mass median diameters of antiperspirant particulates as a function of the distance from the actuator, with particulate size decreasing with increasing distance from the actuator. At a distance of 6 in., the count median diameter was reported to be 3·9 μm and the mass median diameter

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was 16 μm. Vos & Thompson (1974) used a Royco particle counter to obtain (in the range of 0·25–20 μm) count mean diameters of 1·3–2 μm and mass mean diameters of 9–12 μm for a hair spray and an antiperspirant preparation. Mokler, Wong & Snow (1979a) used a simulated breathing zone model and a cascade impactor to determine the mass median aerodynamic diameters of a hair spray, an antiperspirant, and other products. They reported diameters of 6–7 μm for the two cosmetic aerosols. These inconsistencies may be due in part to differences in valve/actuator designs and formulation variables as suggested by Pengilly & Keiner (1977). They found that particulate sizes decrease with increasing propellant level and increasing distance from the actuator. Also, variations in sampling and sizing techniques are probably a major factor in the inconsistency of reported data, as suggested by Cambridge (1973). Mokler *et al.* (1979b) studied the influence of various parameters such as the fullness of the aerosol can and brand-to-brand variability and found that variations in these factors did not significantly alter the mass median diameters.

The purpose of the present study was to determine the size spectra of respirable particulates from five cosmetic aerosol products using three different techniques: microscopy, cascade impaction and relaxation-time analysis. The same aerosol chamber was used in all phases of the study and differences in sampling systems were minimized in order that the results could be compared.

## EXPERIMENTAL

*Cosmetic aerosols.* The cosmetic aerosols evaluated in this study included a pressurized hair spray and four types of antiperspirant sprays: a pump spray, a fluorocarbon propelled spray, an isobutane–propane propelled spray, and a spray powder.

*Aerosol chamber.* An aerosol chamber (60 × 60 × 60 cm) with Plexiglas sides and aluminium removable top and bottom panels was used for aerosol sampling. The volume of the chamber was 216 litres. The aerosols were introduced into the chamber through a 23 × 23 cm door in one wall of the chamber. A 13-cm opening in the bottom panel permitted sampling of the chamber contents. Aerosol was introduced into the chamber by opening the door and spraying into the chamber.

*Microscopy.* An electrostatic precipitator (Mercer, 1973) was used for collecting aerosol particulates on microscope slides. Clean microscope slides were treated with L-1428, an experimental fluorochemical manufactured by the Minnesota Mining and Manufacturing (3M) Company. The L-1428 rendered the glass slides oil and water repellent to minimize flattening of the liquid aerosol droplets. For each aerosol, a slide was placed inside the electrostatic precipitator. Aerosol was drawn from the chamber through the electrostatic precipitator at 3 litres/min.

The slides were examined using a Ziess model RA Research Microscope adjusted for phase contrast (magnification ×400). A Minolta model 102 camera was used for photomicrography. Photomicrographs of a stage micrometer were taken to determine exact magnification. Photomicrographs were taken of each slide at three randomly selected sites. For each aerosol product, two hundred particulates were selected at random from the photomicrographs for sizing and statistical analysis.

*Cascade impaction.* A Sierra model 216 cascade impactor was used in this phase of the study. The collection substrates were placed in a desiccator overnight and then weighed. The collection substrates were then placed inside the impactor and the latter was placed inside the aerosol chamber. Aerosol was introduced into the chamber and the particulate-laden air was drawn through the impactor at a rate of 20 litres/min. After 1 hr of sampling, the collection substrates were removed and placed in a desiccator to dry overnight. The substrates were again weighed. This procedure was repeated for each aerosol.

*Relaxation-time analysis.* Aerosol was introduced into the Plexiglas chamber and drawn through the single particle aerodynamic relaxation time (SPART) analyser (Mazumder, Ware, Wilson, Renninger, Hiller, McLeod, Raible & Testerman, 1979) at a rate of 52 ml/min. Data from the SPART analyser were recorded on paper tape. The chamber was aerated and the other aerosols were sampled in the same manner.

### *Statistical analysis*

*Geometric size measurement.* The two parameters that define the size distribution of aerosol particulates are the median size and the standard deviation. The $\log_{10}$ of these parameters are the geometric mean and geometric standard deviation, respectively. These parameters are generally determined by plotting the cumulative size distribution on logarithmic-probability paper.

The particulates were measured and their diameters were multipled by a factor of 0·66 in order to compensate for their non-sphericity (Liu, Whitby & Yu, 1966). The data were then classified into the number of particulates in successive size intervals (0–1·00 μm, 1·01–2·00 μm etc.). For each size interval, the frequency or percentage of particulates by count was determined. The cumulative percentage of particulates that were equal to or smaller than a given size was plotted against the upper limit of the class interval for each aerosol. Ideally, this should result in a straight line if the distribution is log normal. A least-squares summation was performed to determine the best line through the points. The median or geometric mean size is the 50% size. The spread of the distribution, or geometric standard deviation ($\sigma_g$), is defined by the slope of the line as indicated by the ratio 50% size/15·8% size or 84·13% size/50% size.

*Aerodynamic size measurement by impaction.* Cascade impactor analysis also yields data that may be plotted on log-probability paper. The difference is that size-by-weight information is obtained instead of size-by-count. The mass on all stages of the impactor, including the back-up filter, was summed. The percentage less than $D_p50$ (i.e., the diameter for which the collection efficiency is 50%) for each stage was plotted against $D_p50$. This relationship should be linear if the size distribution is log-normal, so a least-squares fit through the lines was determined. In this case, the

50% size is the mass median aerodynamic diameter (MMAD) of the aerosol. The standard deviation was calculated as described above.

*Aerodynamic size measurement by SPART analysis.* The data from the SPART analyser were analysed with the use of an Interdata minicomputer. Values were computed for the count median aerodynamic diameter (CMAD), MMAD, and count and volume weighted geometric standard deviations ($\sigma_c$ and $\sigma_v$, respectively).

## RESULTS

The results of the three phases of this study are summarized in Tables 1–3. The count median diameter (CMD) values in the microscope study are in good general agreement (well within 1 $\mu$m) with the CMAD values in the SPART analysis, with all the aerosols having a CMD between 0·6 and 1·5 $\mu$m. The MMAD values obtained in the cascade impaction study are also in general agreement (within 1 $\mu$m) with those in the SPART study, with the exception of the value for the pump spray antiperspirant. In both procedures, the MMADs were in the range of 2·2–3·2 $\mu$m, except for the pump spray, which had an MMAD of 12·8 $\mu$m in the impactor study.

## DISCUSSION AND CONCLUSION

The sampling method used and the limits of resolution of the optical microscope limited the size range that could be determined in the microscope studies to approximately 0·3–5 $\mu$m. Particulates in this size range are a cause for concern because of the likelihood of their deposition in the lower respiratory tract. All the products studied, including the pump spray, produced particulates in this size range. However, the aerodynamic behaviour of these particulates cannot be predicted from their geometric size without knowledge of their density and shape. The shape factor was not a significant problem in most cases because the particulates of all of the products except the spray powder appeared to be spherical. Determination of the density is complicated by the likelihood that the particulates of each spray product vary in composition.

Although the cascade impactor has only six stages and does not make a sharp size separation, it yields aerodynamic size information on a larger size range of particulates (approximately 0·2–20 $\mu$m). Four of the aerosols studied produced particulates with MMADs well within the respirable range. The large MMAD of the pump spray indicates that the greatest mass of its particulates is probably beyond the respirable range.

Table 1. *Results of microscope studies: count median diameter (CMD) and geometric standard deviation ($\sigma_g$) for each cosmetic aerosol*

| Aerosol | CMD ($\mu$m) | $\sigma_g$ |
|---|---|---|
| Antiperspirant 1 (pump spray) | 0·72 | 2·50 |
| Antiperspirant 2 (fluorocarbon propellant) | 0·82 | 2·13 |
| Antiperspirant 3 (isobutane–propane propellant) | 1·40 | 2·04 |
| Antiperspirant 4 (spray powder) | 1·53 | 2·29 |
| Hair spray | 0·60 | 2·40 |

Table 2. *Results of cascade impaction studies: mass median aerodynamic diameter (MMAD) and geometric standard deviation ($\sigma_g$) for each cosmetic aerosol*

| Aerosol | MMAD ($\mu$m) | $\sigma_g$ |
|---|---|---|
| Antiperspirant 1 (pump spray) | 12·80 | 3·46 |
| Antiperspirant 2 (fluorocarbon propellant) | 3·20 | 2·06 |
| Antiperspirant 3 (isobutane–propane propellant) | 2·85 | 2·00 |
| Antiperspirant 4 (spray powder) | 2·90 | 2·00 |
| Hair spray | 2·90 | 2·21 |

Table 3. *Results of SPART analysis of cosmetic aerosols showing count median aerodynamic diameter (CMAD), mass median aerodynamic diameter (MMAD) and count and volume weighted geometric standard deviations ($\sigma_c$ and $\sigma_v$, respectively) in the range 0·1 to 10 $\mu$m*

| Aerosol | CMAD ($\mu$m) | MMAD ($\mu$m) | $\sigma_c$ | $\sigma_v$ |
|---|---|---|---|---|
| Antiperspirant 1 (pump spray) | 1·033 | 2·497 | 1·766 | 1·611 |
| Antiperspirant 2 (fluorocarbon propellant) | 0·736 | 2·191 | 1·665 | 1·553 |
| Antiperspirant 3 (isobutane–propane propellant) | 0·750 | 2·183 | 1·733 | 1·485 |
| Antiperspirant 4 (spray powder) | 1·220 | 2·831 | 1·700 | 1·983 |
| Hair spray | 0·790 | 2·497 | 1·698 | 1·617 |

It is not known what effect the solvent evaporation from the impactor substrates had on the apparent median diameters of the aerosols. Many of the particulates may have consisted almost entirely of solvent material which evaporated before the substrates were weighed. However, it was necessary to dry the substrates because they are hygroscopic and tend to acquire moisture from the air. Cascade impactors of this type are best suited for sizing solid particles.

The SPART analyser has a spectral range of 0·1–10 $\mu$m, which is thought to cover the major mass fraction of respirable aerosol. Computer analysis of the SPART-analyser data yields both count and mass median aerodynamic diameters. Again, all the products studied yielded particulates in the lower part of the respirable range (CMAD 0·7–1·2 $\mu$m; MMAD 2·2–2·8 $\mu$m). Because of the real-time, *in situ* sampling of the SPART analyser, no alteration of the particulates occurs in the sampling process because of condensation or evaporation or flattening of the particulates. The instrument has been shown to be suitable for sizing unstable aerosols such as medical aerosols (Hiller, Mazumder, Wilson & Bone, 1978).

The results obtained using the three different sizing techniques agreed in general. The exception was the pump spray, which yielded a large MMAD in the cascade impaction study. These results indicate that under the sampling conditions used in this study, most of the products produced a very large quantity of small particulates in the range of 3 $\mu$m or less and not many large particulates. In the case of the pump spray, small particulates were produced, but the major mass fraction was beyond the range of the microscope analysis and SPART analysis. In general, when the particulates are small, the three techniques appear to yield similar results under similar sampling conditions. When the major mass fraction is larger than a few micrometers, it is reflected in the cascade impaction analysis, but not in the microscope and SPART analyses. At this point, the differences in sizing techniques become important. Although a sizing technique which encompasses a larger size range is valuable in determining total aerosol content, techniques that focus on the respirable range are more appropriate for use in assessing potential inhalation hazard.

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Fd Cosmet. Toxicol. Vol 19. pp. 89 to 92

0015-6264 81 020089-04$02.00 0

# SHORT PAPERS

# EFFECTS OF BUTYLATED HYDROXYTOLUENE AND ACETYLAMINOFLUORENE ON NADPH-CYTOCHROME *P*-450 REDUCTASE ACTIVITY IN RAT LIVER MICROSOMES

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**Abstract**—Cytochrome *P*-450 content and NADPH-cytochrome *P*-450 reductase activity were measured in liver microsomes prepared from male weanling rats fed low-fat, high-saturated fat or high-polyunsaturated fat diets with or without butylated hydroxytoluene (BHT) and 2-acetylaminofluorene (AAF). The inclusion of BHT and/or AAF in the diets consistently produced marked decreases in cytochrome *P*-450 reductase activity, regardless of the amount and type of dietary fat. In contrast, there was no inhibition of reductase activity when the compounds were added *in vitro* to liver microsomes.

## Introduction

Elevated levels of dietary fat increase the incidence of hepatomas in rats fed 2-acetylaminofluorene (AAF) while dietary supplementation with the antioxidant butylated hydroxytoluene (BHT) delays this tumour development (McCay, King, Rikans & Pitha, 1980; Ulland, Weisburger, Yamamoto & Weisburger, 1973). It is possible that the interrelationships between dietary fat, antioxidants and AAF-induced carcinogenesis may be explained on the basis of effects on the microsomal monooxygenase system, since this system is involved in both the activation and inactivation of many chemical carcinogens, including AAF. The selective induction of several liver monooxygenase activities by BHT is well established (Allen & Engblom, 1972; Creaven, Davies & Williams, 1966; Gilbert & Golberg, 1965; Kahl & Wulff, 1979; Walker, Rahim & Parke, 1973). Dietary administration of BHT to male rats apparently produces a change in the relative proportion of individual forms of microsomal cytochrome *P*-450 without altering the total concentration of the cytochrome(s) (Kahl & Wulff, 1979). With female rats, however, dietary BHT produces an induction of liver microsomal cytochrome *P*-450 content in animals consuming diets high in polyunsaturated or saturated fat (King & McCay, 1981).

AAF also produces significant alterations in the liver monooxygenase system. Single or multiple injections of AAF to male and female Sprague–Dawley rats increased microsomal *N*-hydroxylation of AAF by 3–12 times; *C*-hydroxylation was increased also. This induction was attributed to an effect on an unidentified component of the hepatic monooxygenase system, since no increase in cytochromes *P*-450 or NADPH-cytochrome *P*-450 reductase activity was found (Malejka-Giganti, McIver, Glasebrook & Gutmann, 1978).

The effects of AAF and BHT on microsomal enzymes have not been studied in animals fed diets which differ in amount and type of fat. Numerous studies have shown that dietary lipid is a major factor in determining microsomal monooxygenase activity; furthermore, lipid has major effects on the response to inducing compounds (McLean, 1977; Wade & Norred, 1976). Previous results from our laboratories indicated that the antitumour effects of BHT were also modified by the amount and type of fat in the diet (King, Bailey, Gibson, Pitha & McCay, 1979; McCay *et al.* 1980). It may be that dietary lipid is an important determinant of BHT protection because the protective action involves an induction of detoxification pathways. However, BHT has multiple biological actions and the influence of dietary fat could entail some other mechanism. The present investigation was carried out to determine the effects of AAF and BHT ingestion on the liver microsomal cytochrome *P*-450 system in rats fed low-fat, high-saturated fat or high-polyunsaturated fat diets.

## Experimental

*Animals and diets.* Male weanling Sprague–Dawley rats were placed on their respective diets at 21 days of age and fed *ad lib.* The low-fat diet contained 2% linoleic acid, the high-saturated fat diet contained 18% coconut oil + 2% linoleic acid, and the high-polyunsaturated fat diet contained 20% corn oil (Table 1). Where indicated, AAF was included in the diet at a level of 0·05% and/or BHT at 0·3%. The diets were fed for 2 wk and the animals were housed in barrier-sustained cages.

Table 1. *Composition of diets fed to rats for 2 wk*

| Component | Concn (%) of components in test diets*: Low in fat | High in saturated fat | High in polyunsaturated fat |
|---|---|---|---|
| Casein | 23 | 23 | 23 |
| Total fat | 2 | 20 | 20 |
| Linoleic acid | *2* | *2* | — |
| Coconut oil | — | *18* | — |
| Corn oil | — | — | *20* |
| Sucrose | 64 | 46 | 46 |
| Alphacel† | 6 | 6 | 6 |
| Salt mixture‡ | 4 | 4 | 4 |
| Vitamin mixture‡ | 1 | 1 | 1 |

*When included, 2-acetylaminofluorene was incorporated at 0·05% and butylated hydroxytoluene at 0·3%.
†Non-nutrient bulk.
‡Hubbell, Mendel and Wakeman Salt Mixture (modified to contain zinc chloride) and Vitamin Diet Fortification Mixture from ICN Nutritional Biochemicals, Cleveland, OH 44128.

*Assays.* Microsomes were isolated from liver homogenates by differential centrifugation (Rikans, Gibson & McCay, 1979) and the enzyme assays were performed on the same day. The reduction of cytochrome *P*-450 in microsomes was measured at 15°C by following the formation of the reduced cytochrome *P*-450–CO complex after NADPH addition under anaerobic conditions. The procedure used was a modification (Zannoni, Flynn & Lynch, 1972) of the method originally developed by Gigon, Gram & Gillette (1968). Activities were expressed as nmol cytochrome *P*-450 reduced/min/mg microsomal protein at 15°C. In some experiments, BHT and AAF were dissolved in methanol and added to the microsomes (final concentrations 1·0 and 1·1 mM respectively) prior to analysis of reductase activity. Cytochrome *P*-450 was determined from the reduced carbon monoxide difference spectrum (Omura & Sato, 1964). Binding spectra (Schenkman, Remmer & Estabrook, 1967) were recorded using a Pye Unicam SP8-100 spectrophotometer; BHT and AAF in methanol were added to the sample cuvette (final concentrations, 1, 2, 4 and 8 mM) and equal volumes of methanol were added to the reference cuvette. Protein determination was by a modification of the Lowry procedure (Markwell, Haas, Bieber & Tolbert, 1978). The significance of the difference between two means was established by Student's *t* test.

## Results

### In vivo *effects of BHT and AAF*

The effects of the different diets with or without BHT and AAF on components of the microsomal monooxygenase system are presented in Table 2. Marked decreases in NADPH-cytochrome *P*-450 reductase activity were consistently seen when BHT or AAF were included in the diet. Reductase activities in liver microsomes from animals receiving both BHT and AAF were only 30–35% of those in microsomes from animals fed the unsupplemented diets. This effect was not related to the type and amount of fat in the diet. A smaller effect of BHT and AAF on the specific content of cytochrome *P*-450 was also observed. Decreases that were small (9–18%) but significant ($P < 0·05$) resulted when both BHT and AAF were included in the diets, regardless of the amount and type of fat. However, the inclusion of either agent by itself did not produce consistent increases or decreases in cytochrome *P*-450 concentrations.

### *Effects of amount and type of dietary fat*

The specific content of cytochrome *P*-450 and NADPH-cytochrome *P*-450 reductase activity were significantly greater in liver microsomes from rats fed the high fat diets compared with the low fat diet. However, the type of fat in the diet (saturated *v.* polyunsaturated) did not affect these microsomal components. In addition, the effects of BHT and AAF supplementation were not consistently affected by the type and amount of fat in the diet (Table 2).

### In vitro *effects of AAF and BHT*

The results of *in vitro* addition of BHT or AAF on NADPH-cytochrome *P*-450 reductase activity are given in Table 3. BHT and AAF were dissolved in methanol and added to microsomes prior to analysis of cytochrome *P*-450 reductase activity in the usual fashion. Methanol itself inhibited reductase activity and AAF addition produced no further change in the rate of cytochrome *P*-450 reduction. However, BHT addition significantly increased reductase activity compared with the methanol control. It is probably worth mentioning that of all the solvents tested, including methanol, ethylene carbonate, glycerol, propylene glycol, dimethylformamide, triethanolamine, 2-methoxyethanol and isopropyl ether, none were found that did not significantly increase or decrease reductase activity.

Binding spectra were obtained by adding BHT or AAF to liver microsomes from animals fed unsupplemented diets. The difference spectrum produced by BHT addition was a 'type I' binding spectrum, with a

Table 2. *Effects of dietary fat, butylated hydroxytoluene (BHT) and 2-acetylaminofluorene (AAF) on microsomal monooxygenase components in male rats*

| Diet | Additions to diet‡ | No. of animals§ | Concn of cytochrome *P*-450 (nmol/mg protein) | Activity of cytochrome *P*-450 reductase‖ |
|---|---|---|---|---|
| LF | — | 10 | 0·77 ± 0·03 | 1·01 ± 0·09 |
| | BHT | 5 | 0·82 ± 0·02 | 0·77 ± 0·04** |
| | AAF | 6 | 0·75 ± 0·05 | 0·44 ± 0·05** |
| | BHT/AAF | 6 | 0·66 ± 0·03* | 0·32 ± 0·03** |
| SF | — | 10 | 0·99 ± 0·03†† | 1·52 ± 0·11†† |
| | BHT | 6 | 0·85 ± 0·06* | 0·57 ± 0·06** |
| | AAF | 6 | 1·08 ± 0·04†† | 0·66 ± 0·07** |
| | BHT/AAF | 6 | 0·90 ± 0·03*†† | 0·52 ± 0·06** |
| PUF | — | 10 | 1·03 ± 0·05†† | 1·46 ± 0·11†† |
| | BHT | 6 | 0·98 ± 0·02†† | 0·83 ± 0·08** |
| | AAF | 6 | 0·86 ± 0·04* | 0·53 ± 0·03** |
| | BHT/AAF | 5 | 0·84 ± 0·06*†† | 0·42 ± 0·06** |

LF = Low-fat diet SF = High-saturated fat diet
PUF = High-polyunsaturated fat diet

‡AAF was fed at 0·05% and BHT at 0·3%.
§Male Sprague–Dawley rats fed the appropriate diet for 2 wk from weaning and then killed. Assays were performed on freshly-prepared microsomes.
‖Expressed in nmol cytochrome *P*-450 reduced/min/mg protein.
Values are means ± SEM for the numbers of the animals shown. Those marked with asterisks differ significantly (Student's *t* test) from the value for the corresponding unsupplemented control (*$P < 0·05$; **$P < 0·01$) and those marked †† differ significantly from the value for the group fed the low-fat diet with the same additions: ††$P < 0·01$.

maximum at 390 nm and a minimum at 425 nm. The binding spectrum produced with AAF was a 'type II' spectrum, with a minimum at 410 nm and a maximum at 430 nm.

## Discussion

The predominant effect of feeding AAF and/or BHT on the hepatic cytochrome *P*-450 system was a marked decrease in NADPH-cytochrome *P*-450 reductase activity. NADPH-cytochrome *P*-450 reductase is generally considered to be the rate-limiting component of the microsomal monooxygenase system and this decrease in its activity could have profound effects on the metabolism of foreign chemicals. BHT and AAF produced dramatic decreases in reductase activity regardless of whether the rats were fed the low fat, high saturated fat or high polyunsaturated fat diet. It is not clear how this decrease in reductase activity may be related to the carcinogenic process, especially in the light of evidence that the carcinogenic effect of AAF and the protective effect of BHT are both markedly influenced by the fat content of the diet. The extent to which BHT inhibits the binding of [$^{14}$C] AAF to liver DNA *in vivo* is also significantly affected by the type and amount of fat in the animals' diets (McCay *et al.* 1980). Nevertheless, the possibility remains that BHT competes with AAF for some site in the microsomal monooxygenase system and inhibits the metabolic activation of AAF.

Table 3. In vitro *effects of butylated hydroxytoluene (BHT) and 2-acetylaminofluorene (AAF) on microsomal cytochrome* P-*450 reductase activity*

| Additions† | Activity‡ |
|---|---|
| None | 1·08 ± 0·09 |
| Methanol | 0·81 ± 0·06 |
| BHT in methanol | 1·29 ± 0·07** |
| AAF in methanol | 0·85 ± 0·12 |

†BHT and AAF were dissolved in methanol and added to microsomes at final concentrations of 1·0 and 1·1 mM, respectively, prior to the measurement of cytochrome *P*-450 reductase activity.
‡Expressed in nmol cytochrome *P*-450 reduced/min/mg protein.
Values are means ± SEM from six experiments using six different preparations of freshly-prepared microsomes pooled from two rat livers. The value marked with asterisks differs significantly ($P < 0·01$ by Student's *t* test) from the methanol control.

It would appear that BHT and AAF do not inhibit cytochrome *P*-450 reduction directly since the *in vitro* addition of the compounds produced no decrease in reductase activity. One possibility that we had considered was that BHT and AAF might act by binding to cytochrome *P*-450 and subsequently inhibiting its reduction by the reductase since this type of interaction has been reported for several 'type II' substrates (Gigon, Gram & Gillette, 1969). However, *in vitro* additions did not inhibit reductase activity and only AAF gave a 'type II' binding spectrum. A 'type I' binding spectrum has been reported previously for BHT (Yang, Strickhart & Woo, 1974) and is consistent with the stimulation of cytochrome *P*-450 reductase activity caused by *in vitro* BHT addition. The

'type II' binding spectrum observed for AAF contrasts with the findings of Gutmann's group which reported a 'type I' spectrum for AAF binding to cytochrome(s) *P*-450 in microsomes from 3-methylcholanthrene-treated rats and no binding spectrum with microsomes from untreated, AAF-treated or phenobarbital-treated rats (Malejka-Giganti *et al.* 1978). The reason for this discrepancy is not known but it may be related to differences in relative proportions of cytochromes *P*-450 between microsomes from rats fed commercial rations in the above studies and those from rats fed semipurified diets in ours. The absence of inhibition with *in vitro* additions of BHT and AAF in the reductase assay may also reflect a situation in which the compounds are unable to reach a particular 'site' within the membrane. Recent evidence suggests that microsomal phospholipid plays a major role in the formation of a binary complex between cytochrome *P*-450 and the reductase (Miwa & Lu, 1980; Miwa, West, Huang & Lu, 1979); thus, the site involved may be membrane lipid rather than the cytochrome and/or its reductase. Another important possibility is that metabolism of BHT or AAF to other compounds may be required for the inhibitory effect. In this situation, the *in vitro* effects could not correspond to effects occurring after *in vivo* biotransformation.

In conclusion, dietary BHT and AAF produce marked decreases in NADPH-cytochrome *P*-450 reductase activity, but these effects are not seen when the compounds are added *in vitro* to liver microsomes. These results emphasize the need to study dietary effects on the carcinogen-metabolizing system in an *in vivo* model which can be correlated directly with tumour-incidence data.

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Fd Cosmet. Toxicol. Vol. 19, pp. 93 to 96

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# EFFECT OF DIET ON OESTROGEN BIO-ASSAY IN MICE

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**Abstract**—The uterotrophic effect of an oestrogen in the mouse uterine-weight bio-assay was increased by at least 50% when the oestrogen was fed in a semi-synthetic diet instead of in ground rat cubes; when it was administered sc, the change in diet had no effect. Whichever diet was fed, the same maximum uterine weight was achieved. These observations suggest that there was probably a more rapid or complete absorption of the oestrogen from the gut of animals on the semi-synthetic diet, and highlights the difficulty of estimating the oestrogenic activity of an unknown sample by feeding it to mice and comparing the uterotrophic effect with that of known amounts of a standard oestrogen, e.g. diethylstilboestrol, added to the regular diet.

## Introduction

A recent report from this laboratory (Drane, Patterson, Roberts & Saba, 1980) described problems of slight oestrogenicity in the rat cubes (Porton Rat Diet; PRD) used as control diet in mouse uterine-weight oestrogen assays. This activity was traced to the soya-meal constituent of the diet and, in view of the possibility that a high proportion of soya meals may be oestrogenic (Drane *et al.* 1980), it was decided to test the effect of a soya-free semi-synthetic diet (SSD) on the mouse uterine-weight assay. This paper presents data on the effect of diet on the uterine response to standard doses of oestrogens.

## Experimental

*Animals.* Eighteen-day-old MF1 weanling female mice, weighing 7–9 g, were supplied by OLAC (1976) Ltd., Bicester, Oxon.

*Oestrogen bio-assay.* Groups of mice were housed six to a cage and fed one of three diets over a period of 3·5 days, during which each cage was supplied with 40 g of the appropriate diet, as described previously (Drane *et al.* 1980). The mice were killed on the following day. Initial and final body weights and the uterine weight were recorded. Pairs of test diets (in most cases PRD and SSD) were compared in a series of ten bio-assays, in nine of which the oestrogen was mixed with the feed; in the remaining assay (no. 6), the oestrogen was injected sc.

*Diets.* The calculated analyses of PRD and SSD (both supplied by RHM Labsure, Poole, Dorset) are given in Table 1. PRD cubes were ground in a small food mill before oestrogens were added, but when fed were still of a coarser texture than SSD. In two assays (nos 7 and 8) the comparison was made not between SSD and PRD but between SSD and an alternative commercial rat cube diet (RD).

*Oestrogens.* The oestrogens used were (i) diethylstilboestrol dipropionate (B. Vet. C.; DES), 10 mg/ml in oil, suitably diluted with ether; (ii) zearalenone (Commercial Solvents Corp., Terre Haute, IN, USA), dissolved in methanol; (iii) an ether extract of a 70% ethanolic extract of diseased white clover (Saba, Drane, Hebert & Holdsworth, 1974). For the sc injections (assay no. 6), DES was diluted in arachis oil, to provide graded doses totalling 0·025, 0·05 and 0·1 μg DES/mouse, and administered in three injections of 0·05 ml/mouse. Oral doses of oestrogens are indicated in Table 1 and the Results section.

*Statistical analysis of results.* When evaluating assays of this type, the normal procedure is to estimate relative potencies from the log dose-response curves using the recognized statistical methods for parallel line assays, where the response is the uterine weight transformed to log to the base 10 (Finney, 1978). The dose range selected must be such that the curves have adequate slope and show no significant deviations from parallelism or linearity. Hence it is sometimes necessary to exclude dose levels towards the upper or lower ends of the range tested where curvature occurs.

A log dose-response line for known doses of oestrogen is thus obtained for groups of mice fed the two diets under test. By fitting a common slope to the two curves, the ratio of the doses that give the same response to both test and standard can be determined, together with a measure of the variation involved. This ratio is defined as the relative potency, which is expressed as a percentage. As the lines are parallel, the ratio is constant throughout the length of the curves.

Since a dietary effect was being studied in the present experiments, it was thought that any differences in uterine weight might partly reflect differences in body-weight gain on the two diets. Estimates of relative potency were therefore also determined using the ratio of uterine weight to final body weight ($\times 1000$) as the response. Only doses on the linear portion of each log dose-response curve were selected and in almost all assays these were found to be the same as those used for estimates of potency derived from the log uterine weight.

Table 1. *Calculated analyses of Porton Rat Diet (PRD) and the semi-synthetic diet (SSD)*

| Constituent | Concn (%*) of constituent in PRD | SSD |
|---|---|---|
| Crude oil | 2·78 | 3·3 |
| Crude protein | 19·79 | 18·2 |
| Crude fibre | 5·37 | 10·0 |
| Calcium (as Ca) | 0·72 | 0·9 |
| Phosphorus (as P) | 0·71 | 0·5 |
| Salt (as NaCl) | 1·03 | 0·4 |
| Methionine, cystine | 0·63 | 0·7 |
| Lysine | 1·07 | 1·5 |
| Metabolizable energy (Kcal/kg) | 2570 | 2800 |

*Concentrations are expressed as % of feed unless stated otherwise.

A two-fold dilution range was used in all the bio-assays.

## Results

Mean control uterine weights were in the range 6·6–10 mg. Within any one assay there was no significant difference in the mean uterine weights of control mice fed the two diets under test.

Two typical log dose-response curves are illustrated in Fig. 1. The linear portion of the curve spans the range 0·05–0·40 μg for mice fed PRD and 0·025–0·20 μg for those on the SSD; the mean potency of DES fed in SSD relative to that fed in PRD was 195%, with 95% confidence limits of 161–236%. The maximum uterine weights attained under the different feeding regimes (71·6 mg on PRD, 69·6 mg on SSD) were similar (see Fig. 1). Geometric mean uterine weights and corresponding mean body weights are shown in Table 2 for a series of ten bio-assays in which three separate oestrogens were given. The corresponding relative potencies and their 95% confidence limits are shown in Table 3.

The relative potencies—based on log uterine weight—show some significant variability between assays but the combined relative potency for assays 1–5 was 184% (166–204%).

Diet had no effect on the uterine weight response when DES was given sc. This assay was carried out at the same time as assay no. 5, in which the relative potency was 166% (Table 3).

When a white clover extract or zearalenone was fed instead of DES (assays 9 and 10), their relative potencies in the two diets were 174 and 160%, respectively. SSD tested against another commercial rat cube diet (RD) in assays 7 and 8 gave a combined relative potency of 174% (148–205%), which was close to that shown for assays 1–5, in which PRD was fed.

There was reasonable agreement between those estimates based on the ratio of uterine weight to body weight and those using log uterine weight, showing that body weight was not a factor influencing the uterine weight response.

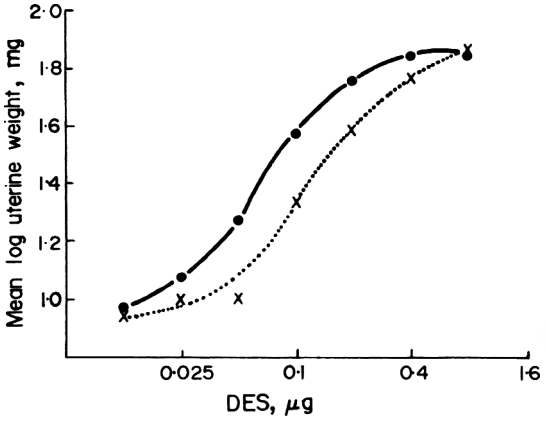


Fig. 1. Typical log dose-response curves for diethylstilboestrol (DES) in mice fed Porton Rat Diet nuts (× – – – ×) or a semi-synthetic diet (●——●).

## Discussion

The results of these studies show that the uterine weight response to orally administered DES can be significantly altered by the nature of the diet and was greater in mice fed the SSD than in those fed conventional rat feed. The observations that the control uterine weights were similar for both diets and that the mean body weights of the mice were not affected shows that the effect is not due to a nutritional factor; this is supported by the results of assay no. 6 in which the response to injected DES was not affected by the diet.

The oestrogenic compounds in white clover are probably coumestans and both these and zearalenone are chemically different from DES. However, from the limited evidence presented it seems that the effect of diet is also observed with these oestrogens.

It is not known why changing the basic diet should have this effect. The protein sources for SSD and PRD are different (SSD contains casein whilst PRD contains cereals and soya meal), but the level of protein in the two diets does not differ sufficiently to relate the dietary effect to an increased rate of liver detoxification on a greater protein intake. The higher percentage of dietary fibre in SSD would decrease transit time in the gut and thus tend to reduce rather

Table 2. *Mean response of groups of six mice to varying doses of oestrogen under different dietary systems*

| Assay no. | Oestrogen Type* | Dose (μg/mouse) | Geometric mean uterine weight (mg)† in mice fed PRD | SSD | RD | Mean body weight (g) of mice fed PRD | SSD | RD |
|---|---|---|---|---|---|---|---|---|
| 1 | DES | 0·05 | 10·8 | 22·8‡ | | 9·1 | 11·1‡ | |
| | | 0·10 | 19·0‡ | 39·7 | | 10·9‡ | 10·8 | |
| | | 0·20 | 33·1 | 70·1 | | 10·2 | 11·4 | |
| 2 | DES | 0·025 | — | 11·8 | | — | 9·7 | |
| | | 0·05 | 10·7 | 18·9 | | 10·2 | 9·6 | |
| | | 0·10 | 21·8 | 37·6 | | 10·3 | 9·8 | |
| | | 0·20 | 37·9 | 56·5 | | 9·9 | 10·1 | |
| | | 0·40 | 57·6 | — | | 10·4 | — | |
| 3 | DES | 0·05 | 11·1 | — | | 9·7 | — | |
| | | 0·10 | 31·5 | 39·3 | | 10·2 | 9·0 | |
| | | 0·20 | 41·3 | 66·9 | | 10·1 | 9·4 | |
| 4 | DES | 0·05 | — | — | | 9·6 | 10·3 | |
| | | 0·10 | 18·8 | 26·1 | | 9·6 | 10·4 | |
| | | 0·20 | 36·2 | 54·6 | | — | — | |
| 5 | DES | 0·03 | 10·4§ | 14·8§ | | 9·4§ | 11·3§ | |
| | | 0·06 | 17·0 | 27·1 | | 9·7 | 10·6 | |
| | | 0·12 | 34·9‡ | 54·9 | | 10·6‡ | 10·6 | |
| 6 | DES (sc) | 0·025 | 17·0 | 17·5 | | 9·8 | 9·4 | |
| | | 0·05 | 35·7 | 37·6 | | 8·9 | 9·8 | |
| | | 0·10 | 51·1 | 56·6 | | 8·5 | 10·4 | |
| 7 | DES | 0·05 | | 17·7§ | 14·6 | | NW | 9·9 |
| | | 0·10 | | 37·6 | 21·3 | | NW | NW |
| | | 0·20 | | 66·5 | 47·3‡ | | NW | 10·8‡ |
| 8 | DES | 0·05 | | 20·4 | 11·6 | | 9·9 | 10·3 |
| | | 0·10 | | 46·6 | 22·5 | | 10·3 | 11·0 |
| | | 0·20 | | 65·6 | 46·4 | | 10·6 | 10·4 |
| 9 | White clover | $0{\cdot}5.10^6$ | 12·2 | 21·4 | | 9·1 | 10·3 | |
| | | $1{\cdot}0.10^6$ | 24·6 | 42·9 | | 9·2 | 10·4 | |
| 10 | Zearalenone | 25 | 8·4 | 11·8 | | — | — | |
| | | 75 | 13·9 | 17·8 | | 8·6 | 10·6 | |
| | | 225 | 36·7 | 51·7 | | 9·2 | 10·2 | |

PRD = Porton Rat Diet SSD = Semi-synthetic diet RD = Alternative rat diet DES = Diethylstilboestrol NW = Not weighed

*All doses were fed in the diet except where indicated otherwise (assay no. 6—sc).

†Mean uterine weights of control mice given no oestrogen ranged from 6·6 to 10 mg and showed no significant difference between the two dietary groups in any one assay.

‡Mean for only five mice.

§Mean for only four mice.

Table 3. *Effect of diet on the response of the mouse uterus to oestrogens*

| Type of oestrogen* | Standard diet | Assay no. | Relative potency† (%) (a) | (b) |
|---|---|---|---|---|
| DES | PRD | 1 | 258 (206–323) | 255 (148–437)‡ |
| | | 2 | 195 (161–236) | 203 (168–245) |
| | | 3 | 160 (124–206) | 225 (146–347) |
| | | 4 | 148 (116–188) | 134 (107–166) |
| | | 5 | 166 (132–208) | 148 (166–188) |
| | | 6 | 107 (87–131) | 93 (75–117) |
| | RD | 7 | 158 (123–201) | NW |
| | | 8 | 189 (152–235) | 200 (160–251) |
| White clover | PRD | 9 | 174 (124–245) | 156 (109–221) |
| Zearalenone | PRD | 10 | 160 (113–226) | 114 (80–162) |

DES = Diethylstilboestrol PRD = Porton Rat Diet RD = Alternative rat diet SSD = Semi-synthetic diet NW = Not weighed

*All doses were fed in the diet except in assay no. 6, in which they were given sc.

†Potency in mice fed SSD expressed (with 95% confidence limits in brackets) relative to that in mice fed a standard rat diet. Estimates of potency were derived from dose-response curves of (a) $\log_{10}$ uterine weight on $\log_{10}$ dose of oestrogen, and (b) uterine weight/body weight ratio on $\log_{10}$ dose of oestrogen.

‡Significant deviation from parallelism in this assay.

than increase the uptake of oestrogens from SSD. The most likely explanation is that the much finer texture of the SSD results in better absorption of the oestrogens administered in it.

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# MONOGRAPHS

## Monographs on Fragrance Raw Materials*

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### *n*-BUTYRIC ACID

*Synonyms*: Ethylacetic acid; *n*-butanoic acid.
*Structure*: $CH_3 \cdot [CH_2]_2 \cdot COOH$.
*Description and physical properties*: *Merck Index* (1976).
*Occurrence*: Normally occurs in butter as a glyceride. Has been reported in the essential oils of citronella Ceylon, *Eucalyptus globulus*, *Araucaria cunninghamii*, *Lippia scaberrima*, *Monarda fistulosa*, cajeput, *Heracleum giganteum*, lavender, *Hedeoma pulegiodes*, valerian, nutmeg, hops, *Pastinaca sativa* and Spanish anise (*Fenaroli's Handbook of Flavor Ingredients*, 1975) and in apple, banana, cherry and morello, citrus fruits, currants, grape, papaya, peach, pear, raspberry and blackberry, strawberry, roasted onion, sauerkraut, tomato, aniseed, white bread, bread and bread preferment, milk and milk products, including blue, Cheddar and Swiss cheeses, fish, heated beef, heated pork, beer, grape brandy, rum, whisky, cider, sherry, wine, cocoa, coffee, tea, barley (roasted), peanuts (roasted), potato chips (American), honey, soya bean, arctic bramble, cloudberry, passion fruit, mushroom, trassi (cooked), plum brandy and pear brandy (CIVO–TNO, 1977).
*Preparation*: By controlled selective fermentation of carbohydrates (Arctander, 1969).
*Uses*: In public use since the 1920s, primarily as an intermediate in the preparation of butyric esters.
Concentration in final product (%):

| | *Soap* | *Detergent* | *Creams, lotions* | *Perfume* |
|---|---|---|---|---|
| *Usual* | 0·001 | 0·0001 | 0·0005 | 0·01 |
| *Maximum* | 0·003 | 0·0003 | 0·001 | 0·02 |

*Analytical data*: Infra-red curve, RIFM no. 77–60.

#### Status

Butyric acid was given GRAS status by FEMA (1965), is approved by the FDA for food use (GRAS) and was included by the Council of Europe (1974) at a level of 400 ppm in the list of artificial flavouring substances that may be added to foodstuffs without hazard to public health. The *Food Chemicals Codex* (1972) has a monograph on butyric acid. CAS Registry No. 107–92–6.

#### Biological data†

*Acute toxicity*. The oral $LD_{50}$ in rats has been reported as 2·94 g/kg (Smyth, Carpenter & Weil, 1951) and as 8·79 g/kg (Smyth, Carpenter, Weil & Pozzani, 1954). The oral $LD_{50}$ of a 1% aqueous solution was reported as >400 mg/kg in rats (Fassett, 1963). The dermal $LD_{50}$ in rabbits has been reported as 6·35 ml/kg (Smyth *et al.* 1954) and as 2·1 g/kg (1·2–3·6 g/kg) (Moreno, 1977). The ip or sc $LD_{50}$ for butyric acid in white mice was 3·18 g/kg, with death occurring within 1 hr (Senior & Sherratt, 1969).

In carp treated with butyric acid for 24 and 48 hr, the median tolerance values were 85 and 65 ppm, respectively (Funasaka, Ose & Sato, 1976). The median tolerance limit of butyric acid over a 24-hr period was found to be 200 mg/litre water for the fish, *Lepomis marochirus* (Dowden & Bennett, 1965).

*Subacute toxicity*. Acetonemic ketosis was observed in a dairy herd when the feed contained an excessive amount of butyric acid; the sick cows demonstrated lack of appetite, weight loss, a decrease in milk yield and a decrease in rumination (Glawischnig, 1969).

*Inhalation*. An 8-hr inhalation of vapour saturated with butyric acid caused no deaths in rats (Smyth *et al.* 1951).

*The most recent of the previous sets of these monographs appeared in *Food and Cosmetics Toxicology* 1980, **18** (pp. 649–682).
†Literature searched from 1962 through June 1977.

*Irritation.* Butyric acid has been reported to be a moderately strong primary irritant in the guinea-pig (Fassett, 1963). Applied full strength to intact or abraded rabbit skin for 24 hr under occlusion, it was moderately to severely irritating (Moreno, 1977). Tested at 1% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Epstein, 1977).

*Sensitization.* A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material (RIFM no. 77–60) was tested at a concentration of 1% in petrolatum and produced no sensitization reactions (Epstein, 1977).

*Absorption and metabolism.* The absorption of butyric acid in man, as determined in buccal absorption tests, appeared to be a true passive transfer into the lipid membrane of the mouth and no specialized transport system was observed (Beckett & Moffat, 1968).

Production of butyric acid by bacterial fermentation of dietary carbohydrates has been noted in ruminants and non-ruminants, including man. Butyric acid appears to be absorbed through the epithelium of the rumen, caecum and colon, and species-specific transport mechanisms may be involved (Schmitt, Soergel & Wood, 1976). For example, in ruminants, butyrate is absorbed directly from the rumen into the portal system (Lane & Moss, 1971).

Human volunteers were used to determine the absorption of butyric acid as the sodium salt and its effects on $H_2O$ and $Na^+$ absorption (Schmitt *et al.* 1976). Intestinal perfusions indicated that butyric acid absorption in the jejunum was rapid. A rise in net $H_2O$ absorption was observed with increasing concentration of butyrate. The net $Na^+$ transport in the jejunum paralleled the changes in net $H_2O$ absorption. Butyric acid did not appear to stimulate jejunal secretion in areas adjacent to the perfused segment. It was also noted that the presence of butyric acid in the jejunum did not cause marked increases in gastric, biliary or pancreatic secretion.

The absorption of butyric acid from the colon was studied in a 55-yr-old woman following perfusion of a mixture of several fatty acids into the gut and colon (Dawson, Holdsworth & Webb, 1964). The relative absorption rates for the fatty acid mixture were similar for both the small bowel and the colon. Jejunal absorption was also observed in seven patients.

Dogs with vagally denervated pouches of the oxyntic-gland area of the stomach were irrigated for periods of 2–30 min with 100 mM-mannitol. Butyric acid moved rapidly through the mucosa and fluxes of $Na^+$, $K^+$ and $Cl^-$ from the mucosa were increased. Subsequent irrigation with 100 mN-HCl indicated mucosal damage because of maintained increased $H^+$ flux (Davenport, 1964).

In monogastric mammals, ingested butyric acid is primarily absorbed from the small intestine in non-esterified form by the portal route and is consequently transported directly to the liver, where it is oxidized in the presence of octanoyl coenzyme A synthetase to the butyryl coenzyme A ester (White, Handler & Smith, 1973). The major portion of this ester is catabolized to acetyl coenzyme A via $\beta$-oxidation, while the remaining butyric acid undergoes $\omega$-oxidation to form succinic acid.

*Pharmacology.* Butyric acid was shown to affect gastric emptying by its action on duodenal receptors when administered intragastrically to human subjects (Hunt & Knox, 1969). After iv infusion of butyric acid into the abomasum of goats and cows, the emptying rate of the abomasum was decreased (Poulsen & Jones, 1974).

When a 0·1 mM mixture of volatile fatty acids containing butyric acid was infused into the caecum of fistulated rams, there was an approximately 70% increase in caecal motility, but when the concentration of the mixture was increased to 1·0 and 10 mM there was a reduction in motility of 40 and 80%, respectively (Svendsen, 1972). When butyric acid was infused alone as a 10 mM solution, a 50–75% decrease in caecal motility was observed (Svendsen, 1972). Intra-abomasal infusion of 50 ml butyric acid (300 mM) in sheep was associated with a marked decrease in abomasal action potential activity and also in the emptying rate (Bolton, Merritt, Carlson & Donawick, 1976). Infusion of sheep with a mixture of short-chain fatty acids, including butyric acid, was accompanied by an increase in plasma-insulin levels and enhanced jejunal electrical activity (Ruckebusch & Fioramonti, 1975). A cow with a parotid fistula exhibited a significant increase in salivary secretion when fed hay sprayed with butyric acid (Hagemeister & Kaufmann, 1970). However, in an experiment in lactating cows, a significant decrease in the plasma-glucose level was noted when butyrate was infused, a finding attributed to enhanced insulin secretion (Reynaert, De Paepe, Marcus & Peeters, 1975). A decrease in serum free fatty acids was observed during the butyrate infusion, and there was also a pronounced increase in growth hormone levels in the serum.

Butyric acid in concentrations $\leqq 0{\cdot}1\%$ accelerated jejunal-villi movement in anaesthetized dogs, while concentrations $>0{\cdot}1\%$ tended to inhibit this movement (Nanba & Okada, 1973).

The bidirectional flux of $Na^+$ across the gastric mucosa in dogs was increased when butyric acid was present in the contents of the stomach (Ventura, Schlegel & Code, 1972; Ventura, Schlegel, LaForce & Code, 1973). Butyric acid, intestinally perfused as a soap, or butyric acid dispersed in $\alpha$-monolein and bile did not stimulate pancreatic secretion when administered to dogs with a gastric or pancreatic fistula (Meyer & Jones, 1974). Following the microinjection of 1–10% butyric acid into various parts of the dog brain, the cortical EEG of dogs with hepatic insufficiency but not of normal dogs, changed suddenly to the slow pattern (Kiyosaki, 1963). The effect of butyrate on sleep patterns in the cat has been studied by Hoshi (1970). Intraventricular injection of butyric acid produced hyperthermia in cats (Varagic & Beleslin, 1973).

The absorption of butyric acid across the intestinal lumen of anaesthetized Wistar-strain rats was

not affected by the perfusion of anisole, butylated hyroxyanisole or safrole (Fritsch, Lamboeuf, de Saint Blanquat & Canal, 1975).

In mice receiving 5 or 7% butyric acid in the diet, increased butyrylcholinesterase activity was noted (Mezincesco & Ghetie, 1974). Butyric acid in an ip dose of 0·8–14 mequiv/kg induced changes in EEG patterns in rats (Marcus, Winters, Mori & Spooner, 1967). These changes included abnormal slow-wave sleep at concentrations between 10·3 and 14·0 mequiv/kg, and desynchronization at all the concentrations tested. When butyric acid at 0·8–58 mequiv/kg was administered ip or iv, there was no induction of REM sleep. Butyric acid at 0·8–14 mequiv/kg ip induced ketosis in these rats, an effect that appeared to be unrelated to the neurophysiological action. The ketosis ranged from slight at the lower dosages to marked at the intermediate and higher dosages. Butyric acid in ip doses of 250 mg/kg was without significant effect on spontaneous locomotor activity or on co-ordination of movement when administered to male albino mice (Weiner & Olson, 1977). When sleep was induced in rats by intracerebral injection of [$^{14}$C]butyrate, binding to neural membrane lecithin was demonstrated (Rizzoli & Galzigna, 1970).

Vaginal secretions from sexually excited women contained several short-chain fatty acids, including butyric acid, which had been shown to be crucial pheromonal components in higher primates (Sokolov, Harris & Hecker, 1976). Analysis of the pheromone composition in the vaginal secretions of minks (Sokolov & Khorlina, 1976) and of rhesus monkeys, (Keverne, 1976) revealed that butyric acid was one of several volatile carboxylic acid constituents.

Butyric acid elicited an apparently sex-related change in the taste response of black-tailed deer to water and ethanol extracts of various plants (Rice & Church, 1974), was among many compounds that produced an alteration in the electrophoretic mobility of plasma α-lipoproteins when administered to chicks (Darcel, Bide & Merriman, 1968), and initiated olfactory receptor-cell action potentials in frogs when circulated at a level of 45% in air over exposed nasal mucosa (Gesteland, 1976).

*Mutagenesis.* Butyric acid was found to be non-mutagenic when assayed in the Sd-4-73 strain of *Escherichia coli* (Szybalski, 1958).

*Teratogenesis.* Butyric acid (0·001 M), was associated with marked abnormalities of the diastematic zone and fibrillar structure of the achromatic apparatus in eggs of the newt, *Triturus helveticus* (Sentein, 1967). Butyric acid treatment of the eggs at the two- or four-blastomere stage caused dispersion of the monocentric or monopolar mitotic fibril systems into the vitellin cytoplasm at telophase, concentration or reassembling of the fibril system into coherent multipolar groups at the centre of each blastomere during metaphase and anaphase, and appearance of tubular spherical mitotic fibril systems at prophase (Sentein, 1968).

*Effects on cell differentiation.* In A/J strain mice inoculated sc with C1300 murine neuroblastoma tumour cells, butyric acid was found to be ineffective in arresting the development of the neuroblastomas, if administered ip when the implanted tumours had reached 1·0–1·5 cm in diameter (Finklestein, Tittle, Meshnik & Weiner, 1975). The acid has been found to elicit differentiation in various cancer cell lines, and the effects of butyrate on mammalian cells in culture has been reviewed by Prasad & Sinha (1976).

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*Pharmacology*

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*Essential oils and cosmetics*

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## ETHYLCELLULOSE

*Synonyms*: Cellulose ethyl ether; Ethocel.
*Description and physical properties*: *Merck Index* (1976).
*Occurrence*: Has apparently not been reported to occur in nature.
*Preparation*: Prepared from wood pulp or chemical cotton by treatment with alkali and ethylation of the alkali cellulose with ethyl chloride (*Merck Index*, 1976).
*Uses*: In public use since the 1950s. Use in fragrances in the USA amounts to less than 1000 lb/yr. The maximum concentration used in perfumes is 1·2% of the final product.
*Analytical data*: Infra-red curve, RIFM no. 77–158.

### Status

Ethylcellulose is approved by the FDA for food use (21 CFR 172.868). Both the *Food Chemicals Codex* (1972) and the *National Formulary* (1980) have a monograph on ethylcellulose. The Federation of American Societies for Experimental Biology (1974) states "there is no evidence in the available information on ethyl cellulose... that demonstrates or suggests reasonable grounds to suspect, a hazard to the public when used in food packaging materials as now practised or as might be expected to be used for such purposes in future". Affirmation of the GRAS status of ethylcellulose and other cellulose derivatives as indirect ingredients of human food has been proposed (Food and Drug Administration, 1979). C.A.S. Registry No. 9004–57–3.

### Biological data*

*Acute toxicity*. Both the acute oral $LD_{50}$ in rats and the acute dermal $LD_{50}$ in rabbits exceeded 5 g/kg (Moreno, 1977).

*Subacute toxicity*. No adverse effects were reported in a group of 80 rats maintained for 8 months on a diet containing 1·2% ethylcellulose, which amounted to an average dose of 182 mg/rat/day (Hake & Rowe, 1963).

*Irritation*. Ethylcellulose applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was slightly irritating (Moreno, 1977). Tested at 12% in diethylphthalate (DEP), it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1977).

*Sensitization*. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material (RIFM no. 77–158) was tested at a concentration of 12% in DEP and produced no sensitization reactions (Kligman, 1977).

*Absorption*. When aspirin coated with ethylcellulose was administered orally to rats, absorption of the combination from the rat stomach was slower than that of aspirin alone (Niwa & Nakayama, 1971). These authors discussed the pharmacodynamics of aspirin–ethylcellulose in the rat and rabbit.

*Physiology*. Ethylcellulose has been described as physiologically inert (Hake & Rowe, 1963).

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## ETHYL CROTONATE

*Synonyms*: Ethyl *trans*-2-butenoate; crotonic acid, ethyl ester; 2-butenoic acid, ethyl ester.
*Structure*: $CH_3 \cdot CH{:}CH \cdot COO \cdot CH_2 \cdot CH_3$.
*Description and physical properties*: A colourless liquid.
*Occurrence*: Reported to be found in *Fragaria vesca* (*Fenaroli's Handbook of Flavor Ingredients*, 1975), and in apples, grapes, papaya, strawberry, wine, cocoa and passion fruit (CIVO–TNO, 1977).
*Preparation*: By esterification of ethanol with crotonic acid under azeotropic conditions (Arctander, 1969).
*Uses*: In public use since the 1940s. Use in fragrances in the USA amounts to less than 1000 lb/yr.
Concentration in final product (%):

| | *Soap* | *Detergent* | *Creams, lotions* | *Perfume* |
|---|---|---|---|---|
| Usual | 0·005 | 0·001 | 0·003 | 0·04 |
| Maximum | 0·05 | 0·01 | 0·02 | 0·4 |

*Analytical data*: Gas chromatogram, RIFM no. 76–100; infra-red curve, RIFM no. 76–100.

### Status

Ethyl crotonate was given GRAS status by FEMA (1977), is approved by the FDA for food use (21 CFR 172.515) and was included by the Council of Europe (1974) at a level of 1 ppm in the list of artificial flavouring substances that may be added temporarily to foodstuffs without hazard to public health. CAS Registry No. 623–70–1; 10544–63–5.

### Biological data*

*Acute toxicity.* The acute oral $LD_{50}$ in rats was reported as 3·0 g/kg (Smyth & Carpenter, 1944). The dermal $LD_{50}$ in guinea-pigs has been reported as $>5$ g/kg (Moreno, 1978) and as $>10$ ml/kg (Smyth & Carpenter, 1944).

*Subacute toxicity.* Ethyl crotonate produced no neuropathy in rats when fed alone in the diet at a concentration of 18,400 ppm for 5 wk (373 mg/rat/day), and its co-administration with acrylamide did not affect the development or progress of acrylamide-induced neuropathy in rats (Edwards, 1975).

*Inhalation.* No deaths were produced in rats that inhaled the saturated vapours of ethyl crotonate for 8 hr (Smyth & Carpenter, 1944).

*Irritation.* Ethyl crotonate was severely irritating to the rabbit eye and was irritating to rabbit skin when applied undiluted for 24 hr (Smyth & Carpenter, 1944). Ethyl crotonate applied full strength to intact or abraded guinea-pig skin for 24 hr under occlusion was slightly irritating (Moreno, 1978). Tested at 4% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Epstein, 1977).

*Sensitization.* A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 26 volunteers. The material (RIFM no. 76–100) was tested at a concentration of 4% in petrolatum and produced no sensitization reactions (Epstein, 1977).

*Nutrition.* The availability of ethyl crotonate was low, when the ester was fed to chicks as 5% of the diet (Yoshida, Morimoto & Oda, 1970).

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*Toxicology*

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*Arrangement is based on section headings from *Chemical Abstracts*.

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# Review Section

## REVIEWS OF RECENT PUBLICATIONS

**Developments in Food Colours—1.** Edited by J. Walford. Applied Science Publishers Ltd., London, 1979. pp. xi + 259. £18.00.

The aim of the *Developments Series* is to draw together a selection of papers reviewing the latest developments in a specific field and to publish them quickly. This book provides an overview of developments in research, technology and legislation concerning food colourings.

The introduction contains a brief review of the history of the use and legal status of colourings and includes a rationale for the addition of colourings to food. The next chapter is devoted to a description of modern theory and practice of food colorimetry. In later chapters developments in both traditional and novel synthetic organic colourings are discussed. A major innovation in the synthetic colourings field has been the concept of polymeric colourings which was initiated by the Dynapol Corporation. By this approach it is argued that the potential toxic effects of a chromophore can be substantially reduced by fixing it to a polymer so that the molecular weight of the resultant colouring is too great to permit absorption.

There is a continuing trend to replace synthetic colourings with naturally derived or nature-equivalent colourings. Research concerning naturally-occurring colourings and the synthetic carotenoids is reviewed and the technological and legislative aspects of these materials are discussed. A further chapter considers the contribution of various expert committees towards effective legislation on food colourings and the difficulties faced by these committees, since human data are rarely available and the extrapolation of animal data to variable human populations is often further complicated by imprecise chemical specifications or problems of instability during processing or storage.

The final chapter includes further speculation on the future use of natural colourings which is said to be hindered at present by technical inadequacy, high costs per colour unit, lack of toxicological information and the unpredictability of legislative bodies. The composition (subject to further qualification) of a worldwide permitted list of colourings is suggested and a request is made for clear thinking in the emotive area of food additive regulations when considering both the need for and the formulation of legislation. Thus this book does not deal exclusively with recent trends. Its indications of areas of likely future activity and descriptions of major past developments in food colouration should make it useful both to those involved in the use and manufacture of food colourings and to those wishing to gain up-to-date general knowledge in this field.

**Toxicological Aspects of Food Safety.** Proceedings of the European Society of Toxicology Meeting held in Copenhagen, June 19–22, 1977. Edited by B. J. Leonard. Archives of Toxicology, Supplement 1. Springer-Verlag, Berlin, 1978. pp. xi + 392. DM 78.00.

From these proceedings emerges a truly heterogeneous collection of nearly 90 contributions extending beyond the confines of food safety (implicit in the Symposium title) to the more distant pastures of clinical pharmacology and drug toxicology. Particularly welcome, however, are the many contributions emanating from the host country and its Scandinavian neighbours. Although the review and research communications, ranging in length from half-page abstracts to 15-page contributions, appear in a random order, there are many to whet the toxicologist's appetite—despite the lack of systematic presentation.

Early on we are treated to a diet of several reviews on the general aspects of food-additive use and toxicology, on pesticides and heavy metals as food contaminants, on toxicokinetics, on problems of evaluating carcinogenic risk (exemplified by the occurrence of nitrosamines in foods, chloroform in drinking-water and vinyl chloride in PVC food packaging), on liver-enzyme induction and its toxicological significance and on regulatory controls.

This then gives way to a 'free-for-all' which at best can be put into several categories roughly identified as the metabolism or toxicology of certain food components (e.g. rapeseed oils), food additives (e.g. Orange RN, tartaric acids) and food contaminants (e.g. PCB, heavy metals) and the pharmacology or toxicology of various drugs (e.g. halothane, the potential anti-inflammatory agent 3-aryl-triene-steroidal-16,17-acetonamide, the tranquillizer lonetil, and the veterinary antibiotic turimycin).

A constantly recurring theme in this maze of presentations is hepatotoxicity, with the full repertoire of cellular and subcellular studies furthering our gradual understanding of the hepatotoxic response of animals and man to a variety of agents. However, no contribution makes a real attempt to correlate the sequential biochemical, histochemical, histological and ultrastructural changes underlying the pathogenesis of liver lesions. Problems encountered in assessing toxicity, such as organ weight differences and the effect of diet and strain on kidney damage, are also discussed, but such important topics as the influence of dietary restriction and hormonal status on tumour development in animals are not considered. Assays for styrene oxidase, vinyl epoxidase and other enzymes and analytical techniques appropriate, for example, for estimating residues of growth promoters in animal tissues add to the variety.

It can be argued that greater benefit could be gained from such important international meetings if the subject coverage were restricted to more specific priority areas rather than to addressing such a wide toxicological spectrum. On the other hand, the many interrelating facets of toxicology may justify the occasional programme that is sufficiently comprehensive to bring a wide range of specialists together and remind them of their mutual dependence.

**Asbestos Killer Dust. A Worker/Community Guide: How to Fight the Hazards of Asbestos and its Substitutes.** A. J. P. Dalton. BSSRS Publications Ltd., London. 1979. pp. 287. £2.25.

This generously and sometimes amusingly illustrated paperback is aimed at the scientific layman and at £2.25 is reasonably priced. It gives a detailed, but readable, account of the long-term hazards associated with asbestos poisoning, the precautions that should be taken and the procedures that should be followed if safeguards appear insufficient. In this respect, the book more than adequately fulfils its purpose, since it provides much practical advice to those who come into contact with asbestos at work or at home.

Its main drawback, however, is that the information is presented in a markedly one-sided manner, which results in the loss of much of the book's impact. Whilst the author appears to have appreciated that there are great difficulties in diagnosing asbestos poisoning and that a disease such as mesothelioma may take several years to become apparent, he does not seem to have accepted that these facts in themselves could have been at least partly responsible for the delay in implementing adequate safeguards. On the contrary, one is left with the impression that this delay was, and is, due entirely to the irresponsible attitude of industrial management and to the apathy of scientists and government representatives alike. While this may be true in part, the unbalanced tone of the text leads the reader to question the authenticity of the details given, especially as the author favours a sensational approach, as exemplified by the frequent repetition of the phrase "the deadly asbestos cancer—Mesothelioma".

Asbestos poisoning is an intolerable hazard and publicity on its far-reaching consequences is vital in order to make people aware of the dangers involved. The objective of this book, namely the improved safety of industrial workers and the general public, should therefore be praised. In a field such as this, however, results are likely to be more readily obtained through the co-operation of everyone concerned, a co-operation which, unfortunately, is not encouraged by this publication.

**Environmental Health Criteria 7. Photochemical Oxidants.** Published under the joint sponsorship of the United Nations Environment Programme and the World Health Organization. WHO, Geneva, 1979. pp. 110. Sw.fr. 10.00 (available in the UK through HMSO).

To our good fortune, the stratosphere is endowed with a protective layer of ozone shielding us from harmful ultraviolet rays from the sun. To our considerable misfortune, however, we are faced with the down-to-earth problem of assessing the potential health hazard of low environmental concentrations of ozone derived both from natural sources and from industrial activities. Photochemical oxidants are formed as a result of sunlight-induced oxidation of precursor pollutants (notably nitrogen oxides and various hydrocarbons) emitted into the atmosphere. Major sources of these oxidant precursors include motor vehicles, space heating, power plants and industrial processes. The principal oxidants are ozone, nitrogen dioxide and peroxyacylnitrates. The major occupational sources of ozone are welding and hydrogen peroxide production but the use of ultraviolet lamps, electrostatic precipitators and photocopying machines may also generate ozone.

Nitrogen dioxide is an important air pollutant, as evidenced by an earlier appraisal of its biological significance in this joint UNEP/WHO series (*Cited in F.C.T.* 1979, **17**, 291). The present volume is devoted mainly to ozone (and oxidants) and represents the deliberations of a WHO Task Group on Environmental Health Criteria for Photochemical Oxidants, who met in Tokyo in September 1976.

The WHO Group acknowledged the fact that photochemical air pollution contained other substances besides ozone, such as nitrogen dioxide, peroxyacylnitrate and possibly many other gaseous and particulate products of atmospheric photochemical reactions. But so little was known about the composition of photochemical pollution, the concentrations of individual components and their possible impact on human health that no attempt could be made to estimate exposure limits for any compound apart from ozone.

The criteria document contains succinct reviews of the chemical and physical properties of ozone and peroxyacylnitrates and methods for their analysis, the natural sources of these oxidants and man-made sources of oxidant precursors, and the environmental atmospheric concentrations and exposures encountered in remote, rural and urban areas (invariably in the developed Western World). The survey of effects in experimental animals is concerned especially with effects on the respiratory system, the principal target for ozone as for nitrogen dioxide. Finally the review of effects in man is concerned mainly with ozone and considers controlled exposure studies *in vivo* and *in vitro*, industrial exposure and community exposure.

In its evaluation of the health risks to man of exposure to these photochemical oxidants, the WHO Group found that the lowest atmospheric levels of ozone associated with adverse effects in man are in the range 0·1–0·25 ppm, a finding compatible with observations in animals. However, the 1-hr exposure limit of 0·05–0·1 ppm for ozone (0·06 ppm for oxidants) proposed by the WHO Group to serve as a guideline for the protection of public health contains no built-in safety factor because of its approximation to the naturally-occurring concentration of ozone (0·005–0·05 ppm even in areas remote from sources of pollution), not to mention the higher levels of 0·15–0·4 ppm in large urban areas. Ozone concentrations in excess of 0·06 ppm are deemed to be related to man-made activities.

The WHO Group expressed some concern over the

possible carcinogenic/mutagenic potential of ozone although no adequate data were available to allow any definitive conclusions to be drawn. It was also noted that most studies of occupational exposure to ozone were difficult to interpret because of co-existing pollutants and that no threshold concentration for ozone toxicity had been determined.

Areas recommended for further investigation include studies of the combined action of ozone with other agents (e.g. nitrogen dioxide, sulphur dioxide or tobacco smoke) on respiratory function in man and animals and study of the carcinogenic/cocarcinogenic/mutagenic potential of ozone and the effects of ozone on humoral and cellular immunity.

**Environmental Health Criteria 8. Sulfur Oxides and Suspended Particulate Matter.** Published under the joint sponsorship of the United Nations Environment Programme and the World Health Organization. WHO, Geneva, 1979. pp. 108. Sw.fr. 10.00 (available in the UK through HMSO).

Number 8 in what is becoming an impressive series from the UNEP/WHO stable provides an excellent review and critical evaluation of the available data on the biological effects of sulphur oxides and suspended particulate matter, including suspended sulphates and sulphuric acid aerosols. It is noteworthy that sulphur oxides and suspended particulate matter are the most widely monitored air pollutants.

As a result of the WHO Task Group meeting in Geneva in 1976, subsequent reappraisal in 1978 and the collaborative efforts and views of various national and international organizations, guidelines for public health protection have been recommended for sulphur dioxide on the basis of short-term exposure (24-hr mean 100–150 $\mu g/m^3$*) and long-term exposure (annual mean 40–60 $\mu g/m^3$). Similar guidelines were issued for smoke but due to insufficient data only interim guidelines were developed for total suspended particulates (60–90 $\mu g/m^3$ for annual arithmetic means and 150–230 $\mu g/m^3$ for 24-hr values). In general these limits reflect a two-fold safety factor in relation to the lowest estimated adverse-effect levels. No guidelines were possible for sulphuric acid or sulphates because of the lack of data.

This environmental health criteria document follows the now familiar pattern of an opening summary and recommendations for further research and action, followed by sections on chemistry and analytical methods, sources of sulphur diodes and particulate matter, dispersion and environmental transformations, environmental concentrations and exposures, absorption, distribution and elimination, effects on experimental animals, effects on man, and finally an evaluation of health risks to man. The Group noted that on a global scale, sulphur emissions derived from natural sources and man-made activities (mainly combustion of fossil fuels) are of the same order, although in large urban areas the latter's contribution predominates. Levels in the workplace can be 100 times greater than average urban levels.

Studies in animals and man have concentrated attention on the respiratory tract, the primary target. Studies in various species of experimental animals have used exposure levels up to 835 ppm sulphur dioxide for 1 hr and 0·53–7 ppm for up to 20 months. In man, controlled exposure studies have been limited to below 24-hr duration at levels up to 25 ppm. Epidemiological studies not surprisingly have run into the inevitable difficulty of separating the effects of sulphur dioxide, sulphuric acid mists, sulphate salts and particulate matter in ambient air. Curiously the criteria document, in reviewing animal data, relates short-term exposure to exposure up to 24 hr (usually a few minutes to a few hours) and long-term exposure to exposures greater than 24 hr but usually of several months duration. Considerable differences in response were encountered in animal studies which make assessment and extrapolation to man difficult.

The WHO Group did not undertake a thorough evaluation of any possible associations between lung cancer and exposure to these air pollutants and recommended that it be the subject of a separate evaluation. However, reference is made to a conclusion drawn at an International Symposium on Air Pollution and Cancer held in Stockholm in 1978 that "Combustion products of fossil fuels in ambient air, probably acting together with cigarette smoke, have been responsible for cases of lung cancer in large urban areas, the numbers produced being of the order of 5–10 cases per 100.000 males per year".

Before our thoughts turn to numbers 9–11 in this series, dealing with DDT, carbon disulphide and mycotoxins respectively, it should not be overlooked that critical evaluation of two well-known naturally-occurring environmental contaminants (ozone and sulphur dioxide) has afforded relatively low safety factors in terms of public health protection, far removed from the considerable safety margins demanded of food additives and other agents. The need remains to maintain perspectives in toxicology as in other spheres!

**Biological/Biomedical Applications of Liquid Chromatography II.** Edited by G. L. Hawk. Marcel Dekker, Inc., New York, 1979. pp. xiii + 504. Sw.fr. 100.00.

The publication of 24 of the papers presented at the second "Liquid Chromatography Symposium: Biological/Biomedical Applications of LC" held in Boston, Massachusetts in October 1978 represents a significant addition to the literature on this rapidly developing subject.

By careful selection of papers and the inclusion of a comprehensive subject index (a rarity in publications of this kind) the editor has succeeded in conveying the core of an obviously fascinating conference into a readable and useful reference book. Not only is the breadth of subject likely to ensure a wide readership among scientists interested in particular analyses but also the developmental details and comments found in many of the papers are likely to prove useful in the elaboration of a much wider range of methods. Quantitation of prostaglandins in human semen may not, for example, immediately enliven the interest of all

*1 ppm $SO_2$ = 2856 $\mu g/m^3$

biologically orientated high-pressure liquid chromatography (HPLC) users but few will fail to gain from the discussion of sample preparation in this unusual material since sample preparation is so often the central problem in biological applications of HPLC.

Papers concentrating solely on the development of methods are few but the inclusion of Rolf Schwarzenbach's discussion of the problems of carbohydrate analysis was wise. Most of the authors have a particular biological assay in view and details of method development are very much secondary to the use and application of a given analysis. Thus the strengths of the volume are numerous. Its weaknesses are those involved in publishing the proceedings of most symposia, namely a somewhat arbitrary subject range and some heterogeneity of style and standard.

Nevertheless this is a useful book which will be well received.

**Diagnostic Electron Microscopy of Tumours.** By F. N. Ghadially. Butterworth & Co., London, 1980. pp. ix + 251. £32.00.

This book, together with Professor Ghadially's earlier work "Ultrastructural Pathology of the Cell", published in 1975, goes a considerable way towards establishing the art of electron microscopy in histopathology, both as an adjunct to light microscopy and as a diagnostic tool in its own right. In common with the earlier volume, the present text is directed primarily to those concerned with human pathology, but although this may seem to detract from its usefulness in animal pathology, the principles outlined apply equally to both disciplines.

The book has an interesting format in which most of the chapters deal with specific problems in the context of their solution by electron microscopy. The first two chapters, however, are concerned with tissue processing and the choosing of a suitable electron microscope. Although methods are treated fairly superficially, adequate references together with a discussion of the pitfalls accompanying tissue preparation justify their inclusion. The next two chapters (part 2 of the book) provide an assessment of the ultrastructural parameters by which a neoplastic tissue can be distinguished from a normal tissue. The third section deals with specific problems of pathological diagnosis in which the electron microscope has proved of importance. Here, the chapters on the differential diagnosis of leukaemias and of schwannoma from fibrosarcoma are outstanding. The final section describes the ultrastructural features of some known tumours, including the clear cell tumour of the lung and acinar cell tumours of the pancreas and salivary glands.

The book is well illustrated with micrographs of the majority of tumours mentioned, and each chapter is adequately supplied with references. Based on Professor Ghadially's wealth of experience in the ultrastructural diagnosis of tissue pathology, it provides a valuable insight into the rational use and interpretation of electron micrographs in a field where, all too often, the electron microscope is regarded as an expensive white elephant.

**Hartley's Microscopy.** By W. G. Hartley. Senecio Publishing Co. Ltd., Oxford, 1979. pp. x + 220. £9.75.

The author of this book admits in his foreword that the revision of the original text (published in 1962) has presented considerable difficulty. In view of the progress made in microscope design and manufacture during the intervening 17 years it might have been better to write a new book. However, a revision is what we have, complete with outdated references to "the ordinary man... from the leather soles of his shoes to the crown of his felt hat" and "the ordinary photographic plate".

The early chapters of the book deal with the microscope in its basic form, light theory and illumination. The later ones are devoted to specialized techniques such as phase contrast, interference and polarizing microscopy, photomicrography and fluorescence and quantitative microscopy. An index and a bibliography conclude the book. The index should, presumably, provide an alphabetical listing of all the words in bold type within the text. The many omissions noted indicate that insufficient care has been taken in the preparation of the index, and consequently its usefulness is limited.

The conventional layout of the book's contents is not reflected in the layout of the text within each chapter. For example, advice to the complete beginner on how to use a microscope for the first time is sandwiched between the ruling that total magnification should never exceed 1000 × numerical aperture of the objective and a discussion of the diminuation [*sic*] of focal depth by an increase in numerical aperture. Further advice to the beginner follows, with a consideration of the "high power objective" which turns out to be an oil immersion lens.

The paragraphs purporting to distinguish between resolving power and resolution are very confusing. Neither resolving power nor magnifying power is clearly defined and it is not difficult to imagine the student rapidly losing interest in a subject presented in this manner. That this should occur on p. 5 does not augur well for his chances of completing the book.

Certain omissions in what is meant to be an instructional text are hard to overlook. In the chapter concerned with photomicrography, a whole paragraph is taken up with a description of the best way to demonstrate the hexagonal lattice of *Pleurosigma angulatum* (a diatom), whilst reciprocity failure, the single cause of the majority of photomicrographic failures, is not even defined, let alone discussed.

One might have anticipated that the newly added chapter on quantitative microscopy would offer some consolation for the confused and incomplete text of the revised chapters. The statement in the author's foreword that it is "probable that quantitative microscopy will become the prime activity before long" suggests a worthwhile coverage of the subject, but after mentioning a computer in the second paragraph, the author writes at great length about eyepiece and stage micrometers. A point counter is illustrated but not mentioned in the text, whilst a flying spot system is mentioned but not further described. Hardly anything

of substance is presented here to justify the comments in the foreword.

The book does contain a great deal of useful information, and some excellent advice. Unfortunately, the gems are somewhat hidden and the index is insufficiently comprehensive to facilitate their complete retrieval. At a price of £9.75, this paperback offers questionable value. It cannot be recommended for student use but parts may be of interest to more experienced microscopists.

**Banbury Report. 1. Assessing Chemical Mutagens: The Risk to Humans.** Edited by V. K. McElheny & S. Abrahamson. Cold Spring Harbor Laboratory, New York, 1979. pp. xiii + 367. $38.00.

**Banbury Report. 2. Mammalian Cell Mutagenesis: The Muturation of Test Systems.** Edited by A. W. Hsie, J. P. O'Neill & V. K. McElheny. Cold Spring Harbor Laboratory, New York, 1979. pp. xiv + 504. $45.00.

The field of genetic toxicology has expanded enormously in recent years. The number of scientific meetings convened to discuss the problems of detecting genetically active chemicals and of estimating the risks to man posed by such chemicals has increased accordingly. In such a rapidly developing field it is not surprising that many of the published proceedings of these conferences contain a rather disorganized mass of information, much of which rapidly becomes outdated. Against this background the appearance of a new series of reports dealing with this topic might not be viewed with enthusiasm. However, the first two Banbury Reports, published by the Cold Spring Harbor Laboratory, have a number of features indicating that this series may make a substantial contribution to genetic toxicology. Each of the reports contains the proceedings of a conference dealing with a specific topic and attended by a small group of scientists, all eminent in the field (although almost exclusively from the USA).

The first Banbury Report, on assessing the risk to humans from chemical mutagens, deals with the most controversial aspects of genetic toxicology. The meeting, held in May 1978, was sponsored by the EPA's Office of Toxic Substances. The formal presentations cover topics such as the current genetic disease burden in man (J. V. Neel), the usefulness of short-term test systems for mutagenicity (W. G. Flamm, E. Eisenstadt, G. C. Walker, R. Setlow), and the quantitative aspects of radiation- and chemical-induced mutagenesis (J. G. Brewen, J. W. Baum, D. Hoel, S. Abrahamson, L. Ehrenberg, W. R. Lee). The most interesting feature of the book, however, is the extraordinary amount of discussion which is reported. Apart from questions interrupting or following the formal papers, there is a general discussion running to 100 pages. A wide range of problems was dealt with in this discussion and heavy emphasis was put on the problems of risk assessment. Methods of estimating chemical-induced mutation frequencies, extrapolation from acute high-dose exposure to chronic low-dose exposure and the relevance of results from model systems are consistent themes.

The detailed report of scientific debate is welcome particularly on such an important and knotty problem as genetic risk assessment. However, for the layman, the discussion is likely to prove very heavy going. It is doubtful, therefore, whether this publication will fulfil the aim, stated by J. D. Watson in the preface, of "providing factual data which should help the public make rational responses to the dangers that these agents [mutagens] may or may not present". In general, the book is well produced and sticks admirably to its central theme.

The second Banbury Report, *Mammalian Cell Mutagenesis: The Maturation of Test Systems*, records the proceedings of a second symposium, sponsored by the NCI, held in May 1979. Although similar in format to the first in the series, this report deals with a quite different subject, concentrating on methods of detecting mutagens by means of mammalian-cell systems. Three cell mutation tests, using respectively CHO, V79 and L5178Y cells are picked out as front-runners in the race to provide a reliable mutagenicity screening system. Papers dealing with the genetic basis, the detailed methodology and the practical applications of each of these techniques are spread throughout the whole book. Supporting these presentations are papers on cell mutation in general, including excellent reviews by T. T. Puck and L. Siminovitch, detailed analyses of the biochemical aspects of specific mutations and descriptions of some test systems involving cultured human cells. The contributions are very well organized and seem to move progressively towards the rational choice of a well characterized test system for use in routine screening of chemicals for mutagenicity. The failure to reach a final conclusion is not at all surprising, even if it is perhaps a little disappointing.

The papers are backed up by a good deal of useful discussion, although there are some puzzling and annoying gaps, such as after an interesting paper by P. O. P. T'so on neoplastic transformation. Overall, the book contains a wealth of information and is thoroughly recommended to all those working in cell genetics. Some of the presentations will be of interest to anyone with even a passing interest in cell genetics and mutation. However, the majority of the book is undeniably for the specialist.

**Handling Chemical Carcinogens in the Laboratory: Problems of Safety.** IARC Scientific Publications no. 33. Edited by R. Montesano, H. Bartsch, E. Boyland, G. Della Porta, L. Fishbein, R. A. Griesemer, A. B. Swan & L. Tomatis. International Agency for Research on Cancer, Lyon, 1979. pp. vii + 32. Sw.fr. 12.00 (available in the UK through HMSO).

Increasing concern about the safety of chemicals has led to the proliferation of laboratories involved in investigating their properties and a concomitant rise in the number of people exposed to such chemicals. The general lack of information about the handling of hazardous materials may mean that some workers do not fully appreciate the potential risks involved, particularly that of cancer. This booklet, aimed primarily at laboratories which handle a variety of carcinogens in small quantities, has been prepared in an attempt to overcome some of these deficiencies and to aid those who wish to implement more stringent safety measures.

The general introduction is followed by an outline of procedures that are needed to avoid the exposure of workers and the contamination of laboratories. The specific areas that are considered include staff training and supervision, the personal hygiene and protective clothing of those handling carcinogens, the efficiency of methods and of equipment such as fume cupboards, contamination monitoring, the protection of cleaners and maintenance staff, and emergency procedures. The chapter on storage, dispensing and disposal of carcinogenic material is helpful and the section giving guidance on the thorny problem of disposal is particularly valuable. Chemical methods of destruction of several groups of carcinogens are outlined and some very useful references are included. The final chapter outlines the individual responsibilities of the scientific investigator and others involved in a programme that includes the handling of carcinogens.

The authors have included a list of areas that urgently require additional investigations. Methods of monitoring exposure and contamination and of decontamination, destruction and disposal are all on the list, thus highlighting the potential inadequacies of many of the methods and procedures that are currently available.

The authors have made it clear that this booklet is not a substitute for a detailed safety code of practice which should be tailored to meet the needs of individual laboratories. It is of value however as a guide in the compilation of such a document and as a source of reference to more detailed literature.

## BOOKS RECEIVED FOR REVIEW

**Meyler's Side Effects of Drugs. An Encyclopaedia of Adverse Reactions and Interactions.** 9th Ed. Edited by M. N. G. Dukes. Excerpta Medica, Amsterdam, 1980. pp. xx + 857. Dfl. 250.00.

**Toxic Metals and their Analysis.** By E. Berman. Heyden & Son Ltd., London, 1980. pp. ix + 293. £12.00.

**Environmental Carcinogens—Selected Methods of Analysis.** Vol. 3. **Analysis of Polycyclic Aromatic Hydrocarbons in Environmental Samples.** Edited by M. Castegnaro, P. Bogovski, H. Kunte & E. A. Walker. IARC Publications No. 29. International Agency for Research on Cancer, Lyon, 1979. pp. ix + 240. Sw.fr. 50.00 (available in the UK from HMSO).

**Pathology of Tumours in Laboratory Animals.** Vol. II. **Tumours of the Mouse.** Edited by V. S. Turosov. IARC Scientific Publications No. 23. International Agency for Research on Cancer, Lyon, 1979. pp. xii + 669. Sw.fr. 100.00 (available in the UK through HMSO).

**Handbook of the Toxicology of Metals.** Edited by L. Friberg, G. F. Nordberg & V. B. Vouk. Elsevier/North-Holland Biomedical Press, Amsterdam, 1979. pp. xxxix + 709. Dfl. 240.00.

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# *Information Section*

## ARTICLES OF GENERAL INTEREST

### DIOXIN: TERATOGENICITY AND REPRODUCTIVE EFFECTS

The question of the teratogenic and reproductive effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (dioxin) in man is highly controversial. In cases such as the study in Oregon (*Cited in F.C.T* 1980, **18,** 541), where exposure to 2,4,5-T contaminated with dioxin is alleged to be at the root of an increased abortion rate, it is extremely difficult to gauge the likely exposure levels of individuals or groups and the importance of other environmental factors. Reports from the Seveso region do not seem to indicate the conclusive increases in either spontaneous abortions or birth defects that might have been expected (*ibid* 1980, **18,** 542). Results from animal studies, however, are very much more clear and indicate that the possible dangers of dioxin in this respect cannot be treated lightly.

In a recent three-generation reproduction study in Sprague–Dawley rats (Murray *et al. Toxic. appl. Pharmac.* 1979, **50,** 241), dioxin administered in the diet was shown to disrupt the breeding performance of the F1 and F2 animals at daily doses as low as 0·01 μg/kg body weight. Fertility was significantly decreased in these generations, as were litter size at birth, gestation survival (proportion of pups born alive) and postnatal body weights. Postnatal survival (to day 21 after birth) was significantly decreased in the F1 and F2 but not in the F3 animals. Decreases in fertility and in postnatal survival also occurred in the F0 animals—treated with dioxin for 90 days prior to mating—but the lowered fertility was found only at the top dose of 0·1 μg/kg; there were so few offspring that this treatment was discontinued in subsequent generations. Among rats receiving the lowest dose of 0·001 μg/kg there were no effects on fertility, litter size at birth or postnatal body-weight gain in any of the three generations, and although there were statistically significant effects on gestation survival and postnatal survival these did not occur consistently.

Significant increases in the average length of time from cohabitation to parturition were seen in the F1 and F2 rats, but not in the F0 generation, at the 0·01-μg dioxin/kg/day level. This may indicate some interference with the oestrous cycle. Kociba *et al.* (*ibid* 1976, **35,** 553) reported morphological alterations in the ovaries and uterus that suggested suppression of the oestrous cycle in rats given 1 μg dioxin/kg/day for 90 days. Such alterations were clearly evident in the results of Barsotti *et al.* (*Cited in F.C.T.* 1980, **18,** 549). Female monkeys given a total of 3 μg dioxin/kg body weight in the diet over 9 months did not show any alterations in their menstrual cycles, during their first 6 months of treatment. However, after 6 months, anovulatory patterns for both progesterone and oestradiol were seen in two animals, reductions in the levels of the two steroids to about half of their pretreatment values were seen in two other animals and a considerable decrease in progesterone but not oestradiol was seen in a fifth.

Dose-related foetal abnormalities were not seen in the study by Murray *et al.* (*loc. cit.*) and it may be that in some species such abnormalities are associated with dose-levels of dioxin appreciably higher than those that affect general reproductive efficiency. When given daily by gavage on days 6–15 of gestation, dioxin produced a significantly increased incidence of cleft palate at 1 and 3 μg/kg (the highest dose level tested) and dilated renal pelvis at 3 μg/kg in the offspring of CF-1 mice (Smith *et al. Toxic. appl. Pharmac.* 1976, **38,** 517). The percentage of resorptions was significantly different from that in the control animals only at the 1-μg/kg dose level and there were no other significant differences in the reproductive parameters. Dioxin increased foetal mortality and early and late resorptions and produced foetal intestinal haemorrhage and subcutaneous oedema at doses of 0·125 μg/kg and above in the offspring of Sprague–Dawley rats treated by gavage on days 6–15 of gestation (Sparschu *et al. Fd Cosmet. Toxicol.* 1971, **9,** 405). In the mouse study, there was no evidence of teratogenicity at oral doses of 0·1 μg/kg/day and below, while in the rat no adverse effects on the foetus were observed at 0·03 μg/kg/day (the only dose level below 0·125 μg/kg evaluated).

Subcutaneous administration of dioxin, again on days 6–15 of gestation, has given similar results; 3 μg/kg/day resulted in cleft palate and kidney abnormalities in CD-1, DBA/2J and C57B1/6J mice (*Cited in F.C.T.* 1972, **10,** 722). Daily doses of 1 μg/kg were similarly tested in the CD-1 strain and caused the same malformations. In the same study, kidney abnormalities but no cleft palates were found in the offspring of CD rats given 0·5 μg/kg/day sc on days 6–15 of gestation.

Zingeser (*Teratology* 1979, **19,** 54A) administered dioxin in corn oil by gavage to four female monkeys in nine doses between days 20 and 40 of gestation. Three of the animals received a total dose of 0·2 μg/kg and one was given a total of 1 μg/kg. All four foetuses showed abnormalities of the soft palate, the most severe damage being seen in the high-dose foetus.

Only one of the seven treated monkeys in the study by Barsotti *et al.* (*Cited in F.C.T.* 1980, **18,** 549). carried her infant to term. The animal gave birth to a well-developed infant, which remained healthy during the 4 months of nursing with respect to gross appearance and haematological and immunological parameters. Two other animals conceived, but both aborted early in pregnancy.

An abstract by Moore *et al.* (*Toxic appl. Pharmac.* 1976, **37,** 146) outlines studies of the transfer of dioxin from pregnant Fischer 344 rats to their foetuses. Dioxin labelled with $^{14}C$ was given to each dam in a single oral dose of 5 μg/kg. The tissues of the progeny were examined for radioactivity on day 14, 18 or 21 of gestation or day 3, 7, 10 or 14 *post partum.* Cross-fostering techniques were used to distinguish dioxin transferred in the milk. At all the sampling times, low concentrations of dioxin were found in the foetuses. Preferential concentration of dioxin in the foetal liver was first seen on gestation day 21. Postnatally, high concentrations of dioxin were found in all the young, indicating continuous mobilization of dioxin from maternal tissues and secretion in the milk. The relatively high concentrations in the neonate compared with the maternal dose could account for increased neonatal sensitivity such as that seen, for example, in the three-generation study by Murray *et al.* (*loc. cit.*).

Dioxin is clearly a potent teratogen in animals but the recent work suggesting that it may also have serious reproductive effects at extremely low doses is perhaps even more disconcerting.

## NICKEL AND NASAL CANCER

The incidence of cancer affecting the lungs, larynx and nasal cavity in workers exposed to nickel (Ni) is high (*Cited in F.C.T.* 1974, **12,** 428). It has been shown that exposure to Ni reduces ciliary activity and leads to nasal epithelial damage in hamsters (*ibid* 1980, **18,** 103). Changes in the histology of the nasal epithelium were observed in a preliminary study on Ni-exposed workers (*ibid* 1977, **15,** 362) and the same group of investigators has now published a number of papers covering various studies on workers at a Norwegian Ni refinery.

To determine the distribution of Ni between the tissue components of the nasal mucosa several histochemical techniques can be used, but most lack specificity. Torjussen *et al.* (*Acta otalar.* 1978, **86,** 449) considered that the sulphide-silver staining method was probably the most sensitive although it could not distinguish between different heavy metals. These authors compared the concentrations of various metals in the nasal mucosa of Ni-exposed and unexposed individuals with the sulphide-silver staining pattern in order to determine whether variations in the metal concentrations and particularly in that of Ni would affect the staining pattern. The levels of a number of metals (Ni, Cu, Co, Zn and Fe) in biopsy specimens of the nasal mucosa of 30 Ni workers and six controls were determined by atomic absorption spectrophotometry. The sulphide-silver staining method was also used on microscopic sections of the tissue. The Ni concentration in the mucosal samples ranged from 15 to 2250 μg Ni/100 g wet wt in exposed workers and from 6 to 45 μg Ni/100 g wet wt in controls. Mean values expressed in μg Ni/100 g wet wt were 270 in electrolysis workers, 630 in roasters and smelters, 124 in other refinery workers, and 21 in controls. The higher mean Ni concentration among the Ni-exposed groups than among the controls was not statistically significant. However the difference between the mean Ni concentrations of roasters and smelters and those of other workers was significant. Sulphide-silver staining showed relatively dense metal deposits in basal cells of pseudostratified or stratified columnar epithelium, and more moderate deposits at more superficial levels. There were variations in the staining of stratified cuboidal epithelium, and stratified squamous epithelium (not found in the controls) was largely unstained. Cancerous epithelium from two nasal tumours was virtually devoid of staining. There was no material difference between the staining characteristics of corresponding types of epithelium from exposed workers and controls. It was concluded that abnormal Ni deposits could not consistently be revealed by this staining method.

Further studies (Torjussen & Andersen, *Annls clin. Lab. Sci.* 1979, **9,** 289) were directed to determining the influence of occupational Ni exposure on the concentration of Ni in the nasal mucosa, plasma and urine of 318 refinery workers, 15 retired workers and 57 unexposed controls. In the control group, there was no correlation between Ni levels in the plasma and those in either the urine or the nasal mucosa. However in both the active workers and the retired group there was a significant correlation between plasma Ni levels and urinary Ni levels and in the retired workers, both plasma and urinary values were also significantly correlated with the values for the nasal mucosae. In both of these exposed groups Ni concentrations in mucosa, plasma and urine were elevated. Although the means of all three measurements were greater in the active group than in the retired group, this was significant only in the case of the plasma. Workers exposed to nickel subsulphide and oxide dusts in the roasting and smelting areas (where atmospheric nickel levels were highest) showed the highest mean mucosal concentration (467·2 μg Ni/100 g wet tissue). Those exposed to less concentrated aerosols of nickel chloride and sulphate (electrolysis workers) had the highest mean Ni concentrations in the plasma and urine—8·1 and 73·3 μg Ni/litre, respectively. It therefore seemed that the nasal mucosa might provide the best measure of exposure of the upper respiratory tract to Ni. This is in agreement with the conclusions of previous workers (*Cited in F.C.T.* 1979, **17,** 311) who found that urinary Ni levels were high in Ni workers exposed to aerosols of soluble Ni salts but that this elevation was much less evident in other groups of workers exposed to atmospheric Ni. Tola *et al.* (*J. occup. Med.* 1979, **21,** 184) studied urinary and plasma Ni concentrations in four workers from an electroplating shop and concluded that these indicators could be used to assess exposure to soluble Ni compounds.

Torjussen & Andersen (*loc. cit.*) also found that Ni concentrations in mucosa, plasma and urine correlated with the duration of exposure. There was a significant negative correlation between mucosal Ni and

length of retirement. Deposits of Ni in the nasal mucosa were released with an estimated half-life of 3·5 yr.

In a preliminary histopathological study of a part of the workforce of the same factory, Torjussen *et al.* (*Cancer, N.Y.* 1979, **44,** 963) found carcinoma and epithelial dysplasia exclusively in roasting, smelting and electrolysis workers with at least 10 yr employment. As a result, a more extensive survey was carried out (Torjussen *et al. Br. J. Cancer* 1979, **40,** 568) on the same 318 refinery workers, 15 retired refinery workers and 57 controls as were included in the Ni-analysis study (Torjussen & Andersen, *loc. cit.*). Biopsy specimens were taken from the anterior curvature of the middle nasal turbinate. The surface epithelia were given a score from zero to seven according to their characteristics, a score of six corresponding to epithelial dysplasia, and one of seven indicating carcinoma or carcinoma *in situ*. Stratified cuboidal epithelium was the most common type both in controls and in active Ni workers, whereas stratified squamous epithelium was most frequent in retired nickel workers. Two subjects from the roasting and smelting department, who had each worked at the refinery for 28 yr, had nasal carcinomas. One of them had an anaplastic carcinoma and the other a squamous-cell carcinoma; in this latter individual an adjacent carcinoma *in situ* was detected. Epithelial dysplasia was found in 12% of active and 47% of retired exposed workers but only in one of 57 controls. The average histological score was significantly lower among controls than among all the exposed groups and significantly higher in the retired workers than in the active group. There was a significant correlation between epithelial dysplasia and age and between average histological score and age. Histological score was also significantly correlated with time since the first Ni exposure, but epithelial dysplasia was not. However only two out of 38 Ni workers with epithelial dysplasia had been working at the plant for less than 10 yr. When the three different job categories (work in the smelting/roasting, electrolysis or non-process areas) were considered, there were no significant differences in the frequency of epithelial dysplasia. Average histological scores were highest for workers in the roasting/smelting areas. There was a statistically significant correlation between histological score and work category for roasting/smelting and electrolysis but not for non-process work.

The percentage of smokers was nearly equal among active Ni workers and controls but lower for retired Ni workers. There was no correlation between tobacco smoking and epithelial dysplasia, but the correlation between histological score and tobacco consumption was statistically significant. No significant correlation was found between epithelial dysplasia and Ni concentrations in nasal mucosa, plasma or urine.

These investigators did not consider that the epithelial dysplasia seen indicated a clearly premalignant state. Their results did suggest, however, that some of these changes may well represent early premalignant lesions. Epithelial dysplasia seemed to be clearly connected with Ni processing.

The authors suggest that Ni exposure should be kept to a minimum, and that regular health controls should include X-ray examinations to detect pulmonary cancer, Ni measurements in plasma and urine and examination of nasal biopsy specimens or cytological smears. Subjects with nasal epithelial dysplasia should be transferred to work involving minimal Ni exposure and should be followed up regularly so that any malignant disease of the respiratory tract can be detected at an early stage.

[P. Cooper—BIBRA]

## RELEVANCE OF IMMUNOLOGY TO TOXICOLOGY

Whilst hypersensitivity and other allergic responses to a number of drugs and food additives have been known for some years, it is only recently that various chemicals have been shown to affect the immune response. As the immune mechanism has evolved to enable the host to survive in an environment that includes not only opportunist pathogens such as bacteria and viruses but also foreign particles such as industrial pollutants and environmental chemicals, any agent that adversely affects the immune response can seriously alter the quality of life. It is for this reason that the discipline of immunology is playing an increasingly important role in the safety assessment of drugs, chemicals and food additives.

The immune response, or immunocompetence, is the capacity to recognize, isolate and reject foreign agents and also to maintain homeostatic control. This aspect of immunology includes the removal of worn-out 'self'-components and the recognition of 'self', as well as the recognition and disposal of mutant cells, and is generally termed immuno-surveillance. The two main cell populations involved in maintaining immunocompetence are the mononuclear phagocytes (which include monocytes, macrophages, histiocytes and Kupffer cells) and the lymphoid cells, the T and B lymphocytes.

The immune response functions in a number of ways to protect the host. The initial response is often non-specific—the foreign agent or antigen is ingested by macrophages or other phagocytic cells and an inflammatory response ensues. Once the irritant is removed or sequestered the lesion subsides. More specific responses are the humoral response, which is the synthesis and release of free antibody into the blood and other body fluids, and the cellular, or cell-mediated, responses. Cellular immunity includes responses mediated by mononuclear phagocytes, which are the first to recognize the antigen in the host, and by sensitized (immunologically committed) T lymphocytes. This cell population proliferates, thus increasing the pool of 'memory' cells, that is cells receptive to that particular antigen. Other cells produce soluble

factors called lymphokines, or directly attack certain target cells and destroy them, or interact with B lymphocytes to bring about the production of antibody.

These cell populations all interact with each other, as well as with other populations such as neutrophils and mast cells, to amplify and diversify the host defences. If the macrophages and lymphocytes are immunocompetent, the cells will also react in a more effective manner to sequential exposures to a particular antigen, because there will be a store of memory cells after the initial encounter. The manifestations of these responses may be localized or systemic, acute or chronic, reversible or irreversible, and among the factors influencing the immune response will be the antigen, the individual immunocompetent cell, the cell populations involved and the state of the host at the time of the encounter.

Thus it is evident that a substance causing damage to the cells involved in the immune response or interfering with their function could lead to a state of suppression. We agree, therefore, that whenever toxicological studies give reason to suppose that a material affects the immune response (such as by evoking changes in the lymphoid organs), appropriate tests should be carried out. These investigations should ideally evaluate the potential risk of the substance to the immune system as well as enable us to gain insight into the mode of action of the chemical.

In the field of toxicology, recent investigations have pinpointed several areas of particular relevance. First, a number of reports have shown that some industrial chemicals as well as some naturally occurring compounds have a selective effect on lymphoid organs in experimental animals. Secondly, a better understanding of how the local immune systems of the intestinal epithelial surfaces and the respiratory tract act as a first line of defence has demonstrated that immunological factors are associated with both pulmonary disease and gastro-intestinal disorders.

Of particular interest in connection with the effect of some compounds on lymphoid organs is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). TCDD was first reported to cause thymic atrophy in 1972 (Bau-Hoï *et al. Naturwissenschaften* 1972, **4,** 174) and later investigations confirmed TCDD's ability to cause thymic atrophy in all the mammalian species that were studied (Vos, *Crit. Rev. Toxicol.* 1977, **5,** 67; Faith *et al.* in *Reviews in Biochemical Toxicology*, Vol. 2, edited by E. Hodgson, J. R. Bend & R. M. Philpot, Elsevier, Amsterdam, 1980). Some of these studies also demonstrated impairment of certain T-cell-mediated functions in rats and mice, such as a depression of the response of splenic lymphocytes to mitogen stimulation and a delayed ability to reject allografts. Of particular interest is the finding that rat and mouse pups exposed to TCDD during the perinatal period as a result of sub-toxic maternal treatment showed only minor liver pathology but severe effects in the thymus. As only certain T-cell-mediated functions were depressed in TCDD-exposed offspring, it has been suggested that the maturation of specific T-lymphocyte subpopulations may have been impaired (Thomas & Hinsdill, *Drug Chem. Toxicol.* 1979, **2,** 77).

Since 1976, several publications have described an adverse effect elicited in the rat thymus by organotin compounds, particularly dibutyltin dichloride (DBTC) and dioctyltin dichloride (DOTC). Seinen & Willems (*Toxic. appl. Pharmac.* 1976, **35,** 63) demonstrated that rats fed DOTC at a dietary level of 50 or 150 ppm for 4 wk exhibited severe reductions in thymus weight. Later the same group (Seinen *et al. Immunopharmacology* 1979, **1,** 343) reported the suppression of various parameters of cell-mediated immunity. Seinen *et al.* (*Toxic. appl. Pharmac.* 1977, **42,** 197) pointed out that the severe reduction in thymus weight was followed by a fast recovery when the animals were returned to the stock diet. The major pathological finding in these studies was a marked depletion in the cells of the thymus combined with little evidence of cell destruction (Seinen & Willems, *loc. cit.*). This effect was also observed in an earlier (unpublished) study carried out at BIBRA on groups of rats fed various dose levels of DOTC for 90 days. There was a difference between these two studies, however, in that Seinen and his co-workers did not demonstrate any pathological effect of the compound on peripheral blood lymphocytes *in vivo*, whereas in the BIBRA study a reduction in circulating lymphocytes and a possible effect on erythrocyte precursor cells was found. It is possible, therefore, that the compound has an effect not only on the maturation of thymocytes but also on circulating cells, as well as on the stem-cell population.

These investigations are particularly relevant because naturally occurring substances such as theobromine also affect the thymus; a decrease in thymic weight precedes any other organ lesions, suggesting that the immune system may be the most sensitive indicator of toxic effects. It is not known at present whether the pathways by which two such different substances as an octyltin derivative and a methylxanthine exert their effect on this lymphoid organ are the same, or whether different mechanisms are involved.

An important physiological route for the entry of antigens and foreign particles into an organism is through the gastro-intestinal tract. Available evidence suggests that although the amount of macromolecular material persorbed across the gut epithelial barrier into the lamina propria is relatively small and therefore unlikely to constitut an acute toxic hazard, the entry of a potentially allergenic or antigenic compound may result in local or systemic tissue damage due to the activation of various immunological mechanisms (Walker & Isselbacher, *Gastroenterology* 1974, **67,** 531). Indeed in man, macromolecular absorption has been implicated in the pathogenesis of a number of immunologically-related disease states, including gastro-intestinal allergy, inflammatory bowel disease and coeliac disease.

Absorbed particulates are sequestered by Peyer's patch macrophages or sometimes by macrophages that migrate from the lamina propria into the intestinal epithelium ('intestinal macrophages'). Whilst the effects of particle-laden macrophages on the proliferating cells in Peyer's patches are not known, these macrophages are considered to be in a state of activation (LeFevre *et al. J. Reticuloendothel. Soc.* 1979, **26,** 553). Activated macrophages have the potential to influence surrounding cells and tissues by the release of chemical mediators, leading to inflammatory and/or immune responses. Activated macrophages

can also give rise to sensitized lymphocytes, leading to the induction of cell-mediated reactions.

It is apparent, therefore, that the immunological aspects of persorption may be of significance in the assessment of poorly degradable macromolecular substances, such as carboxymethylcellulose and guar gums, used in food.

As for the other main route of entry into the body, the immunological functions of the respiratory tract have already been associated with exposure to a number of industrial pollutants, mineral dusts such as asbestos and organic materials such as cotton dust. This is not surprising, since not only is lymphoid tissue present throughout the tract, but the alveolar macrophage plays a major role in both non-specific and specific immune responses (Kaltreider, *Am. Rev. resp. Dis.* 1976, **113**, 347). The alveolar macrophage is not only the resident phagocyte of the alveolar membrane and a participant in the immunological responses of the lung but is itself affected by injury or disruption of the normal lung architecture (Green *et al. ibid* 1977, **115**, 479). Surfactant has also been shown to modulate macrophage activity and thus an alteration in the constituents or amount of surfactant could impair several of the complex functions of the alveolar macrophage, including those initiating the pulmonary immune response. It is relevant, therefore, to determine whether inhaled particles and vapours affect the lung in such a manner as to damage or alter macrophage functions. Again the postulate is that the immune system may be the most sensitive indicator of toxic effects and that changes in immunological competence occur prior to clinical or pathological changes.

Finally it must be emphasized that in toxicology testing it is particularly important to discriminate between direct effects on the immune system and indirect effects due to impaired absorption or utilization of some nutrients, to changes in endocrine balance or to the influence of pathogenic organisms. Altered or impaired immune responses represent a hazard that should should not be underestimated in the evaluation of the toxicity of drugs, chemicals and other environmental substances.

[K. Miller—BIBRA]

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# ABSTRACTS AND COMMENTS

## FOOD ADDITIVES

### Metabolism of aspartate and aspartame

Oppermann, J. A. & Ranney, R. E. (1979). The metabolism of aspartate in infant and adult mice. *J. envir. Path. Toxicol.* **2**, 987.

Ranney, R. E. & Oppermann, J. A. (1979). A review of the metabolism of the aspartyl moiety of aspartame in experimental animals and man. *J. envir. Path. Toxicol.* **2**, 979.

The first of these papers describes a pharmacokinetic study of the absorption and metabolism of aspartic acid after the oral or intraperitoneal (ip) administration of 0, 10, 100 or 1000 mg L-aspartate/kg body weight to 15-day-old and adult mice. It was found that massive oral doses of L-aspartate led to higher plasma concentrations in the young mice than did equivalent doses in adults, and it is suggested that this might explain the increased susceptibility of infant mice to the hypothalamic damage produced by large oral doses of aspartate (Olney, *Fd Cosmet. Toxicol.* 1975, **13**, 595; Olney & Ho, *Nature, Lond.* 1970, **227**, 609; Reynolds *et al. J. Toxicol. envir. Hlth* 1976, **2**, 471).

At doses of up to 100 mg monosodium L-aspartate/kg by either the oral or ip route, plasma levels of aspartic acid were not appreciably altered. However, plasma aspartic acid concentrations 30 min after oral or ip treatment with 1000 mg/kg were greatly elevated—to 554 or 718·3 μg/ml, respectively, in the infants and to 141 or 1435 μg/ml, respectively, in the adults. Thereafter the plasma levels declined rapidly in both 15-day-old and adult mice in this dose group. The plasma concentration of aspartic acid observed 30 min after ip administration of 1000 mg L-aspartate/kg to infant mice was 180 times the concentration in the controls, which were given saline. The marked increases in plasma aspartic acid concentrations after doses of 1000 mg L-aspartate/kg suggest that the pharmacokinetics of aspartate became altered at the high dose. Measurement of the areas under the plasma concentration–time curves for the high oral and ip doses showed that in infants the systemic availability of aspartic acid was similar after administration by either route, while in adults the bioavailability was much less after oral administration than after ip injection. The authors suggest that gut-wall metabolism in adult mice might account for this effect. A lower rate of metabolism in the gastro-intestinal tract might also account for the higher peak plasma concentrations after oral doses of L-aspartate in the younger mice. The higher peak concentrations could not have resulted from slower systemic metabolism or excretion since the decline in plasma aspartic acid concentration was faster in neonates (half life 0·15 hr) than that observed in the adult group (half life 0·26 hr).

The rates of $^{14}CO_2$ excretion after oral or ip doses of [$^{14}$C]-L-aspartate were also determined. In both adult and 15-day-old mice there was a pronounced decrease in the rats of $^{14}CO_2$ excretion during the 30 min following oral or ip dosing at 1000 mg/kg. Such dramatic decreases were not apparent after doses of 10 or 100 mg/kg. Therefore the decrease in the rate of $^{14}CO_2$ excretion may reflect the saturation of an enzyme-mediated metabolic process, and this saturation may have contributed to the elevated plasma aspartic acid concentrations that were observed at the high dose level.

The second paper cited above is a useful adjunct to the first. It reviews the literature concerning the metabolic paths followed by aspartate in its conversion to $CO_2$ or its incorporation into body constituents (*Cited in F.C.T.* 1978, **16**, 293). It appears that the aspartate moiety of the sweetening agent aspartame is metabolized in a similar manner to dietary aspartic acid.

### Three new BHT metabolites

Yamamoto, K., Tajima, K. & Mizutani, T. (1979). Identification of new metabolites of butylated hydroxytoluene (BHT) in rats. *J. Pharmacobio-dyn.* **2**, 164.

Various products of BHT metabolism have been identified in man (*Cited in F.C.T.* 1979, **17**, 551) and the rat (*ibid* 1973, **11**, 1141; Takahashi & Hiraga, *Fd Cosmet. Toxicol.* 1979, **17**, 451). In the paper cited above three newly identified metabolites of BHT in the rat are described. Urine and faeces collected from male Wistar rats for 48 hr following intraperitoneal administration of 500 mg BHT/kg body weight were extracted with ether and fractionated on a silica-gel column. One fraction (I) was eluted from the column with hexane–benzene while the other (II) was eluted with benzene–ether. Gas chromatograms of I revealed the presence of the known metabolite 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde, along with three previously unrecognized metabolites. By high-resolution mass spectrometry it was indicated that these might be 2,6-di-*tert*-butyl-*p*-benzoquinone (III), 2,6-di-*tert*-butylhydroquinone (IV) and 2,6-di-*tert*-butyl-4-[(methylthio)methyl]phenol (V). These identities were confirmed by comparison with the authentic materials using gas chromatography–mass spectrometry. Fraction II contained no new metabolites. Metabolite III was predominant. During the 5 days following BHT administration, 0·048 and 1·52% of the original dose was excreted as III in the urine and faeces, respectively. The maximum rate of excretion occurred on day 2. During the same 5-day period, 0·003% of the initial dose was excreted in the urine as V.

During the 72 hr following the ip administration of 100 mg 2,6-di-*tert*-butyl-4-hydroperoxy-4-methyl-2,5-

cyclohexadien-1-one (BHT hydroperoxide)/kg, III and 2,6-di-*tert*-butyl-4-hydroxy-4-methyl-2,5-cyclohexadien-1-one were detected in the urine and faeces. The authors consider that these results and those of previous studies indicate that, *in vivo*, III may be produced from BHT *via* hydroperoxide. Studies indicating that BHT hydroperoxide induces haemorrhagic death in rats similar to that induced by BHT provide a further indication that the hydroperoxide may be an important intermediate in the metabolic transformation of BHT.

[Also of possible relevance in this connection is the recent demonstration of 2,6-di-*tert*-4-methylene-2,5-cyclohexadienone in the livers of rats treated orally with BHT (Takahashi & Hiraga, *loc. cit.*). This could give rise to 2,6-di-*tert*-butylhydroquinone (metabolite IV, above) and thus indirectly to the *p*-benzoquinone (metabolite III).]

## PROCESSING AND PACKAGING CONTAMINANTS

### Hepatotoxicity of vinylidene fluoride

Conolly, R. B., Szabo, S. & Jaeger, R. J. (1979). Vinylidene fluoride: acute hepatotoxicity in rats pretreated with PCB or phenobarbital. *Proc. Soc. exp. Biol. Med.* **162**, 163.

Although vinylidene fluoride (VDF) is a widely used monomer in the plastics industry, comparatively few studies of its toxicity have been done. Rat inhalation studies have shown it to be of low acute toxicity (Carpenter *et al. J. ind. Hyg. Toxicol.* 1949, **31**, 343; Lester & Greenberg, *A.M.A. Archs ind. Hyg. occup. Med.* 1950, **2**, 335) and it has been shown to be non-mutagenic in bacteria (Bartsh *et al. Proc. Am. Ass. Cancer Res.* 1976, **17**, 17). However, a recent long-term study has suggested that it may be carcinogenic in rats (*Pesticide and Toxic Chemical News* 1980, **8** (21), 6). VDF is structurally related to ethylene, vinyl fluoride, vinyl chloride and vinyl bromide, which, like VDF, have low acute toxicity, but which show acute hepatotoxicity in rats pretreated with polychlorinated biphenyl (Conolly *et al. Expl. mol. Path.* 1978, **28**, 25). Since VDF has been shown to be metabolized *in vivo* (Dilley *et al. Toxic appl. Pharmac.* 1974, **27**, 582), the present investigation was carried out to determine whether pretreatment of test animals with polychlorinated biphenyl (PCB) or phenobarbital (PB), another mixed-function oxidase inducer, would render VDF acutely hepatotoxic.

Fasted male rats were administered 100 mg PCB/kg by gavage for 3 consecutive days or 0·1% PB w/v in their drinking water for 7 days. PCB-pretreated rats were then exposed by inhalation to 5000, 15,000 or 25,000 ppm VDF for 4 hr, whilst rats pretreated with PB were exposed to 25,000 ppm VDF for 6 hr. Hepatotoxicity was evaluated by measurement of liver weight and serum sorbitol dehydrogenase (SDH) activity (a sensitive and specific index of acute liver injury), and by examination of the liver using a light microscope. Acute hepatotoxic effects developed within 24 hr of VDF exposure in the pretreated rats, but not in rats exposed to VDF without a pretreatment. In the animals pretreated with PB there was no increase in SDH activity but liver weight was significantly increased and there were some histological effects. Hepatocytes in the subscapular areas had pale cytoplasm and chromatin grouped along the nuclear membrane. There were also occasional foci of inflammatory reaction and necrosis surrounded by balloon cells. Mild congestion was seen in the deep hepatic parenchyma. In the PCB-pretreated animals increases in both liver weight and SDH activity were seen at all three dose levels but the increase in liver weight was not significant at the lowest dose. Histological alterations were similar to those seen in the animals pretreated with PB but were considerably more severe, particularly at the higher dose levels. PCB was far more effective than PB in sensitizing rats to the acute hepatotoxicity of VDF, and the VDF toxic effects observed following PCB pretreatment were found to be dose-related.

Since acute VDF hepatotoxicity is apparently dependent on pretreatment with mixed-function oxidase inducers, the authors conclude that toxic VDF metabolites are responsible for the observed acute hepatotoxicity. Furthermore, as metabolic activation of the structurally related chlorinated ethylenes probably occurs by epoxidation, the authors suggest that the observed hepatotoxicity of VDF could be at least partly due to VDF epoxide.

## NATURAL PRODUCTS

### Dihydrosafrole and stomach tumours

Reuber, M. D. (1979). Neoplasms of the forestomach in mice ingesting dihydrosafrole. *Digestion* **19**, 42.

Safrole is hepatotoxic in mice and rats, and at high dose levels also produces liver tumours (*Cited in F.C.T.* 1974, **12**, 155; *ibid* 1977, **15**, 645). A long-term rat feeding study revealed that its derivatives isosafrole and dihydrosafrole also damaged the liver, but only isosafrole was weakly carcinogenic to that organ (five hepatic tumours being found in 50 rats fed 5000 ppm), while dihydrosafrole at 2500–10,000 ppm induced a high incidence of oesophageal tumours (*ibid* 1965, **3**, 857; Hagan *et al. Fd Cosmet. Toxicol.* 1967, **5**, 141). In an NCI bioassay of 120 pesticides and industrial chemicals in two hybrid strains of mice, safrole, isosafrole and dihydrosafrole were used as positive control compounds and all three were reported to have increased the incidence of hepatomas. However, in the case of isosafrole the increase was significant only in one of the strains, and was apparently made so only by a lack of tumours in female controls, while dihydrosafrole produced such

an increase only in males. The latter compound also significantly increased the lung tumour incidence (Innes *et al. J. natn. Cancer Inst.* 1969, **42**, 1101). The results of this last study have now been analysed in more detail, and stomach tumours have for the first time been reported from dihydrosafrole.

The two hybrid mouse strains, (C57BL/6 × C3HAnf)$F_1$ and (C57BL/6 × AKR)$F_1$ (referred to as strains X and Y, respectively), were given safrole, isosafrole or dihydrosafrole at the maximum tolerated dose levels of 464, 215 and 464 mg/kg, respectively, in distilled water by stomach tube on days 7–27 of age. From 28 days of age the mice were given maximum tolerated doses of 1400 ppm safrole, 517 ppm isosafrole or 1400 ppm dihydrosafrole in the diet. There were 15–18 mice in each treatment group and the animals were killed after 82 wk of treatment. In mice fed dihydrosafrole, hyperplasia and carcinomas of the forestomach were found in 18% of the males and 88% of the females of strain X and in 41% of the males and 78% of the females of strain Y. One of the strain Y females had a haemangiosarcoma that metastasized to the peritoneum. Of the controls, 23% of the males and 28% of the females of strain X had stomach hyperplasia or carcinoma, while there was a zero incidence among the strain Y controls. The increase in such tumours produced by dihydrosafrole was thus highly statistically significant in all groups except the strain X males. The neoplasms were sessile growths with an increase in squamous and basal cells; the latter extended downwards into the submucosa and were often atypical in appearance. The incidence of stomach tumours was not increased in mice fed safrole or isosafrole.

Carcinomas of the liver were very frequent in safrole-fed mice, 60% of the males and 100% of the females of strain X and 11% of the males and 75% of the females of strain Y being affected. Dihydrosafrole also caused a significant increase in such tumours in males: 60 and 41% of the strain X and Y males, respectively, developed liver carcinomas, compared with 6 and 0% of the corresponding controls. However, in dihydrosafrole-fed females the incidence of liver tumours was no higher than in the controls. In isosafrole-treated male mice liver carcinomas were somewhat more frequent than in the controls but the difference was not statistically significant. Only one strain X female given dihydrosafrole developed liver carcinoma.

Mice with neoplasms of the forestomach generally did not have liver tumours, a finding similar to that in chlorobenzilate-treated female mice (Reuber, *Digestion* 1977, **16**, 308). It is noted that in other, unpublished, FDA studies oesophageal hyperplasia occurred in dogs fed dihydrosafrole for 2 yr, and carcinomas of the skin and tongue developed in dogs given safrole for 6 yr. The author cites previous studies in hamsters that have indicated that vitamin A deficiency may be involved in the aetiology of tumours of the forestomach and other squamous epithelia.

## AGRICULTURAL CHEMICALS

### Effect of feeding BHC on testicular tissue

Nigam, S. K., Lakkad, B. C., Karnik, A. B., Thakore, K. N., Bhatt, D. K., Babu, K. A. & Kashyap, S. K. (1979). Effect of hexachlorocyclohexane feeding on testicular tissue of pure inbred Swiss mice. *Bull. envir. Contam. Toxicol.* **23**, 431.

Commercial benzene hexachloride (lindane; BHC) consists predominantly of the $\gamma$ isomer but also contains some $\alpha$ and $\beta$ isomers. Testicular atrophy has previously been reported in rats ingesting lindane (100% $\gamma$-BHC; Reuber, *Envir. Res.* 1979, **19**, 460). In addition, severe hypertrophy and the total arrest of spermatogenesis with the appearance of multinucleated cells have resulted from the testicular injection of 0·25 mg lindane (98% $\gamma$-BHC) in rats (Dikshith & Datta, *Acta pharmac. tox.* 1972, **31**, 1). However a three-generation reproduction study involving the feeding of lindane to rats at levels of 25–100 ppm showed no effects on reproductive function (Palmer *et al. Toxicology* 1978, **10**, 45).

This paper describes the testicular changes observed following the dietary administration of 500 ppm technical grade BHC to 150 male inbred Swiss mice over a period of 10 months. Six controls and six test animals were killed each month so that the organs could be weighed and a histological examination of the tissues could be made. From the third month onward the testes of the mice from the test group were significantly heavier than those of the controls and various histopathological changes were observed. These included degeneration and shrinking of the epithelia of the seminiferous tubules, some of which had become completely hyalinized, accompanied by the appearance of oedematous fluids in the lumen. Active cell proliferation of the interstitial tissue was noted along with severe damage in the spermatogonic cells and a sparcity of spermatocytes. In extreme cases the seminiferous tubules consisted of large numbers of multinucleated giant cells. The authors stress that these findings may be of importance to those involved in the manufacture and use of BHC.

### Pentachlorobenzene metabolism in monkeys

Rozman, K., Williams, J., Mueller, W. F., Coulston, F. & Korte, F. (1979). Metabolism and pharmacokinetics of pentachlorobenzene in the rhesus monkey. *Bull. envir. Contam. Toxicol.* **22**, 190.

Pentachlorobenzene is a contaminant of commercial hexachlorobenzene, which has been widely used as a fungicide. However in its 1978 recommendation that pentachlorobenzene be added to the list of substances for testing under the TSCA, the Interagency Testing Committee pointed out the lack of data on the effects of this compound on animals and man and cited a study that indicated that it was foetotoxic and teratogenic in rats (*Federal Register* 1978, **43**, 50630). The metabolism and pharmacokinetics of penta-

chlorobenzene in rhesus monkeys were investigated in the study cited above.

Two male and two female rhesus monkeys were each given a single oral dose of 0·5 mg [$^{14}$C]pentachlorobenzene/kg body weight. It was found that about 95% of the dose was absorbed, with peak blood levels of pentachlorobenzene occurring between 2 and 4·5 hr after treatment. The highest tissue concentrations of pentachlorobenzene 40 days after treatment were in the fat and bone marrow, followed by those in the lymph nodes, thymus, adrenal cortex and large intestine. Only about 40 and 33% of the dose had been excreted by the males and females, respectively, 40 days after treatment and it was estimated that the half-life of pentachlorobenzene in the rhesus monkey was 2–3 months. The amount excreted in the faeces was about twice that in the urine. The blood and tissue distributions of pentachlorobenzene were similar to those reported in rhesus monkeys given a single oral dose of hexachlorobenzene, but pentachlorobenzene was metabolized more rapidly than hexachlorobenzene (Rozman *et al. Toxic. appl. Pharmac.* 1978, **45**, 293). Pentachlorophenol, 2,3,4,5-tetrachlorophenol, 2,3,5,6-tetrachlorophenol and 1,2,3,4-tetrachlorobenzene were identified as metabolites of pentachlorobenzene in both males and females. The phenolic metabolites seemed to be restricted to the blood, kidney and urine, while 1,2,3,4-tetrachlorobenzene was detected only in the liver. This suggested that biodegradation of pentachlorobenzene does not occur in the liver.

## OCCUPATIONAL HEALTH

### Arsenicals and lung cancer

Mabuchi, K., Lilienfeld, A. M. & Snell, L. M. (1979). Lung cancer among pesticide workers exposed to inorganic arsenicals. *Archs envir. Hlth* **34**, 312.

Epidemiological studies have revealed a correlation between the incidence of lung, lymphatic and skin cancers and exposure to inorganic arsenic. The mortality study cited above was carried out following a preliminary investigation that suggested an excess mortality from cancers of the lung and lymphatic tissues among workers who had retired from a factory in which they had been exposed to high atmospheric levels of inorganic arsenicals during the manufacture and packaging of pesticides.

The incidence of mortality was determined among 86·9% of 1050 men and 66·8% of 343 women who had been employed at the plant for varying periods between 1946 and 1977. The workers were grouped according to estimates of their degree of exposure to arsenicals and non-arsenicals. The observed number of deaths from all causes (197 males and 43 females) was not greater than expected, but amongst males significantly higher standard mortality ratios (SMRs) for lung cancer and anaemia were recorded (although there were only two cases of anaemia). Unlike the preliminary study, this study did not indicate an excess of deaths from lymphosarcoma. Significantly increased SMRs for lung cancer occurred among men employed predominantly in producing arsenicals (although not in the group employed entirely in such production) and also among those first employed before 1946 and those employed for 25 yr or more. No data concerning smoking habits were available but the SMRs for causes of death, other than lung cancer, that are strongly associated with smoking were not significantly increased. Furthermore, the pattern of SMRs for lung cancer was unlikely to be explained by smoking habits alone. An increasing gradient in the SMR for lung cancer among workers predominantly exposed to arsenicals was observed with increasing length of exposure both among those first employed before 1946 and among those first employed between 1946 and 1954. Such a dose-response relationship was not seen in those employees predominantly exposed to non-arsenicals. The lack of a demonstrable excess of lung cancer in those exposed to arsenicals alone suggested a synergistic effect between arsenicals and non-arsenicals although further analysis of the data did not support this indication.

### Monitoring nickel levels

Morgan, L. G. & Rouge, P. J. C. (1979). A study into the correlation between atmospheric and biological monitoring of nickel in nickel refinery workers. *Ann. occup. Hyg.* **22**, 311.

The hazards to workers in the nickel industry are well documented (*Cited in F.C.T.* 1974, **12**, 428; *ibid* 1977, **15**, 362). Monitoring personal exposure to carcinogens such as nickel makes an important contribution to the assessment of the carcinogenic risk to individuals. Biological monitoring can also play an important role in preventing toxicological problems such as the dermatitic effects associated with nickel metal or salts (*ibid* 1976, **14**, 366) and the acute toxicity of nickel carbonyl gas. Previous studies have indicated a lack of direct correlation between atmospheric exposure and nickel levels in the urine (*ibid* 1979, **17**, 311). The study identified above further investigates the extent to which urinary nickel levels reflect exposure.

The study involved 242 workers in a nickel refinery and 44 employees in a nearby petrochemical installation (controls). The test subjects worked in different departments between which the degree of exposure and type of nickel compound varied considerably. Each manual worker participating wore a personal air sampler for the duration of his shift. Urine samples were carefully collected at the end of each shift. In the control group the mean urinary nickel content (corrected to 1·6 g creatinine/litre) was 28 μg/litre which compared well with the in-house control group (research department staff) exposed to a mean of 0·02 mg Ni/m$^3$ and having a mean urinary nickel content of 30 μg/litre. Workers in the chemical products department (examined twice) were exposed to concen-

trations of 0·45 and 0·5 mg Ni/m$^3$ (as soluble salts), values reflected in the urinary levels of 49 and 65 μg/litre respectively. The calciners, exposed to a mean of 0·28 mg Ni/m$^3$ (as dust of low solubility) and the nickel plant workers, exposed to nickel dusts and carbonyl gas at a mean level of 0·25 mg Ni/m$^3$, were found to have urinary nickel concentrations of 52 and 68 μg/litre respectively. Workers exposed to nickel powder and nickel carbonyl gas at a mean level of 3·71 mg/m$^3$ in the nickel powder manufacturing plant had urinary levels of 70 μg Ni/litre. The only significant correlation between urine and air levels found were those for the chemical products department, but even here there were enormous individual variations.

Total urinary Ni was also assessed in two employees for one complete week; one was an office worker (no exposure), the other was exposed to nickel nitrate in the plant. The results reveal that while exposure to soluble salts was reflected by urinary nickel levels, unexplained variations of 10–50 μg/litre were observed even in the urinary nickel levels of the office worker. However, a marked nickeluresis was induced when, on day 6 of the study, the unexposed worker was given 0·5 g sodium diethyldithiocarbamate, a specific nickel chelating agent.

The authors conclude that whilst on a group basis there was a correlation between exposure to soluble nickel salts and their rate of excretion, the range of ratiation between individuals is considerable. Methods for monitoring nickel exposure require further investigation.

**The cause of MBK neuropathy?**

Eden, A., Flucke, W., Mihail, F., Thyssen, J. & Kimmerle, G. (1979). Toxicological and metabolic studies of methyl *n*-butylketone, 2,5-hexanedione, and 2,5-hexanediol in male rats. *Ecotoxic. envir. Safety* **3,** 204.

Methyl *n*-butylketone (MBK) has been implicated in cases of occupational peripheral neuropathy and induces axonal degeneration of the central nervous system in experimental animals (*Cited in F.C.T.* 1979, **17,** 682). The metabolites of MBK include 2,5-hexanediol (HDL) and 2,5-hexanedione (HDE), both of which produced peripheral neuropathy when given to rats at 0·5% in their drinking water for 3 months (Spencer & Schaumburg, *Proc. R. Soc. Med.* 1977, **70,** 37). Other animal studies have confirmed the potency of HDE in this respect (*Cited in F.C.T.* 1979, **17,** 683; Schaumburg & Spencer, *Science, N.Y.* 1978, **199,** 199; Spencer & Schaumburg, *J. Neurol. Neurosurg. Psychiat.* 1975, **38,** 771), leading to suspicions that it is the ultimate neurotoxic metabolite of MBK. Some further support for this hypothesis has now emerged.

Rats were treated by stomach tube with HDE, HDL or MBK, each at a dose level of 400 mg/kg/day, for 5·5, 10·5 or 40 wk respectively. Hindlimb weakness developed in the HDE-, HDL- and MBK-treated groups after 3, 5 and 17 wk respectively, and bodyweight gain decreased after 3–4, 8 and 17 wk, respectively. HDE and HDL caused paresis after 5 and 10 wk respectively, but MBK-treated rats gradually recovered from their hindlimb weakness from wk 28 onwards. HDE was thus the most potently neurotoxic of the three compounds.

In HDE-treated rats, HDE blood levels were high 1 hr after administration, and were still appreciable after 7 hr; the highest 1-hr levels were recorded on days 29–33, just before the onset of paresis. HDE was excreted in the urine largely in a conjugated form, although the amount of conjugate decreased after the first 2 wk. In HDL-treated rats, HDL rapidly disappeared from the blood, persisting for as long as 7 hr only during wk 1. HDE was present in the blood after 1 hr and reached a peak after 5–7 hr (after 3 hr from day 59 of treatment onwards). HDE and HDL were excreted in the urine of this group in both free and conjugated forms, the amounts of free HDL and of conjugated HDE declining somewhat after 6 wk. In MBK-treated animals, MBK blood levels reached a peak after 1 hr, and HDE was also present in the blood at this stage. Maximum blood HDE concentrations (reached after 5–7 hr) were only about half those attained after HDL administration, and less than one-quarter of those after HDE treatment. HDL could not be detected in the blood at any stage, but 2-hexanol was found at low levels for up to 3 hr after administration. The onset of hindlimb weakness after 17 wk coincided with a peak in urinary levels of free MBK, free 2-hexanol and free HDL and with a trough in the urinary level of conjugated 2-hexanol. The levels of conjugated 2-hexanol rose again as the animals recovered. Urinary HDE (largely in a conjugated form) reached a maximum after 7 wk, with a subsidiary peak after 16 wk.

The changes in metabolism with time were suggested to be due to an initial inhibition of the uridinyl diphosphate glucuronyl transferases, later compensated for by enzyme induction, but this possibility has still to be investigated.

[Recent studies carried out at the Chemical Industry Institute of Toxicology (*Chemical Industry Institute of Toxicology 1979 Annual Report and Scientific Review: Science in the Public Interest*, p. 12) have shown that 2,5-hexanedione (HDE) is selectively retained in the sciatic nerve of rats after inhalation exposure to 1000 ppm *n*-hexane for 6 hr. It has also been found that biosynthesis of both cholesterol and ubiquinone decreased in the sciatic nerves of rats ingesting HDE in their drinking-water. These observations have important implications with regard to *n*-hexane-induced neurotoxicity, since cholesterol is important in maintaining both the axon membrane and neurofilaments and cholesterol and ubiquinone are both required for energy production within the cells. Clearly the role of HDE in solvent-induced neuropathies needs further investigation.]

**More isocyanate sensitization**

White, W. G., Morris, M. J., Sugden, E. & Zapata, E. (1980). Isocyanate-induced asthma in a car factory. *Lancet* **I,** 756.

It has proved difficult to establish an atmospheric concentration of toluene diisocyanate (TDI) that will not induce sensitization, and consequently asthmatic reactions, in susceptible individuals (*Cited in F.C.T.* 1980, **18,** 545). As the following account demonstrates,

concentrations of the order of 0·003 ppm TDI associated with fabrics containing polyurethane foam may give rise to sensitization.

Women working in a factory making car-seat covers complained of shortness of breath or wheezing after the introduction of a new fabric, cropped nylon backed with flame-bonded polyurethane foam. Sixty-eighty women were examined on their first day of work after a week's holiday and again at the end of a working week. Of these, 31 were currently machining the fabric containing polyurethane. The 37 other workers not involved in machining this fabric were divided into two groups depending upon whether they had or had not previously machined the fabric. These groups reflected levels of exposure rather than subjects and controls since none were completely free of TDI exposure. A total of 17 women claimed to have asthmatic symptoms; ten attributed their onset to the introduction of the new fabric 3 yr before, and three others said that previous symptoms had been aggravated or had recurred since its introduction. However, no differences in respiratory peak flow rates were detected between the exposure groups, although the incidence of wheezing and/or shortness of breath in the machinists was greater than would be expected. The 13 machinists claiming to have recent respiratory symptoms tended to have lower peak flow rates than did the rest of the group.

In a second study, which included an additional 124 women doing similar work at the same factory, 30% of the 192 subjects interviewed claimed to have wheeziness and/or intermittent shortness of breath. Although the incidence of these symptoms was higher in the group who had machined the polyurethane fabric at some time, the difference was not statistically significant. There was, however, a significantly greater number of women with reduced peak flow rate among the group that had machined the nylon.

A few subjects were studied in greater detail. In one, a fall in peak flow was measured when she returned to work after absence because of asthma. The peak flow of this subject also fell after challenge with the fabric in the laboratory and she showed increased airways resistance in response to TDI challenge on two occasions. Six workers with asthmatic symptoms also showed airflow obstruction and three symptomatic workers given challenge tests with the fabric showed positive responses as determined by airflow resistance measurements. Four of nine women with symptoms had IgE antibodies to TDI.

TDI was detected in all the regions sampled in the trimming shop at levels of roughly 0·0003 to 0·003 ppm. The maximum concentration of airborne fibres was less than 1 mg/litre and the fibres were 50 $\mu$m in diameter and 200 $\mu$m to 5 mm in length.

Medical surveillance of the workers in this factory is now being carried out and ways of handling the fabric to avoid TDI release are being investigated. Ventilation of the trim-shop area is also being re-examined. The authors expressed the hope that a more innocuous backing foam could be found for future use.

**Catalyst for polyurethane foam causes bladder dysfunction**

Kreiss, K., Wegman, D. H., Niles, C. A., Siroky, M. B., Krane, R. J. & Feldman, R. G. (1980). Neurological dysfunction of the bladder in workers exposed to dimethylaminopropionitrile. *J. Am. med. Ass.* **243,** 741.

Keogh, J. P., Pestronk, A., Wertheimer, D. & Moreland, R. (1980). An epidemic of urinary retention caused by dimethylaminopropionitrile. *J. Am. med. Ass.* **243,** 746.

Several industrial chemicals have been identified as neurotoxins in outbreaks of occupational neuropathy, generally affecting the somatic nervous system. However, the authors cited above describe the occurrence of a disorder of the autonomic nervous system, neurological dysfunction of the bladder, among workers exposed to dimethylaminopropionitrile (DMAPN).

The first paper cited above describes a survey of a plant at which DMAPN was used as a catalyst in the manufacture of polyurethane foam. The catalyst was used on one of two factory production lines from August until December 1977 and then on both production lines until March 1978 when the cause of the health problems was identified and use was stopped. Of 230 employees, 208 were studied. From the results of a questionnaire completed by these workers it was determined that 104 out of 116 production- and finishing-room workers had suffered neurological bladder dysfunction. Their symptoms were, characteristically, hesitancy and a need to strain to urinate, decreased urine stream and increased duration of urination. No symptoms occurred among office or warehouse workers. There were no significant age- or sex-related differences in the incidence of dysfunction. However, the incidence was greater among workers who had worn cotton gloves and was lower among workers on the first and third shifts (53 and 58%, respectively) than on the second shift (78%). Nevertheless, when the incidence was calculated as cases per person-month of exposure, the second- and third-shift workers were affected in equal proportions (0·14 and 0·15 cases per person-month, respectively) while the proportion of those on the first shift who were affected was much lower (0·07 cases per person-month). This may have been related to the fact that all production innovations were done on the first shift and this slowed down production, while cleaning-up procedures were deferred until the third shift.

Between 8 and 12 days after DMAPN use was stopped, 21% of the cases had recovered completely and a further 51% showed improvement. Three months later, 86% of the cases were asymptomatic and the remainder were improved. It seemed that the major route of exposure was respiratory, since neuropathy occurred among workers on the second production line before DMAPN was used there and cases occurred among workers with little if any skin contact with the catalyst. Measurements taken 10 days after the use of DMAPN had ceased showed that the compound was present in the air of the production and finishing areas at levels of 0·11 mg/m$^3$ but none could be detected 4 wk later. No measurements were taken during the period that the catalyst was used and so the quantitative exposures associated with the adverse health effects are not known. Twenty three of the affected workers reported sexual difficulties. Only 13 of the 104 affected workers reported symptoms

suggesting limb neuropathy. Eight workers were examined neurologically during recovery and seven of these had subclinical neuropathy or abnormality. The two patients who had the most severe bladder dysfunction had motor and sacral neuropathies as well.

Another survey was carried out by Keogh *et al.* (cited above) as a result of similar complaints at a different plant using DMAPN. At this plant, 85 out of 141 workers were affected by urinary retention and many of them had additional symptoms such as impotence, decreased libido, insomnia, irritability, muscle weakness and paraesthesia. Urinary retention was the predominant symptom and there was strong evidence that DMAPN was the causative agent.

It would seem that the catalyst DMAPN is unique among known neurotoxins in that bladder dysfunction is the primary symptom of the neuropathy.

**Pulmonary haemorrhage from trimellitic anhydride**

Herbert, F. A. & Orford, R. (1979). Pulmonary haemorrhage and edema due to inhalation of resins containing trimellitic anhydride. *Chest* **76,** 546.

Two cases of pulmonary haemorrhage and haemolytic anaemia due to trimellitic anhydride (TMA) were recently described (*Cited in F.C.T.* 1980, **18,** 445) and seven similar cases have now come to light. These were previously healthy young men aged 18–21 yr who 28–76 days after starting work in a steel pipe-coating plant began to experience coughing, nosebleeds or minor haemoptysis and dyspnoea. The symptoms worsened but the occupational cause of the problem was not recognized. Most of the men therefore continued to work until hospital admission was necessary, at which stage most were also suffering from fever, chills, weakness, vomiting and headache. All were hypoxaemic, and two admitted to intensive care units had high alveolar arterial oxygen gradients. There was also evidence of reduced lung volume and diffusing capacity, a bilateral or unilateral pulmonary infiltrate and intra-alveolar haemorrhage, with haemosiderin-laden macrophages and hypertrophy of lining septal cells.

All the patients were anaemic, with changes indicative of red-cell destruction and regeneration, but complement levels were normal in three out of four subjects and no antiglomerular basement membrane antibodies could be found in the two patients examined. Follow-up studies failed to reveal IgE antibodies against TMA or TMA-serum albumin conjugate. It therefore appeared that an immunological response was not involved in the aetiology of the disease in these subjects, unlike the cases previously reported. Hepatic and renal function studies gave normal results. The men had been exposed to dust and fumes from TMA-containing epoxy resins, and TMA levels of 0·14–0·27 mg/m$^3$ were detected in three places 2–40 ft from the powder spray booth. Follow-up studies on six of the men, 3 wk to 1 yr later, indicated apparent recovery. Of 29 currently employed workers at the plant, five reported episodes of severe haemoptysis and/or dyspnoea or coughing, and four had suffered mild nasal irritation, but in only one case was there evidence of haematological changes. Measures were subsequently taken to reduce fume and dust exposure, and masks with a dust prefilter and an acid gas cartridge were provided.

**Benzene and the micronucleus test**

Hite, M., Pecharo, M., Smith, I. & Thornton, S. (1980). The effect of benzene in the micronucleus test. *Mutation Res.* **77,** 148.

The micronucleus test, which is based on the detection of small chromatin particles in the cytoplasm of young erythrocytes from the bone marrow, has not proved among the most accurate of short-term predictive tests for carcinogenicity (*Cited in F.C.T.* 1977, **15,** 646). However, as benzene is a known leukaemogen and induces chromosome changes in bone marrow (*ibid* 1977, **15,** 652; *ibid* 1978, **16,** 299) it was decided to subject it to this test method. A mutagenicity test with benzene in Drosophila gave negative results (*ibid* 1979, **17,** 419) and the mechanisms by which the solvent induces leukaemia is still unclear (*ibid* 1977, **15,** 652).

In the present study, mice (eight to ten at each dose level) were given benzene in peanut oil by stomach tube at levels in the range 0·0625–2·0 ml/kg/day in two doses 24 hr apart, and were killed 6, 18, 24 or 48 hr or 5, 9 or 16 days later. About 3000 polychromatic and normochromatic erythrocytes from the bone marrow of each mouse were then examined for the presence of micronuclei. Normochromatic erythrocytes showed no treatment-related changes, but there was a significant ($P < 0·05$) increase in the number of polychromatic erythrocytes with micronuclei in those killed 6 hr after doses of 0·25 ml/kg/day or more, and after 18 hr a similar increase was found at dose levels from 0·125 ml/kg upwards. After 24 hr no significant increase was found in one experiment involving dose levels up to 0·25 ml/kg/day, but when this experiment was repeated at dose levels of 0·125, 0·25 or 0·5 ml/kg/day there was a significant increase at all three levels. In mice killed 48 hr after doses of up to 0·25 ml/kg/day, the increase was significant only at the highest level. Mice killed 5 days after treatment with 0·125–0·5 ml/kg/day were significantly affected at all levels, but after 9 or 16 days values were similar to those in controls. It was concluded that the micronucleus test can be a useful screening procedure in cytogenetics.

## ENVIRONMENTAL CONTAMINANTS

**Review of chloroform carcinogenicity**

Reuber, M. D. (1979). Carcinogenicity of chloroform. *Envir. Hlth Perspect.* **31,** 171.

A recent review by the US Association of Life Sciences Subcommittee on Epidemiology of twelve epidemiological studies failed either to support or to refute the results of animal bioassays that have suggested that certain trihalomethanes may be carcinogenic (*ALS Lifelines* 1978, **4** (4), 3). This was partly

because of imprecise exposure data and partly because of difficulties in controlling for all of the factors that could possibly affect cancer incidence. In the paper cited above, Dr. Reuber, of the NCI, presents a review of all of the animal studies on the carcinogenicity of chloroform known to him and so provides a useful basis for the interpretation of these data with reference to man.

The studies reviewed include the NCI rat and mouse studies in which chloroform was given by gavage in corn oil for 78 wk on 5 days/wk (*Federal Register* 1976, **41,** 15026). On the basis of his examinations of the histological sections, Dr. Reuber concludes that chloroform induced tumours at many different sites but particularly in the livers of female rats and the kidneys of male rats. There was also a significant increase in the incidence of thyroid tumours among female rats. Other toxic changes in rats included testicular atrophy, polyarteritis and interstitial fibrosis of the kidney. In mice, chloroform induced carcinoma of the liver in all of the animals in the high-dose group (mean dose levels: 277 mg/kg for males, 477 mg/kg for females). Females showed a particular susceptibility to this cancer and among those in the high-dose group there was also a high incidence of an extremely rare lesion, thrombosis of the heart.

Chloroform was also administered to mice in a study carried out at the Huntingdon Research Centre. Four different strains were used in one series of experiments, the chemical being administered in a toothpaste base by gavage 6 days/wk for 80 wk. Renal tumours were induced in only one strain, the ICI-Swiss mice given 60 mg chloroform/kg/day, but male CBA and CF1 mice given the same dose developed significant chronic nephritis. C57BL mice did not show these effects. Chronic renal disease was a "prominent feature" in chloroform-treated and control mice of the ICI-Swiss strain, but the presence or absence of chronic renal disease with tumours was not mentioned. Two further experiments were carried out on ICI-Swiss mice using the same regimen but using groundnut oil as the vehicle in one of the studies. In both experiments renal tumours were observed, indicating that male ICI-Swiss mice are sensitive to the carcinogenic properties of chloroform.

The Huntingdon studies were extended to include a 95-wk study using Sprague–Dawley rats, and an 18-month study on beagle dogs. Male and female beagle were given 15 or 30 mg chloroform/kg/day in a toothpaste base 6 days/wk for 376 wk. An increase in tumour incidence and a variety of liver abnormalities were found in both sexes. Hepatic nodules were observed in some dogs. However, chloroform did not produce any increase in tumour incidence in the rats, which were given 60 mg chloroform/kg/day, 6 days/wk for 95 wk, although it is noted that there was a high incidence of tumours among the control rats. Four other studies in mice and guinea-pigs are also reported briefly. Two of these indicated the development of liver tumours in mice exposed to chloroform, but the numbers of mice used were small and the studies were relatively short. When chloroform was given to mice at low doses subcutaneously, tumour incidence was not significantly increased. Guinea-pigs given doses of 35 mg chloroform/kg developed fatty change, necrosis and cirrhosis of the liver.

The reviewer concludes that there is significant evidence that chloroform is carcinogenic in several mammalian species and that "sufficient documentation is available on qualitative extrapolation of animal data" to conclude that these findings should be deemed relevant to man.

[It is perhaps unfortunate that the author did not consider the extrapolation of the results for animals to man more closely, especially in view of the many objections that were voiced to the FDA on their proposals to ban the use of chloroform in human drugs and cosmetics (*Federal Register* 1976, **41,** 26842). These included the different metabolism of chloroform in mice and men, the excessively high dose levels used in the NCI study and the possibly incorrect interpretation of some of the control-animal data. The conclusions reached in the review differ somewhat from those in the original NCI report (*ibid* 1976, **41,** 15026). No increased incidence of liver tumours in female rats was originally reported, and the increase in thyroid tumours in female rats was not considered to be biologically significant.]

### Nonvolatile mutagens in chlorinated drinking-water

Cheh, A. M., Skochdopole, J., Koski, P. & Cole, L. (1980). Nonvolatile mutagens in drinking water: production by chlorination and destruction by sulfite. *Science, N.Y.* **207,** 90.

A very large number of organic contaminants are known to be present in sources of drinking-water and there has been concern that chlorination of water containing organic compounds may produce trihalomethanes and other compounds suspected of having some carcinogenic potential. A study reported last year (*Cited in F.C.T.* 1980, **18,** 447) emphasized the justification of such concern, in that the effluents from two waste-water treatment plants showed significant mutagenic activity in the Ames test. In the study cited above the effects of chlorination on the production of nonvolatile mutagens in drinking-water were investigated.

Samples (40–80 litres) of water that had been softened with lime were obtained from a US municipal treatment plant. The samples were first treated in the laboratory by the same procedure that would have been used in the plant except that various methods of chlorination were used. Organic compounds present in the treated water were then adsorbed onto a nonpolar resin and subsequently desorbed by washing with acetone and then with methylene chloride. The solvent and the volatiles were evaporated off and the residual organic compounds were dissolved in dimethylsulphoxide and assayed for direct-acting mutagens using the Ames Salmonella plate test. All of the results are for Salmonella strain TA100, without added rat-liver postmitochondrial supernatant (the presence of the activation system reduced the mutagenic activity). No nonvolatile mutagens were detected in the unchlorinated water, but mutagenic activity was present in water that had been treated with chloramine or with free chlorine. The mutagenic activities of the chlorinated drinking-water samples were two- to tenfold (mean fivefold) greater than those of distilled water samples that had been treated

in the same way. Most of the mutagenic activity in the drinking-water appeared to result from the chlorination of the constituents of the water and not from mutagens present in the chlorine or from reactions between the chlorine and the experimental apparatus. Chlorination with free chlorine produced considerably more mutagens than chlorination with chloramine.

When chlorinated drinking-water was dechlorinated using sodium sulphite before it was passed through the nonpolar column there was a 50–80% reduction in mutagenic activity. (Since the mutagenic activity of the chlorinated distilled water samples was only about 20% that of the drinking-water samples, this reduction is greater than could be attributed solely to the chlorination of the resin used to adsorb the organic compounds.) The effectiveness of sulphite in reducing the level of mutagens in treated drinking-water was assessed on a laboratory scale. Water samples were chlorinated with free chlorine, dechlorinated with sodium sulphite and then postchlorinated with monochloroamine. The dechlorinated samples had significantly less mutagenic activity than similarly treated samples that had not been dechlorinated.

It is concluded that the mutagens detected in this study were unlikely to be trihalomethanes since volatiles were removed and since the Salmonella strains used were known not to respond to chloroform, carbon tetrachloride or other common trihalomethanes under the test conditions used. Although the effectiveness of the method would need to be tested on a pilot-plant scale, the authors suggest that the dechlorination of drinking-water with sulphur dioxide or sulphite might be a relatively easy and cheap method of reducing consumer exposure to direct-acting mutagens. Dechlorination might also be used to the same effect in treated industrial effluents.

[The results of the study by Saxena & Schwartz reported earlier (*Cited in F.C.T.* 1980, **18**, 477) demonstrated that the mutagenicity of the effluent fluctuated considerably at different stages in the treatment process. Clearly more studies are needed to define the effects of different water treatments on the presence of mutagens in drinking-water.]

## COSMETICS, PHARMACEUTICALS AND HOUSEHOLD PRODUCTS

### Not only skin deep for NDELA

Edwards, G. S., Peng, M., Fine, D. H., Spiegelhalder, B. & Kann, J. (1979). Detection of *N*-nitrosodiethanolamine in human urine following application of a contaminated cosmetic. *Toxicology Lett.* **4**, 217.

The presence of *N*-nitrosodiethanolamine (NDELA) has been detected in cosmetics, body lotions and hair shampoos (Fan *et al. Fd Cosmet. Toxicol.* 1977, **15**, 423). More recently, NDELA in an aqueous vehicle has been shown to penetrate excised samples of human skin and to be absorbed *in vivo* through monkey skin (*Federal Register* 1979, **44**, 21365). The implications of these findings have now received support from a direct demonstration of absorption of NDELA through human skin *in vivo* following application of a contaminated cosmetic.

The cosmetic was a widely used cream-type facial 'foundation' purchased from a retailer. It contained 77 ppm NDELA, a relatively high level of contamination, although the FDA has reported up to 130 ppm in some cosmetic products. Both a gas chromatograph (GC) interfaced to a Thermal Energy Analyzer (TEA) and a high-pressure liquid chromatography (HPLC)–TEA system were used for determinations of NDELA in the cosmetic and in urine samples. The cosmetic was applied evenly over an area of unblemished skin on the chest and back of a 40-yr-old male (12·7 g over 2090 $cm^2$). It was covered loosely to prevent loss onto clothing and was washed off after a period of 7·75 hr. Care was taken to avoid exposure to other known sources of NDELA for 48 hr before the test and during the exposure, and no food or drink was taken for 12 hr before the application or while the cosmetic was on the skin.

Urine collected immediately before the start of treatment provided some evidence for a possible "basal level" of NDELA in this subject, but the concentration was too low to permit confirmation by other methods. A markedly raised level of NDELA was present in all the urine collected between 1 hr after the cosmetic was applied and 12 hr after its removal. It was calculated that a total of at least 17·3 μg NDELA was excreted during this period, at the end of which the excretion rate was still 0·8 μg/hr (more than half the maximum rate recorded), indicating a fairly long half-life in man. Results of some animal studies have indicated that urinary excretion may not account for all the material absorbed. Moreover, while the treated area was relatively large in this study, the exposure time was shorter than that frequently repeated daily under normal conditions of cosmetic use. These points suggest the possibility of accumulation of NDELA in regular users of contaminated cosmetics. The authors also mention some unpublished work indicating that absorption would have been greater from an oil-based preparation, such as a suntan lotion, or through the skin of the female face.

## MEDICAL DEVICES

### Adverse reaction to PVC haemodialysis tubing

Bommer, J., Ritz, E. & Andrassy, K. (1979). Necrotizing dermatitis resulting from haemodialysis with polyvinylchloride tubing. *Ann. intern. Med.* **91**, 869.

There have been some reports of adverse effects of polyvinylchloride tubing (PVC) used in medical procedures. Duke & Vane (*Lancet* 1968, **II**, 7558) found that the pulmonary blood vessels of isolated perfused cat lungs did not respond in the normal way to

hypoxia when the perfusion circuit was made partly of PVC. Rogers & Dunn (*ibid* 1969, **1**, 1246) reported cases of intestinal perforation in human neonates after exchange transfusion. In the paper cited above, a case of necrotizing dermatitis resulting from haemodialysis with PVC tubing is reported.

A 59-yr-old man with polycystic disease had been undergoing haemodialysis since 1970, and until 1978 PVC tubing obtained from one manufacturer was used for dialysis. In 1974 he began to suffer recurrent bouts of cutaneous necrotizing dermatitis, which first appeared on his scalp and later spread to other parts of his body. The sites of the lesions were not related to exposure to light or to mechanical irritation. The symptoms appeared 1–8 hr after the start of dialysis with the development of an isolated erythematous spot which eventually enlarged into an intensely itching papule with an erythematous flare of up to 2 cm in diameter. The lesions slowly healed over several weeks, but left an atrophic hyperpigmented scar. One to six papules appeared per haemodialysis and older lesions re-erupted regularly during subsequent dialyses. There were no other symptoms and no evidence of visceral involvement. The results of clinical chemical tests were essentially normal. Histological studies showed acanthotic thickening of the epidermis, focal inter- and intracellular oedema, thrombosis of the vessels in the corium with fibrin deposits in the arteriolar walls, perivascular infiltrates consisting of activated lymphocytes and polymorphonuclear granulocytes with eosinophils and occasional mast cells. Immunohistological findings included pronounced precipitates of fibrin in the upper corium and precipitates of immunoglobin G and of complement in the small vessels. Scratch tests and intracutaneous tests were negative for all commercially available heparin preparations and for preservatives used in heparin.

Various types of dialysers were used without effect on the lesions. However when polyurethane-coated PVC tubing was used instead of plain PVC the papules ceased to appear, the existing ones healed and the itching rapidly disappeared. Re-exposure to plain PVC tubing caused an immediate reappearance of the dermatitis. Epicutaneous tests with used, plain-PVC tubing produced a papule that was histologically identical to the spontaneous lesions. An immunopathogenetic origin for the reaction was suggested by the results of this test and also possibly by the histology of the lesions and the deposition of immunoglobulins and complement.

The frequency of reactions to PVC remains to be determined, although the authors state that eosinophilia and itching occur frequently in haemodialysed patients and that this may be related to an allergy to PVC tubing or to a plasticizer in the tubing. Further information on the occurrence of side effects is needed before the use of polyurethane-coated PVC tubing can be generally recommended for haemodialysis.

### MMA in the optical field

Turkish, L. & Galin, M. A. (1980). Methylmethacrylate monomer in intra-ocular lenses of polymethylmethacrylate: cellular toxicity. *Archs Ophthal., N.Y.* **98**, 120.

MMA monomer has been implicated in allergic or irritant reactions associated with the handling of acrylic dough used in dentistry or surgery (*Cited in F.C.T.* 1972, **10**, 569). Polymethylmethacrylate (PMMA) is used also in the manufacture of intra-ocular lenses. It has been recommended that polymer used for this purpose should not contain more than 0·5% residual monomer, but little seems to be known specifically about the intra-ocular toxicity of MMA. The authors cited above therefore determined the toxicity of MMA to cultures of rabbit kidney cells, an *in vitro* system that has been used to assess the safety of the lenses themselves.

Commercial MMA (99·9% pure and containing 1 ppm hydroquinone and 5 ppm monoethyl ether of hydroquinone) was tested at concentrations in the culture of 0·05%–5%. Total cell destruction occurred in less than 1 hr with 2 or 5% MMA and in 24 hr with 1%. Some of the cultures treated with 0·5% were totally destroyed in 72–96 hr but in others, 50% of the cells were viable after 7 days, while with 0·25% MMA, 10–50% of the cells had degenerated by day 7. Cultures containing 0·1 or 0·05% MMA were indistinguishable from the controls up to day 12, and after that cell degeneration was apparent in both groups. From these results, and taking into account the progressive dilution that results from water exchange between the vitreous and aqueous components of the eye *in vivo* and known rates of monomer elution from PMMA samples of relevant dimensions, the authors deduce that the monomer persisting in the aqueous fluid from carefully manufactured intra-ocular lenses must be almost negligible.

## TOXICITY MECHANISMS

### Oxidation–reduction of mercury in mice

Sugata, Y. & Clarkson, T. W. (1979). Exhalation of mercury—further evidence for an oxidation–reduction cycle in mammalian tissues. *Biochem. Pharmac.* **28**, 3474.

The hydrogen peroxide-catalase pathway has been implicated in the oxidation of inhaled metallic mercury vapour ($Hg^0$) to divalent inorganic mercury ($Hg^{2+}$) in mammals (Magos *et al. Toxic. appl. Pharmac.* 1974, **28**, 367). Conversely, injected $Hg^{2+}$ can be exhaled as $Hg^0$ in rats (Clarkson & Rothstein, *Hlth Phys.* 1964, **10**, 115). Ethanol decreases the pulmonary retention of metallic $Hg^0$ and increases its exhalation in rats (Magos *et al. Toxic. appl. Pharmac.* 1973, **26**, 180), and mice treated with $Hg^{2+}$ and ethanol exhaled increased amounts of $Hg^0$ (Dunn *et al. Br. J. ind. Med.* 1978, **35**, 241). These effects might be brought about by the competition of ethanol, a substrate for hydrogen peroxide-catalase, with $Hg^0$, resulting in a

decrease in the rate of oxidation of inhaled $Hg^0$ or of $Hg^0$ formed from $Hg^{2+}$. $Hg^0$ is able to pass membrane barriers more readily than $Hg^{2+}$. The results of the study cited above confirm those of previous investigations and add to the evidence for an oxidation-reduction cycle for mercury in mammalian tissues.

Groups of mice genetically deficient in catalase (acatalasaemic), their corresponding wild type, and CBA/J mice were either exposed to radioactive $Hg^0$ vapour (0·18–0·38 mg/m) for 26 min or given a single intraperitoneal injection of radioactive $Hg^{2+}$ (10 μg/kg body weight) as mercuric chloride in saline. Some of the CBA/J mice were given a single ip dose (1 g/kg body weight) of 3-amino-1,2,4-triazole (AT), an inhibitor of catalase, before or after treatment with the mercury. The exposed mice were kept in plastics bottles and exhaled volatile mercury was collected in a filter. The cumulative amount of exhaled mercury increased continuously for 6 hr after treatment in all the groups of mice. Considerably less $Hg^0$ was recovered from animals killed immediately after exposure, indicating that the main source of $Hg^0$ was exhalation.

Of the mice exposed to $Hg^0$ vapour, those with reduced catalase activities (the acatalasaemic and AT-treated mice) exhaled more $Hg^0$ than those with normal catalase activities. AT given 30 min before or after the dose of $Hg^{2+}$ increased the amount of exhaled $Hg^0$, but only after a 1–1·5 hr time lag. The increased exhalation of $Hg^0$ in the acatalasaemic and AT-treated mice could not be attributed to changes in the tissue distribution of mercury in either the vapour-exposed or the $Hg^{2+}$-dosed mice. Indeed, in those that inhaled $Hg^0$ the lung deposition of $Hg^0$ was reduced.

The amount of $Hg^0$ exhaled by the $Hg^{2+}$-treated mice was much less than that exhaled by the mice exposed to $Hg^0$. However, the exhalation of $Hg^0$ by these mice confirms that $Hg^{2+}$ can be reduced to $Hg^0$ in body tissues and since AT increased this exhalation the hydrogen peroxide-catalase pathway is implicated in the reoxidation of $Hg^0$ derived from $Hg^{2+}$. That the rate of exhalation of $Hg^0$ in the vapour-exposed groups was greater than in the groups injected with $Hg^{2+}$ may be accounted for by the differences in the quantities of available $Hg^0$. In the vapour-exposed mice the residual $Hg^0$ in the tissues should contribute to exhaled $Hg^0$ and the exhalation curve should level off as this reservoir is depleted. Such a levelling off was seen in the groups of mice that had normal catalase activities. In AT-treated and acatalasaemic mice the rate of oxidation of $Hg^0$ was reduced, the rate of depletion of the $Hg^0$ reservoir was decreased and so there was a greater cumulative $Hg^0$ exhalation.

The balance between the oxidation and the reduction of mercury in mammalian tissues has considerable toxicological importance since $Hg^0$ is thought to be the form of mercury which most rapidly crosses the blood–brain and placental barriers.

**The gut flora and liver cancer**

Mizutani, T. & Mitsuoka, T. (1979). Effect of intestinal bacteria on incidence of liver tumors in gnotobiotic C3H/He male mice. *J. natn. Cancer Inst.* **63,** 1365.

The incidence of hepatoma in mice is dependent on a variety of factors, including strain, sex, diet and environment (*Cited in F.C.T.* 1976, **14,** 63). The gut flora may also affect the liver-tumour incidence by converting hepatic carcinogens to their active form. Thus, cycasin failed to produce liver lesions in germ-free rats, presumably because they lacked the bacterial β-glycosidase which normally converts cycasin to its active metabolite (*ibid* 1965, **3,** 508), and germ-free mice were resistant to the induction of liver and lung tumours by 7,12-dimethylbenz[*a*]anthracene (*ibid* 1971, **9,** 612). An attempt has now been made to identify the bacterial species responsible for 'spontaneous' liver tumours in one mouse strain.

The incidence of liver tumours and liver nodules was compared in 14 groups of 48-wk-old C3H/He male mice that had been maintained under either germ-free or normal conditions or that were the offspring of germ-free mice that had been infested before mating with with one or more specific intestinal bacteria and then isolated. In germ-free mice the incidence of liver tumours was 39% and there were only 0·5 tumour nodules per mouse, whereas in normal mice the incidence of liver tumours was 82% and there were 1·6 nodules/mouse. In all but one of the 12 groups infected with specific bacteria the liver-tumour incidence was in the range 62–100%, and the number of tumour nodules/mouse was in the range 1·0–2·9.

Single infection with *Escherichia coli*, *Streptococcus faecalis*, *Bifidobacterium adolescentis*, *Clostridium indolis*, *B. infants infantis* or *Bacteroides multiacidus* progressively raised the liver-tumour incidence, which ranged from 62% in the first group to 100% in the last. The number of tumour nodules/mouse ranged from 1·0 to 1·5 in these groups. Mice infected with both *E. coli* and *C. perfringens* had a higher incidence of liver tumours (88%) and a higher number of liver nodules/mouse (1·7) than did those infected with *E. coli* alone. The incidence of these conditions was also increased in a group infected with *E. coli*, *S. faecalis* and *Bacteroides fragilis* (to 80% and 1·5, respectively), and was increased even more in a group infected with *E. coli*, *S. faecalis* and four strains of *C. paraputrificum* (to 95% and 2·9, respectively). However, in mice infected with the last six organisms and also with *C. perfringens*, *C. indolis*, *C. innocuum*, *C. nexile*, *C. ramosum*, *C. clostridiiforme*, *Veillonella alcalescens* and *V. parvula*, the incidence of liver tumours and nodules/mouse were only 88% and 1·4, respectively, suggesting that bacterial interaction in the intestine might influence liver tumorigenesis in the gnotobiotic mice. A group infected with *E. coli*, *S. faecalis*, *V. alcalescens* and *V. parvula* also had a fairly low incidence of liver tumours and nodules/mouse (67% and 1·4, respectively). In mice infected with *E. coli*, *S. faecalis*, *Lactobacillus acidophilus*, *C. perfringens* and *Bacteroides fragilis* the incidence of liver tumours was only 46% and the number of tumour nodules/mouse was only 0·9, values that were not significantly different from those in germ-free mice. The protective effect in this last group appeared to be due to *L. acidophilus*, since liver-tumour promotion had been observed with the other species alone or in combination.

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# MEETING ANNOUNCEMENTS

## HEAVY METALS CONFERENCE

The third in a series of International Conferences on Heavy Metals in the Environment is to be held in Amsterdam in September 1981. The themes of the Conference are to include health effects, metal speciation, interaction of metals, pathways and cycling, industrial waste management, material balances, sludge treatment/disposal, and analytical techniques. Further information may be obtained from CEP Consultants Ltd, 26 Albany St, Edinburgh EH1 3QH (telephone no. 031-557 2478).

## SOLVENT SAFETY SYMPOSIUM

An International Symposium on the Safe Use of Solvents is to be held at the University of Sussex, Brighton on 23–27 March 1982. The symposium is being organized by the International Union of Pure and Applied Chemistry which has been actively concerned with solvents for some years. This meeting is intended to provide for discussion of current advances in knowledge and problems of safe solvent use and to allow the identification of problems of industrial interest that should be given priority in future projects. The topics to be covered by lecture and poster sessions include identification, toxicology, fire and explosion, monitoring, hazard assessment, control, classification and transport. Further details may be obtained from The Secretariat, International Symposium on the Safe Use of Solvents, 142–144 Oxford Rd, Cowley, Oxford OX4 2DZ.

*Fd Cosmet. Toxicol.* Vol. 19, p. 143
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# CORRIGENDA

*Volume 18 (1980)*

p. 240, The following reference should be listed: Khoudokormoff, B. (1978). Potential carcinogenicity of some food preservatives in the presence of traces of nitrite. *Mutation Res.* **53,** 208.

p. 624, line 3: *For* kidneys *read* proximal tubules.

Fd Cosmet. Toxicol. Vol. 19, p. 145
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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*:

Chronic toxicity of butylated hydroxytoluene in Wistar rats. By M. Hirose, M. Shibata, A. Hagiwara, K. Imaida and N. Ito.

Developmental neurobehavioural toxicity of butylated hydroxytoluene in rats. By C. V. Vorhees, R. E. Butcher, R. L. Brunner and T. J. Sobotka.

Effect of dietary tin on zinc, copper and iron utilization by rats. By J. L. Greger and M. A. Johnson.

Volatile nitrosamines in salt-preserved fish before and after cooking. By D. P. Huang, J. H. C. Ho, K. S. Webb, B. J. Wood and T. A. Gough.

Analysis of 1-methyl-1,2,3,4-tetrahydro-$\beta$-carboline in alcoholic beverages. By O. Beck and B. Holmstedt.

Long-term effects of feeding aflatoxin-contaminated market peanut oil to Sprague–Dawley rats. By L. Y. Y. Fong and W. C. Chan.

The toxicity of T-2 toxin and diacetoxyscirpenol in combination for broiler chickens. By F. J. Hoerr, W. W. Carlton and B. Yagen.

Effect of 2,4,5-trichlorophenoxyacetate on renal function. By F. J. Koschier and S. K. Hong.

Percutaneous penetration of mosquito repellents in the hairless dog: effect of dose on its percentage penetration. By W. G. Reifenrath, P. B. Robinson, V. D. Bolton and R. E. Aliff.

Analyse de la nitrosodiethanolamine dans les produits de l'industrie cosmétique. By D. Klein, A.-M. Girard, J. De Smedt, Y. Fellion and G. Debry.

Ultrastructural lesions of clioquinol toxicity in beagle dogs. By D. M. Hoover, W. W. Carlton and C. K. Henrikson.

A sulphite oxidase-deficient rat model: metabolic characterization. By A. F. Gunnison, T. J. Farruggella, G. Chiang, L. Dulak, J. Zaccardi and J. Birkner.

A sulphite oxidase-deficient rat model: subchronic toxicology. By A. F. Gunnison, L. Dulak, G. Chiang, J. Zaccardi and T. J. Farruggella.

Dose-response functions in carcinogenesis and the Weibull model. By F. W. Carlborg. (Review paper).

[*Contents continued*]



## *Aims and Scope*

The Journal publishes original papers and reviews relating to the interests of the British Industrial Biological Research Association. This is a wide-ranging field covering all aspects of toxicology but with particular reference to food and cosmetics. The Journal aims to be informative to all who generate or make use of toxicological data.

# *Instructions to Authors*

**General.** Authors in North and South America should send original papers and reviews to the American Editor, Dr L. Golberg. All other papers and reviews should be sent to the Assistant Editor or to the appropriate Regional Editor. All 'Letters to the Editor' 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.

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In general the text should be subdivided as follows: (a) Abstract: 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.

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

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

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

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