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INFORMATION SECTION

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Fd Cosmet. Toxicol. Vol. 17, pp. 187 to 193

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Research Section

TWO-YEAR TOXICITY STUDY ON COUMARIN IN THE BABOON

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(*Received 18 December 1978*)

Abstract—Groups of male baboons were fed 0 (control), 2·5, 7·5, 22·5 or 67·5 mg coumarin/kg/day for between 16 and 24 months. No adverse effect was found on histological examination of a wide variety of tissues or in the biochemical or histochemical examinations of the liver. Ultrastructural examination of the liver revealed dilatation of the endoplasmic reticulum, a finding consistent with the early stages of intracellular oedema in hepatocytes, in three of the four animals fed 67·5 mg coumarin/kg/day, and a significant increase in liver weight expressed relative to body weight was recorded in this group. The dilatation was first evident after 10 months and showed an increase in severity and extent at 16 months and 2 yr. This finding indicates a no-effect level of 22·5 mg/kg/day. However, if conventional histological evidence alone were considered, 67·5 mg/kg/day would constitute the no-effect level.

INTRODUCTION

Coumarin (1,2-benzopyrene; *cis-o*-coumarinic acid lactone) is a crystalline solid with a fragrant odour and a burning taste. It has been used as a flavouring in food and tobacco and is widely used in cosmetics and toiletries. First isolated from the tonka bean (*Dipteryx odorata*) in 1820, it is widely distributed in nature and has been in use for over 100 yr (Jacobs, 1953).

Hazelton, Tusing, Zeitlin, Thiessen & Murer (1956) reported that dietary levels of 50 and 250 ppm coumarin had no effect on the weight gain, efficiency of food utilization or organ pathology of rats in a 90-day study. At 2500 ppm, however, there was impaired weight gain, liver enlargement and histological evidence of hepatic damage. Similarly, in 2-yr studies, no adverse effects were noted with dietary levels up to 1000 ppm but a reduction in weight gain and evidence of liver damage was seen at 2500 ppm (Bär & Griepentrog, 1967; Hagan, Hansen, Fitzhugh, Jenner, Jones, Taylor, Long, Nelson & Brouwer, 1967). Liver damage was also produced in dogs when coumarin was given in oral doses of 25 mg/kg/day for 130–330 days (Hagan *et al.* 1967).

Recent work has shown that there is a wide species variation in the routes of metabolism of coumarin. In the rat and rabbit, *o*-hydroxyphenylacetic acid has been shown to be an important urinary metabolite (Kaighen & Williams, 1961; Van Sumere & Teuchy, 1971), whereas in man very little is excreted in this form, the major metabolite being 7-hydroxycoumarin (Shilling, Crampton & Longland, 1969). Therefore, it has been suggested that a species that metabolizes coumarin in a manner similar to that demonstrated in man would be more appropriate for investigating the toxic effects of coumarin. Of a range of species examined, it was found that the baboon was the most suitable in this respect (Gangolli, Shilling, Grasso & Gaunt, 1974). Preliminary studies also showed that when coumarin was given for 3 wk at dose levels of 50 or 100 mg/kg/day (not at 50 or 100 ppm in the diet [S.D. Gangolli, personal communication, 1978] as stated in the cited paper), evidence of liver damage was obtained (Gangolli *et al.* 1974).

A two-year feeding study was planned, therefore, in an attempt to determine a no-effect level for coumarin in baboons. It was decided that particular attention would be paid to histological, biochemical, histochemical and ultrastructural studies. The results of these investigations are reported in this paper.

EXPERIMENTAL

Test material. The coumarin used in the experiment was obtained from the British-American Tobacco Co. Ltd., and conformed to BPC 1934 specifications (*Martindale—Extra Pharmacopoeia*, 1972).

Animals. Thirty-four male baboons weighing between 6 and 13·4 kg were obtained from two separate sources (Shamrock Farms (Great Britain) Ltd., Henfield, Sussex, and Animal Supplies (London) Ltd., Welwyn, Herts.). The animals, approximately equal numbers of Olive (*Papio anubis*) and Hamadryas (*Papio hamadras*) baboons and a single Yellow (*Papio cynocephalus*), had all been treated for worm infestations and were negative when tuberculin tested. They were housed in metal cages (61 × 61 × 100 cm) in a room maintained at 24 ± 2°C.

Diets. The basic diet was a wholly vegetable pellet (BP Nutrition (UK) Ltd., Witham, Essex), which had proved adequate for maintaining growth and health in baboons for up to 4 yr. To incorporate the coumarin in the diet the pellets were ground and a stock mixture was prepared containing 1·4% coumarin. This concentration allowed for a loss of approximately 15% during storage and processing and for the later addition of syrup and sorbic acid. Diets for individual animals were then prepared from the basic and stock

mixtures, the proportions of each being dependent on the intended coumarin intake for each animal. Syrup and sorbic acid were added to give final concentrations of 21·5 and 2%, respectively. Each mixture was then pelleted and dried. Syrup and sorbic acid were added in the same concentrations to the basic diet to provide the control feed.

Experimental design and conduct. The baboons were randomly divided into five groups of eight, eight, eight, six and four animals, fed diet providing a coumarin intake of 0 (control) 2·5, 7·5, 22·5 or 67·5 mg/kg/day, respectively. The animals were allowed free access to tap-water and were fed *ad lib.*, the levels of coumarin in each diet being adjusted every third week, on the basis of current consumption levels, to maintain the coumarin intake at the required level. In addition, each animal was given an orange every morning. Food consumption was recorded daily and the body weight of each animal was determined every 2 months.

After treatment for 6 months, half of the animals from each group were selected at random and anaesthetized with phencyclidine and sodium pentobarbital, and a needle biopsy of the liver was taken through a paracostal incision in the right abdominal wall. This procedure was repeated on the rest of the animals 4 months later. In each case, part of the specimen was used for electron microscopy and the remainder for light microscopy and histochemical examination.

At 16 months, one animal from each of the groups treated with 0, 22·5 and 67·5 mg coumarin/kg/day was killed and examined. At 18 months all the animals fed 2·5 mg/kg/day were killed, together with four of those fed 7·5 mg/kg/day. In addition, the daily intake of coumarin of one of the animals was increased from 22·5 to 67·5 mg/kg/day. All surviving animals were killed at month 24. Similar extensive investigations were performed at each autopsy.

Post-mortem examination. All animals were killed by exsanguination after induction of anaesthesia with sodium pentobarbital. The major organs were weighed and samples of these, together with a wide variety of other tissues, were fixed in 10% formal saline, embedded in wax, sectioned at 5 μm and stained with haematoxylin and eosin for histopathological investigation. In addition, tissue from each liver was taken at random for biochemical, histochemical and ultrastructural studies.

Biochemical study of liver. Homogenates of liver taken at autopsy (0·25 g fresh tissue/ml) were prepared in ice-cold 0·154 M-KCl containing 50 mM-Tris-HCl buffer (pH 7·4), by means of a motorized Teflon-glass homogenizer (A. H. Thomas & Co., Philadelphia, PA, USA). Homogenates were centrifuged at 10,000 **g** av. for 20 min and the post-mitochondrial supernatant fractions were used for the determination of ethylmorphine *N*-demethylase (Holtzman, Gram, Gigon & Gillette, 1968), aniline 4-hydroxylase (Nakanishi, Masumura, Tsukada & Marusmara, 1971) and coumarin 7-hydroxylase (Creaven, Parke & Williams, 1965). Portions of the 10,000 **g** supernatant were further centrifuged at 105,000 **g** av. for 60 min to sediment the microsomal fractions. Washed microsomes were prepared by resuspension of the microsomal pellets in fresh homogenizing medium and further centrifuging for 60 min. Glucose-6-phosphatase (G6Pase) activity (De Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955), cytochromes *P*-450 and b_5 (Omura & Sato, 1964) and protein levels (Lowry, Rosebrough, Farr & Randall, 1961) were determined on the washed microsomal pellet. The spectral interaction of 8 μM-coumarin with hepatic washed microsomes (3 mg protein/ml) was performed as described by Schenkman, Remmer & Estabrook (1967) using a Pye-Unicam SP 1800 dual-beam recording spectrophotometer (Pye-Unicam Ltd., Cambridge). All enzyme assays were checked to ensure linearity of product formation with respect to the incubation time and the homogenate concentration used.

Histochemical study of the liver. Activity of G6Pase (Wachstein & Meisel, 1956) and of aniline hydroxylase (Gangolli & Wright, 1971) was demonstrated on cryostat sections cut at 15 μm. Lysosomal acid phosphatase was demonstrated on liver tissue fixed in formal–calcium using a Gomori technique (Gomori, 1952).

Electron microscopy. Cubes of liver (1 mm^3 were fixed in 1% osmium tetroxide in 0·1 M-phosphate buffer (pH 7·4) for 1 hr at 4°C. The cubes were dehydrated in two changes of acidified dimethoxypropane and embedded in Epon resin. Selected areas were cut on an LKB ultramicrotome and ultrathin sections were stained with uranyl acetate and lead citrate and examined in an AEI EM 6B electron microscope.

RESULTS

The individual body weights of the animals varied widely at the start of the experiment, presumably because of the different ages and species of baboon used. This variation remained evident during the course of the experiment and no effect on weight gain from the feeding of coumarin could be detected.

In the baboon whose daily intake of coumarin was increased from 22·5 to 67·5 mg/kg/day at 18 months, the body and organ weights and the values for the various biochemical parameters were similar to those of the controls, and no abnormality was seen in the ultrastructure of the liver. This animal has not been considered, therefore, in any of the following analyses.

There was wide variation in the terminal organ weights, reflecting the differences in the individual body weights of the animals. The mean liver weights of the groups fed 22·5 and 67·5 mg coumarin/kg/day were heavier than those of the controls. However, the relative liver weights for the group fed 22·5 mg/kg/day were similar to those of the controls and it was only in animals fed 67·5 mg/kg/day that the mean relative liver weight was significantly higher ($P < 0·05$ by Student's t test) than the control value (Table 1).

Histological examination of tissues taken at autopsy and at the 6- and 10-month liver biopsies revealed no differences between control and treated animals. The activity of G6Pase demonstrated histochemically at 16, 18 and 24 months and of aniline hydroxylase at 16 months was the same in control and treated animals. Neither was there any detectable difference between the various groups in the activity of acid phosphatase at 10 months or during the terminal kill. At month 6 there was more acid-phosphatase activity in the liver tissue of the two animals fed 67·5 mg/kg/day than in the corresponding con-

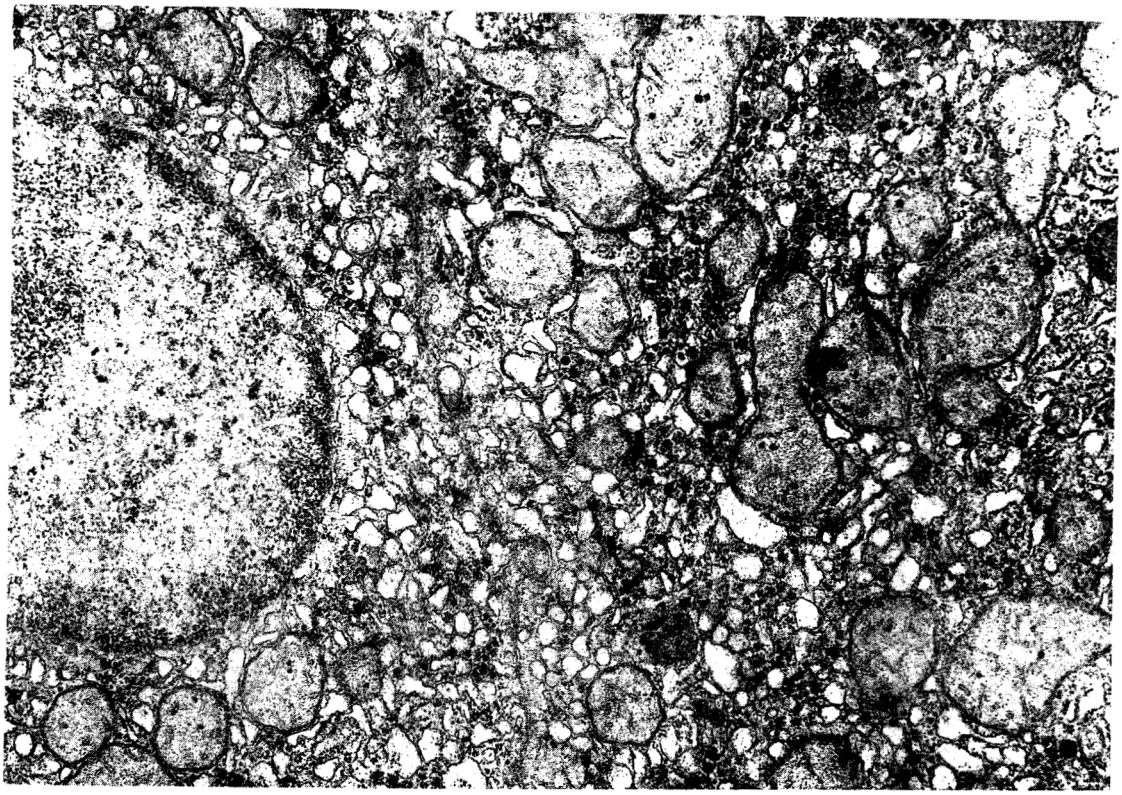

Fig. 1. Ultrastructural appearance of liver from an untreated baboon, showing the prominently vesicular endoplasmic reticulum. × 17,000.

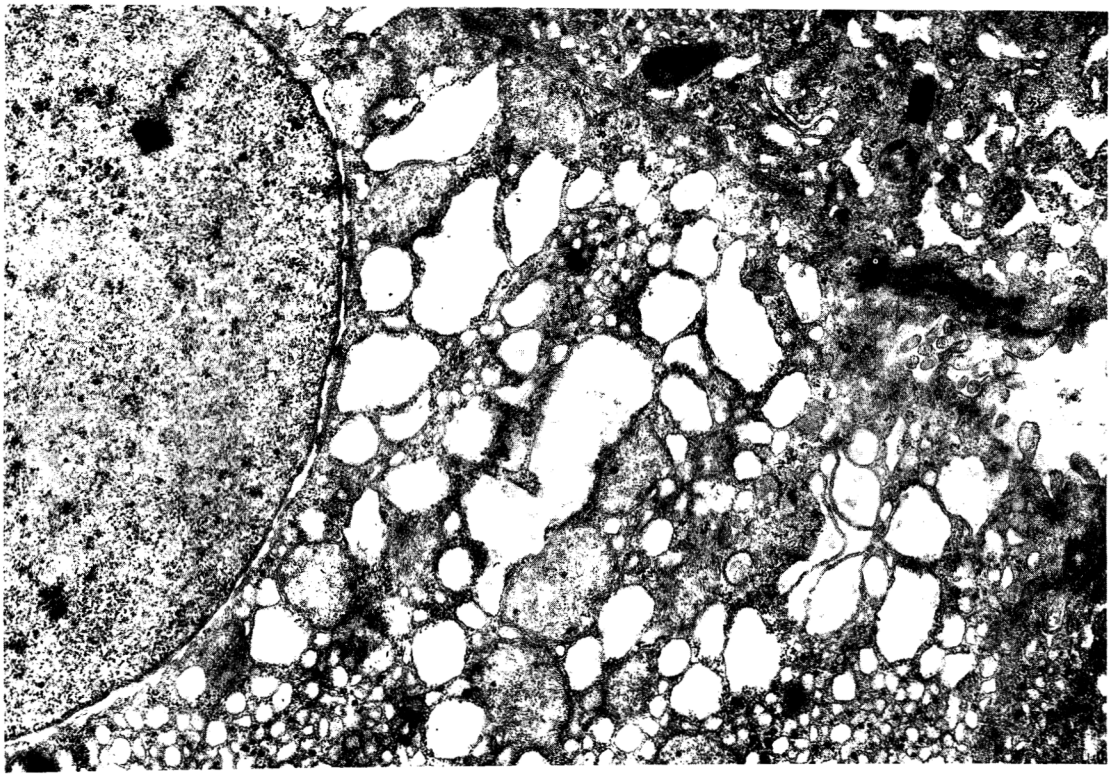

Fig. 2. Ultrastructural appearance of liver from a baboon fed coumarin in a daily dose of 67·5 mg/kg for 16 months, showing, in particular, a pronounced dilatation of the endoplasmic reticulum. × 17,000.

Table 1. *Hepatic biochemical parameters and body and liver weights of baboons for 0–67·5 mg coumarin/kg/day for 2 yr*

Parameter	Values* for baboons fed diets providing a coumarin intake (mg/kg/day) of			
No. of animals examined...	0 7	7·5 4	22·5 4	67·5 3
Ethylmorphine *N*-demethylase (μmol/g liver/hr)	18·1 (13·6–23·5)	18·0 (15·6–19·2)	16·3 (12·0–19·2)	14·1 (7·5–19·7)
Aniline 4-hydroxylase (μmol/g liver/hr)	3·0 (2·1–4·4)	2·9 (2·1–3·7)	2·8 (2·4–3·3)	2·2 (1·9–2·6)
Coumarin 7-hydroxylase (μmol/g liver/hr)	2·0 (0·5–3·2)	2·8 (0·7–4·6)	2·6 (1·8–3·4)	1·4 (0·4–2·4)
Glucose-6-phosphatase (μmol/g liver/hr)	6·7 (4·5–8·1)	6·6 (5·9–8·2)	6·6 (4·8–8·0)	6·5 (5·8–7·0)
Cytochrome *P*-450 (ΔE/g liver)	4·7 (4·0–5·4)	4·6 (3·5–6·4)	4·6 (3·5–5·4)	5·6 (7·7–7·6)
Cytochrome b_5 (ΔE/g liver)	2·8 (2·3–3·1)	2·6 (2·0–3·5)	3·0 (2·8–3·3)	2·8 (2·0–3·3)
Microsomal protein (mg/g liver)	32·8 (29·8–38·4)	30·8 (27·4–35·7)	33·9 (31·8–36·3)	34·6 (28·0–38·2)
Coumarin binding (ΔE/3 mg microsomal protein)†	0·047 (0·031–0·064)	0·050 (0·048–0·052)	0·035 (0·025–0·042)	0·031 (0·013–0·040)
Body weight (kg)	22·3 (12·5–30·0)	17·3 (12·0–19·5)	26·1 (12·5–33·0)	20·0 (15·0–26·5)
Absolute liver weight (g)	327 (262–402)	283 (278–306)	410 (345–520)	403 (346–447)
Relative liver weight	15·4 (10·4–21·0)	17·3 (14·8–23·2)	17·0 (13·0–27·6)	20·75 (17·2–27·6)

*Means for the numbers of animals stated, with the range in parentheses.
†Substrate concentration 8 μM.

trols, but at that time the activity of this enzyme in the control animals was particularly low, while that in the two animals on the top dose was similar to that seen in all groups at 10, 16, 18 and 24 months.

Ultrastructurally the hepatocyte of the untreated baboon was similar to that of man (Rohr, Luthy, Gudat, Oberholzer, Hysin, Stadler & Bianchi, 1976) and there was no difference between the various species of baboon examined. Unlike that of the rat, the endoplasmic reticulum of control baboons was prominently vesicular (Fig. 1). This appearance was also seen in all treated groups with the exception of the group fed 67·5 mg coumarin/kg/day. In this group, the endoplasmic reticulum showed slight hypertrophy in the liver biopsy samples taken from two animals at month 6 and moderate dilatation in the tissue taken from the other two animals at month 10. In the liver of the animal from this group killed at month 16, the dilatation was more pronounced and affected a larger number of cells (Fig. 2). A similar increase was seen in two of the three baboons killed after 2 yr on 67·5 mg/kg/day. No changes were observed in the structure of the lysosomes or mitochondria and there was no increase in the number of autophagic or fat vacuoles.

The values for the various hepatic biochemical parameters for the animals killed after ingesting coumarin for 2 yr are shown in Table 1. The activity of two drug-metabolizing enzymes, ethylmorphine *N*-demethylase and aniline hydroxylase, and the binding of coumarin by cytochrome *P*-450 were lowered in animals fed 67·5 mg coumarin/day for 2 yr. However, because of the wide variation in values from individual animals, the difference from the control values was not statistically significant. In addition, indications at 16 months of a reduction in G6Pase activity in coumarin-treated animals were not seen at 24 months, the mean values for control and treated animals being comparable at that time.

DISCUSSION

Extensive pathological changes have been reported in rats fed coumarin in the diet at levels of 2500 ppm (equivalent to approximately 125 mg/kg/day) and above (Hagan *et al.* 1967; Hazelton *et al.* 1956). These changes involved fatty change and severe cholangiofibrosis. In addition, Bär & Griepentrog (1967) and Griepentrog (1973) claim to have found biliary carcinomas in male rats fed coumarin at a level of 5000 ppm for 2 yr. However, review of the slides from the latter study has suggested that the lesion was probably a marked biliary hyperplasia and fibrosis (P. Grasso and J. G. Evans, unpublished observations 1976), similar to the lesion reported by Hagan *et al.* (1967).

In the present study, no evidence of biliary hyperplasia or fibrosis was seen although coumarin was fed to the baboons at maximum levels compatible with normal food consumption. However, examination of the ultrastructure of the liver showed marked dilatation of the endoplasmic reticulum in three of the animals fed 67·5 mg/kg/day. Such changes are generally accepted as evidence of early cell damage and are among the first structural changes seen in the course of the intracellular oedema induced by a number of compounds, including carbon disulphide and carbon tetrachloride (Butler, Chandra & Magos,

1974; Ghadially, 1975; Reynolds, Ree & Moslen, 1972). That such changes were not seen in all the animals fed 67·5 mg/kg/day may have been due partly to variations in the response of individual animals to coumarin.

Biochemical studies of the liver showed marked variation from animal to animal in both control and treated groups. Hence, although the mean values for a number of parameters, particularly in the group fed 67·5 mg coumarin/kg/day were lower than the corresponding control values by as much as 20%, the differences were not statistically significant.

Histochemical evidence tends to support the view that coumarin, when fed at the levels used in this study, has little or no effect on the activity of liver enzymes. No difference was seen between control and treated animals in the distribution or intensity of reaction of aniline hydroxylase or of G6Pase. In the case of acid phosphatase, wide natural variation occurred in different parts of the same liver, with high activity in the periportal areas. It seems, therefore, that the apparent increase in the activity of this enzyme at month 6 in animals fed 67·5 mg coumarin/kg/day resulted from the taking of biopsy samples from areas of low activity in the control animals.

Thus the increase in relative liver weight seen in animals fed 67·5 mg coumarin/kg/day was associated with hypertrophy and later with dilatation of the endoplasmic reticulum of hepatocytes, and consequent upon these changes there was a marginal reduction in enzyme activity.

The absence of any unequivocal biochemical or histochemical evidence of hepatotoxicity in baboons fed coumarin is in contrast to the situation in the rat, in which a marked depression in G6Pase activity follows dosing with 2–200 mg coumarin/kg/day for 7 days, the reduction being significant with coumarin doses of 10–20 mg/kg/day (Feuer, Golberg & Le Pelley, 1965). Similarly, activity of aniline hydroxylase and lysosomal acid phosphatase is altered in the rat by coumarin treatment, although such changes are restricted to the area of the central vein and only occur when there is evidence of liver enlargement (Grasso, Wright, Gangolli & Hendy, 1974).

That there should be differences in coumarin toxicity is not surprising when the metabolism of the compound by the different species is considered. Coumarin may be hydroxylated at any of positions 3–8 and 3-hydroxylation may be followed by opening of the pyrone ring. There are marked species variations in the quantities of each metabolic product excreted (Gangolli *et al.* 1974; Kaighen & Williams, 1961). The main urinary metabolic product in the rat is *o*-hydroxyphenylacetic acid (Kaighen & Williams, 1961; Van Sumere & Teuchy, 1971), whereas in man and the baboon it is 7-hydroxycoumarin. This difference may partly explain the difference in the effect of coumarin treatment on G6Pase activity in the rat and baboon, as it has been shown that it is the *o*-hydroxyphenolic acids, particularly *o*-hydroxyphenylacetic acid, that depress the *in vivo* and *in vitro* activity of G6Pase, while the hydroxycoumarins, other than 6-hydroxycoumarin, have no inhibitory effect (Feuer, Golberg & Gibson, 1966).

The similarity of the metabolic pathways of coumarin in the baboon and in man suggests that the toxicity study carried out in the baboon is more relevant than those conducted in the rat or the dog to the evaluation of the toxic hazard of coumarin to man. Acceptance of this suggestion would provide, from the current study, a no-effect-level of 22·5 mg coumarin/kg/day.

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Fd Cosmet. Toxicol. Vol. 17, pp. 195 to 200

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RESPONSE OF MALE RATS TO SODIUM SACCHARIN INGESTION: URINE COMPOSITION AND MINERAL BALANCE*

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(*Received 4 November 1978*)

Abstract—Sodium saccharin was fed to weanling male rats for 4 wk at levels up to 7·5% of the diet. The ingestion of saccharin led to dose-related decreases in growth and possibly in feed efficiency. It also caused marked increases in faecal water content, faecal excretion of Na and K, and urinary excretion of Ca, Mg and P. There were dose-related increases in urine volume and decreases in urinary pH. No crystalluria was observed. The concentration of saccharin in plasma was a linear function of the saccharin level in the diet, and at dietary levels of 3% and greater, a dose-related fraction of the saccharin was bound to plasma protein. The blood-cell lysates did not contain measurable levels of saccharin, showing that saccharin does not readily cross cell membranes.

INTRODUCTION

Saccharin can induce bladder cancer in rats when it is added to their diets at high concentrations. This subject has been exhaustively reviewed by Cranmer (1978). A recent report (Arnold, 1977) states that when rats were given diets containing 5% saccharin, they excreted higher than normal amounts of phosphorus in their urine. We have observed, also, that saccharin ingestion causes increased urinary output of magnesium (Anderson, 1977). These increases in urinary phosphorus and magnesium could conceivably result in the formation of crystalline $MgNH_4PO_4$ in the urine (Marshall & Robertson, 1976), an event which could have profound consequences, since compounds that produce crystalluria are frequently associated with bladder carcinoma in rodents (Cranmer, 1978).

When rats consume diets containing enough sodium saccharin to cause bladder cancer (5% in the diet) they also experience massive increases in their intake of sodium (Oser, 1977), and it is possible that such increases in sodium intake may alter the disposition of dietary minerals.

The work described here was designed to reveal whether dietary sodium saccharin affects mineral disposition or urine characteristics during 4 wk of *ad lib.* ingestion by weanling male rats.

EXPERIMENTAL

Animals and diets. Weanling male Charles River rats (from Charles River Breeding Laboratories, Wilmington, MA) were acclimatized to Purina Laboratory Chow (The Ralston Purina Co., St. Louis, MO) for 3 days and were then randomly assigned to five groups of ten rats. Their mean body weight at that time was 59 ± 1 g. They were housed in individual stainless-steel cages designed to separate urine and faeces, and were kept at 22 ± 2°C and 50 ± 10% relative humidity, with alternating 12-hr periods of light and dark. They were allowed food and water *ad lib.* The diets were prepared by mixing sodium saccharin (Lot no. 1648 from Sherwin-Williams Co., Cleveland, OH) with the ground laboratory Chow at levels of 0, 1, 3, 5 and 7·5%. Diets were made isocaloric by adding 7·5, 6·5, 4·5, 2·5 and 0% cellulose (Cellufloor, from Chicago Dietetic Supply House, Chicago, IL).

Conduct of experiment. Weekly records of weight and food consumption were kept. On days 4, 11, 18 and 25 from the beginning of the saccharin feeding, total 24-hr urine was collected from each animal. The appearance and volume of each sample was recorded, its pH was determined on a pH meter, and other characteristics were assessed by testing with Multistix (Ames Co., Elkhardt, IN). On day 25, all animals were fitted with anal cups for collecting faeces. During the next 3 days, daily feed intake and body weights were recorded and faeces and urine were collected. The faeces were dried to constant weight *in vacuo* at 30°C.

On day 28 each animal was anaesthetized with 50 mg sodium pentobarbital/kg given ip. A mid-line incision was made and blood was collected in heparinized syringes via an aortic puncture. The cells were separated by centrifugation, pooled within each dietary group, washed three times with 0·9% saline and lysed with 3 vols distilled water. The cell membranes were removed by centrifugation.

Analyses. Samples of each diet and of faeces, urine, pooled plasma and blood cell-lysate solubles were wet ashed and analysed for sodium, potassium, calcium and magnesium by atomic absorption spectrometry† and for total phosphorus by the autoanalyser method†. Other samples were analysed for saccharin by high-performance liquid chromatography on μBondapak CN (Waters Associates, Milford, MA), 10 μm size. The solvent was 6% aqueous acetic acid

*A preliminary report of this work was presented at the 'Saccharin Update Special Session' of the Toxicology Forum held at Aspen, CO, on 23 July 1978.

†Details of the analytical procedures may be obtained from the author.

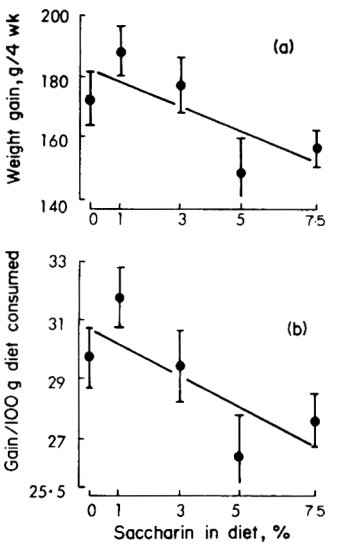

Fig. 1. Effect of dietary sodium saccharin concentration on (a) weight gain and (b) feed efficiency in male rats during a 4-wk feeding period. Regression equations are: for (a), $\hat{y} = 182 - 4{\cdot}1\,X$ ($P = 0{\cdot}009$), and for (b), $\hat{y} = 30{\cdot}7 - 0{\cdot}53\,X$ ($P = 0.007$).

and saccharin was detected by UV absorption at 254 nm. Urine was analysed directly, diets and faeces were extracted with water, and plasma and blood-cell lysates were first treated with nine times their weight of 5% trichloroacetic acid to precipitate proteins. A sample of each pooled plasma was filtered through an Amicon PM10 filter (Amicon Corp., Lexington, MA) and then reanalysed to determine the amount of saccharin that was bound to protein. Urine samples were also analysed for NH_3 and urea, the urea analyses being carried out by VetPath, Hackensack, NJ.

The data were analysed by analysis of variance and for linear regression of each variable with saccharin dosage by means of standard statistical programs*.

RESULTS

Analyses of the diets showed that they contained very nearly the intended quantities of saccharin, and that the concentrations of saccharin did not change while the diets were stored during the feeding period.

Of the animals receiving 7·5% saccharin in their diets, one died on day 4; the others survived all 28 days. Five of these ten animals had haemoglobin in their urine on day 4. All of these animals had diarrhoea for the first 10–14 days of the study, but by day 14 the diarrhoea had abated, although the stools remained soft. Only two urine samples collected on day 11 contained haemoglobin.

Saccharin ingestion at 1% in the diet increased growth but, overall, dietary saccharin resulted in statistically significant dose-dependent decreases in weight gain (Fig. 1a) and in feed efficiency (Fig. 1b). Part of the reduction in feed efficiency, however, may have been caused by the animals' scattering of their diets, rather than by saccharin-induced energy wasting.

*Details of the statistical analyses and programs may be obtained from the author.

Figure 2 shows the daily intake of saccharin as a function of dietary concentration and time. The daily saccharin intake, expressed in g/kg body weight/day, was initially about twice the percentage concentration of saccharin in the diet, and, although it decreased with time, it remained higher than the percentage of saccharin in the diet throughout the entire 28-day feeding period.

The daily urine volume was elevated only among the animals consuming 7·5% saccharin (Fig. 3a). The urine from all animals, including the controls, increased in pH during the study, but the presence of saccharin in the diet diminished this pH change in a dose-related manner (Fig. 3b).

Balance phase (days 26–28). Weight gains during the 3-day balance period were independent of the saccharin concentration in the diet and ranged between 2 and 30 g. The placement of anal cups did not alter diet consumption, since the average daily intake during the balance period did not differ from that in the 4 days before the cups were placed.

Figure 4 shows the mean weights of faeces excreted during the 3-day balance period. The weight of fresh faeces/g of diet increased in a linear manner with increasing saccharin concentration in the diet, but the dry weight was constant at 0·25 g/g diet for all treatments. Thus all of the increase in faecal mass associated with saccharin ingestion was due to an increase in water content. In contrast, the urine volume was affected much less (Fig. 5). Even the animals receiving 7·5% sodium saccharin in their diet voided only 50% more urine than the controls. But the pH of the urine was markedly lowered in animals consuming saccharin (Fig. 5).

Figure 6 shows the effects of feeding saccharin on the recovery of ingested minerals in the faeces and urine during the 3-day balance period. Increasing the concentration of dietary saccharin resulted in a significant dose-dependent increase in the fraction of the ingested Na that was voided in the faeces and a concomitant decrease in the fraction that was voided in the urine.

All levels of saccharin caused a statistically significant, but dose-independent, increase in the fraction of ingested K that was voided in the faeces, and a

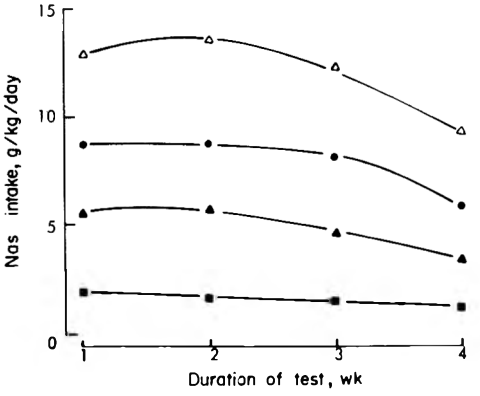

Fig. 2. Sodium saccharin (NaS) ingestion (calculated from the disappearance of diet from the feed cups and the average weekly body weight) by male rats fed diet containing 1 (■), 3 (▲), 5 (●) and 7·5% (△) sodium saccharin in the diet.

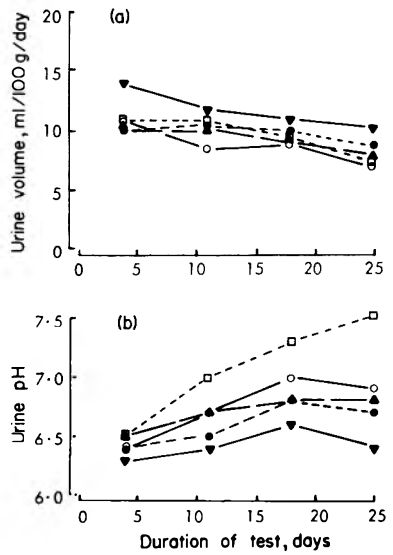

Fig. 3. Volume (a) and pH (b) of urine of male rats as a function of duration of consumption of diets containing 0 (□), 1 (○), 3 (▲), 5 (●) and 7·5% (▼) sodium saccharin. Values are means for groups of ten rats, except that for the 7·5% diet which was calculated on nine.

dose-dependent decrease in urinary K. Saccharin ingestion also resulted in statistically significant dose-dependent increases in the fractions of Ca, Mg and P recovered in the urine, and there were concomitant decreases in the faecal excretion of Mg and P, but not of Ca. The animals receiving the 7·5% saccharin diet excreted about twice as much of their ingested Ca, Mg and P in urine than did the control animals.

Since saccharin ingestion resulted in a dose-dependent decrease in urinary pH, the individual urine samples were assayed for ammonia and pooled samples were assayed for urea. Figure 7 shows that the urinary ammonia decreased and urea increased as a function of saccharin ingestion.

Both the percentages of ingested minerals recovered in the faeces plus urine and the weight gains were independent of saccharin dose except at the 7·5% level, which caused a doubling of the carcass retention of K, Ca, Mg and P, calculated as the intake minus the total excreted in urine plus faeces. Thus retentions of K, Ca, Mg and P per gram of weight gain were approximately constant, but the retention of Na was markedly increased, since its ingestion increased as a function of saccharin concentration in the diet.

The ingestion of saccharin did not result in any dose-dependent shifts in mineral levels of the plasma or of the blood-cell cytosol (Table 1). Although there was an apparent dose-dependent increase in the carcass retention of Na, this was not reflected in the concentration of Na in either the plasma or the blood-cell cytosol.

Saccharin recovery in the urine and faeces as a function of dose is presented in Fig. 8. The saccharin determinations were conducted on pooled samples of urine and faeces so that the variation between individual animals was not indicated. The saccharin recovered in the urine and the faeces was a linear function of the dietary level and the two pools were essentially equal. The sum of the urinary and faecal saccharin accounted for only 65–90% of the ingested load, and the remainder was not accounted for.

The total concentration of saccharin in plasma was approximately a linear function of the dietary concentration (Fig. 9). When the plasma samples were filtered through membranes designed to retain species of mol wt > 10,000, the fraction of the saccharin that passed through the filter was variable. At the lower plasma-saccharin concentrations, all of the saccharin was filterable, but at higher plasma concentrations a significant portion of it was retained with the material of mol wt > 10,000. When control plasma was spiked with sodium saccharin at 80 μg/ml, the filtrate had a saccharin concentration of 73 μg/ml, showing only slight binding of the saccharin to protein.

None of the blood-cell cytosol samples yielded measurable levels of saccharin, so the concentration of saccharin was <0·5μg/ml of cells, even when the plasma contained >90 μg/ml.

DISCUSSION

The one area where the results reported in this study do not agree with other workers (Ball, Renwick & Williams, 1977) is in the percentage of the ingested

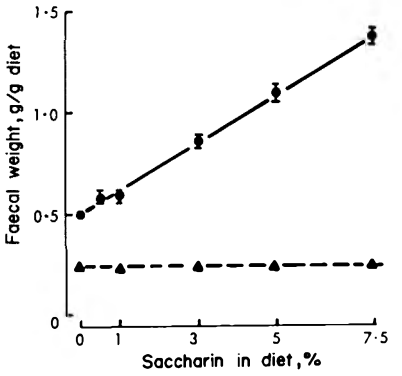

Fig. 4. Fresh weight (●) and dry weight (▲) of faeces of male rats in relation to the saccharin diet consumed. The values (means ± SEM for ten rats, or for nine at 7·5%) were obtained during a 3-day period (days 28–30) when the animals were fitted with anal cups. For the dry weights, the SEM bars are within the symbol in each case.

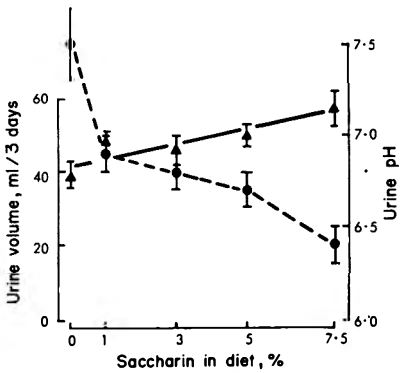

Fig. 5. Volume (▲) and pH (●) of samples collected over the 3-day period when rats fed 0–7·5% saccharin in the diet were fitted with anal cups for faecal collection. Each value is the mean ± SEM for ten rats (nine at 7·5%).

dose recovered in the urine. The recovery of saccharin added to control urines was excellent, suggesting that the assay we used was adequate, and there are four possible causes for the reduced recovery:

(1) Diet scattering may have resulted in an over-estimation of saccharin intake.

(2) The fitting of anal cups may have impaired gastro-intestinal emptying (gastro-intestinal contents were not assayed for saccharin).

(3) The published assessment of urinary saccharin in animals pre-fed saccharin was based on ^{14}C recovery in the urine following oral gavage (Ball *et al.* 1977). It is possible that gavaged saccharin may be more completely absorbed than saccharin entering the gut mixed in a 'diet.

(4) It is possible that the saccharin unaccounted for was in either the faeces or urine in an altered form not detectable by the analytical technique used.

The resolution of this problem will require additional experimentation.

The observation of transient diarrhoea and haemoglobinuria in the animals fed the diet containing 7·5% saccharin suggests that this dose of saccharin may have altered the gut flora. In support of this contention, it has been noted that saccharin feeding does result in a marked increase in the size of the caecum (T. Lawson, personal communication 1978), a response frequently observed when the gut flora are disturbed or eliminated (Gordon, 1968). Further support for the possibility that saccharin may interfere with microbial populations is the finding that, at concentrations >20 mg/ml, sodium saccharin was toxic

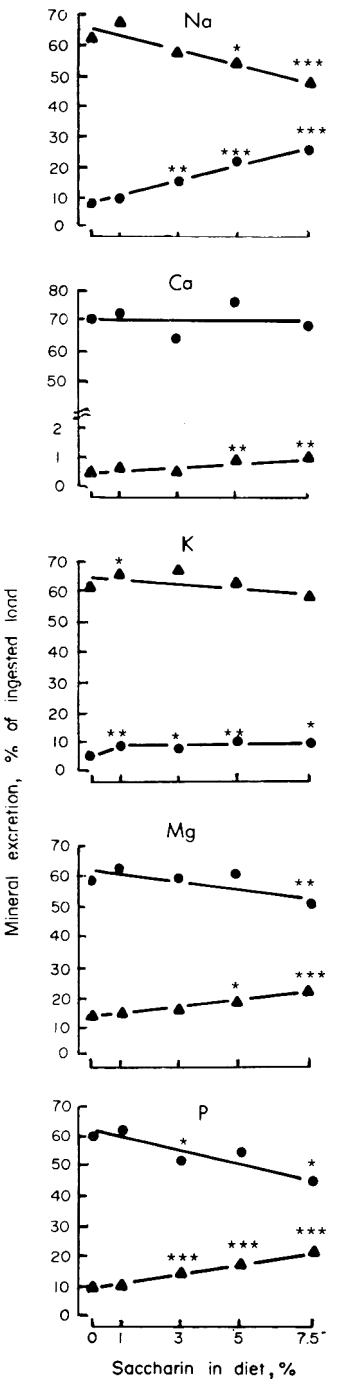

Fig. 6. Effect of dietary sodium saccharin concentration on the percentage of ingested minerals excreted in the urine (▲) and faeces (●) of male rats during a 3-day balance period. Values marked with asterisks differ significantly from the controls: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$).

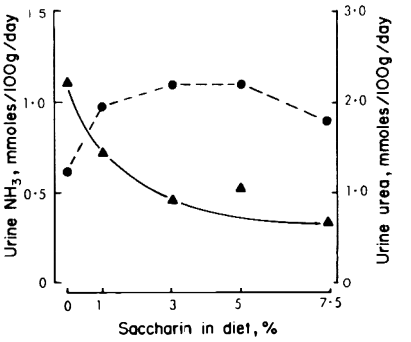

Fig. 7. Effect of sodium saccharin concentration in the diet of male rats on the levels of ammonia (▲) and urea (●) in urines collected during a 3-day balance study when anal cups prevented faecal contamination of the urine.

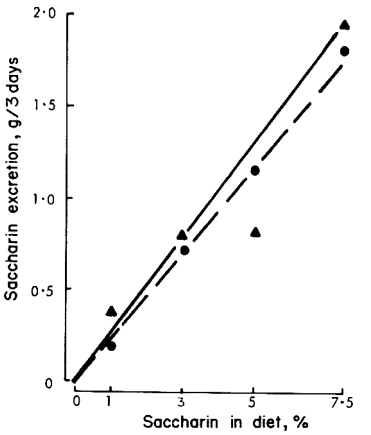

Fig. 8. Mean total urinary (▲) and faecal (●) excretion of sodium saccharin during a 3-day balance period by male rats fed 0–7·5% sodium saccharin in the diet.

Table 1. *Mineral concentrations in plasma and blood-cell lysate from rats consuming diets containing sodium saccharin*

Dietary level of NaS (%)	Concentration of minerals (mM) in pooled sample from each treatment group Na	K	Ca	Mg	P
	In plasma				
0	116	4·6	2·2	0·51	4·7
1	119	4·4	2·2	0·47	4·7
3	116	4·2	2·1	0·45	4·5
5	121	4·2	2·2	0·48	4·3
7·5	117	4·3	2·2	0·52	4·5
	In blood-cell soluble pool*				
0	57·7	102	0·13	2·98	31·5
1	58·6	104	0·18	3·00	31·7
3	57·2	97	0·15	2·92	31·3
5	49·2	94	0·14	2·88	29·5
7·5	51·0	99	0·14	3·03	34·1

NaS = Sodium saccharin
*Cells lysed with 3 vols distilled water and centrifuged to remove membranes.

to the Salmonella mutants used in the Ames assay (Office of Technology Assessment, 1977). If we calculate the saccharin ingestion from diet intake and assume that water intake is equal to faecal water plus urine volume, the potential sodium saccharin concentrations in the gastro-intestinal contents attain levels of approximately 40 mg/ml. This calculated value is based on the assumption that the entire diet and water consumed in a 24-hr period are in the gut at the same time, and is thus probably a minimal concentration value. The effect of saccharin on the gut flora is currently under study.

One hypothesis tested in this study was that sodium saccharin ingestion would result in an increase in the urinary concentrations of Mg and PO_4, thereby causing crystalline $MgNH_4PO_4$ to form in the urine. Table 2 shows that saccharin feeding did result in a dose-associated increase in the urinary concentration of P (presumably PO_4) but not of Mg, and visual inspection did not reveal crystalline material in any of the five urine samples collected on days 4, 11, 18, 25 and 26–28 for each animal. Furthermore, the reduction in urinary pH and the dose-dependent decrease in urinary ammonia would minimize the probability that crystalline $MgNH_4PO_4$ would form. This study does not, however, preclude the possibility that saccharin could result in crystalluria in mature animals when the competition of growth for dietary Mg and P would be reduced.

It was unexpected to find a large fraction of the ingested Na being excreted in the faeces. When the animals in an earlier study (Anderson & Kanerva, 1978) were fed a comparable level of Na as sodium acetate, none of the excess Na ingested appeared in the faeces; thus the high faecal Na in saccharin-fed animals was a consequence of saccharin feeding and not simply a result of excess Na ingestion. This conclusion is strengthened by the approximately 1:1 (molar) ratio of sodium and saccharin in the faeces at dietary saccharin concentrations of 3, 5 and 7·5%.

If one assumes that the minerals ingested but not recovered in either the urine or faeces were retained in the carcass, it can be reasoned that the carcass K/Na ratio was markedly altered by saccharin inges-

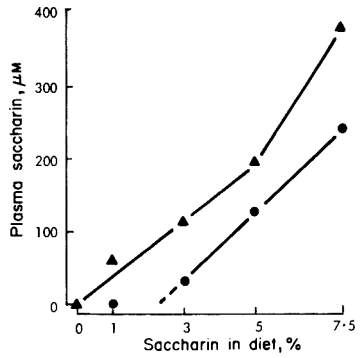

Fig. 9. Total (▲) and filterable (●) sodium saccharin concentrations in the plasma of male rats as a function of dietary concentration of sodium saccharin. Values were determined after *ad lib.* ingestion for 28 days.

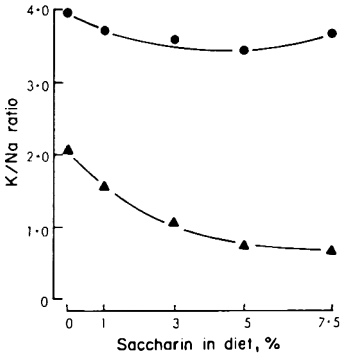

Fig. 10. Calculated K/Na ratios in minerals retained in the carcass (intake minus total excreted in urine and faeces) during a 3-day balance study and in those present in the plasma after sodium saccharin ingestion for 28 days at levels of 0–7·5% in the diet.

Table 2. *Urinary total P and Mg concentrations as a function of dietary sodium saccharin concentration*

Dietary level of NaS (%)	Concentration (mM) in urine	
	Mg	P
0	1·89	4·36
	1·73	4·10
3	1·95	6·14
5	1·79	5·96
7·5	2·10	6·80

NaS = Sodium saccharin

tion (Fig. 10). The plasma mineral analyses, however, do not show such a marked effect of saccharin on the K/Na ratio.

The low level of saccharin in the blood-cell cytosol, even when the animals were fed 7·5% sodium saccharin, shows that saccharin does not easily cross cell membranes, and therefore that most of the systemic saccharin is probably in the extracellular water pool. This distribution should certainly be considered in any evaluation of the possible mechanisms by which extreme doses of saccharin induce cellular perturbations both *in vitro* and *in vivo* (Cranmer, 1978).

The plasma saccharin concentration values (Fig. 9) agree very well with reported values (Renwick, 1977). The plasma binding of saccharin that was noted in this study does not agree with the results reported for iv-infused saccharin (Bourgoigne, Hwang & Bricker, 1977), where only 3% of the plasma pool was protein bound. We have noted a similar difference in plasma-protein binding between iv-infused and dietary forms of another anionic molecule, nitrilotriacetate (R. L. Anderson, unpublished data 1978). Such extensive binding of saccharin to plasma protein (63% of the total at the highest dose) would certainly influence the renal clearance of saccharin. Furthermore, the lack of protein binding at the lowest dose suggests that this phenomenon has a threshold, in the sense that significant binding did not occur until the systemic load exceeded that produced by 1% dietary sodium saccharin. The threshold systemic load is estimated, from the urine saccharin values obtained in this study, to be about 0·85 g/kg/day.

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INTESTINAL ABSORPTION OF TWO POTENTIAL POLYMERIC FOOD ADDITIVES IN MAN

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Abstract—Intestinal absorption of two ^{14}C-labelled polymeric compounds, an antioxidant (D00079) and a red colouring (D00478) being developed for use in foods, was measured in healthy adult male volunteers. Two groups of six subjects each ingested 50 μCi of either compound in gelatin capsules. Blood, urine and faecal samples were collected at intervals for 4 days following ingestion and were analysed for radioactivity by combustion analysis or direct scintillation spectrometry. No radioactivity was detected in any blood samples with either compound. The 96-hr urine samples contained an average of 0·02 and 0·04% of the administered doses of D00079 and D00478, respectively. Faecal recoveries after 96 hr were 93·7% for D00079 and 79·0% for D00478. There was no correlation between faecal recoveries and urinary excretion for either compound. Blood and urine data indicate that both compounds were only minimally absorbed in man, confirming earlier results in rats, mice, guinea-pigs and rabbits.

INTRODUCTION

One approach to the problem of toxic chemicals in our food supply is the systematic removal of current additives and this is being done in some cases. An alternative is the development of new, stable compounds that are only minimally absorbed from the gastro-intestinal tract and, hence, have potentially greater safety. To minimize absorption of certain types of food additives, polymeric derivatives that retain the essential properties of their monomeric equivalents but are more chemically and biologically stable, have been synthesized (Dawson, Otteson, Wang & Wingard, 1978; Furia, 1977; Furia & Bellanca, 1977).

Two of these compounds, D00079 (Poly AO™-79, Dynapol), a polymeric antioxidant for stabilizing fats and oils and D00478 (Poly R™-478, Dynapol), a food colouring, have been selected for further development. ^{14}C-Labelled D00079 was found to be minimally absorbed (0·1–0·6%) in rats and mice (Parkinson, Honohan, Enderlin, Halladay, Hale, de Keczer, Dubin, Ryerson & Read, 1978) and in guinea-pigs and rabbits (Parkinson, Halladay & Enderlin, 1978). Radiolabelled D00478 demonstrated a similar low level of absorption in rats and mice (Furia, 1977) and in guinea-pigs and rabbits (T. M. Parkinson, 1978, unpublished data). Both of these compounds are non-toxic in acute studies in rats, mice and dogs at single oral doses as high as 10 g/kg body weight by gavage and in sub-chronic feeding studies in rats at doses of up to 5% in the diet (T. M. Parkinson, 1978, unpublished data). They are also non-mutagenic in several test systems *in vitro* (Brown, Brown & Roehm, 1977). We therefore determined their intestinal absorption in normal human volunteers for purposes of comparison with results from the experimental animals.

EXPERIMENTAL

Test compounds. ^{14}C-Labelled D00079 and D00478 were synthesized by the Radiochemistry Department, Dynapol, and all polymer characterization was carried out at Dynapol. D00079, a divinylbenzene-hydroquinone-phenols condensation polymer was prepared by the polymerization of hydroxyanisole, *tert*-butylhydroquinone, bisphenol A, *p-tert*-butylphenol and *p*-cresol with ^{14}C-labelled divinylbenzene/ethylvinylbenzene (DVB; Parkinson *et al.* 1978). The apparent peak molecular weight of this preparation by gel permeation chromatography (GPC) was 4500 relative to polystyrene standards, with less than 0·5% of the radioactivity below molecular weight 1000. [^{14}C]D00478 was prepared by covalently linking a [^{14}C]anthrapyridone chromophore to an acetylated polyaminoethylene–sodium ethylene sulphonate copolymer backbone (Brown, Brown, Hyde & Bakner, 1978). Apparent peak molecular weight by GPC was 4×10^4 relative to polystyrene sulphonate standards, with only 0·05–0·2% low molecular weight impurities.

Clinical protocol. Healthy male volunteers, aged 22–34 yr, were selected for the studies. Volunteers were questioned to be sure each had sufficient knowledge of chemistry and radiochemistry to understand the structures of the compounds and the risks of ^{14}C-labelled compounds. They all signed informed consent forms approved by the University Human Subjects Committee. Training sessions were held to

describe procedures, demonstrate equipment and to provide useful hints on remembering collections. Prior to and within 2 wk after completion of the study, all subjects had a physical examination and laboratory examinations including an electrocardiogram, urinalysis, examination of stools for blood, ova and parasites, determination of serum electrolytes, liver function tests, and determination of blood urea nitrogen and creatinine. All subjects collected urine over a 24-hr period and a single pre-study stool specimen for baseline studies. Subjects fasted from midnight on the day before the study until 5 hr after ingesting the compounds, when they were allowed to continue their usual diets *ad lib.*

Six subjects ingested 50 μCi of D00478 as a powder in a hard gelatin capsule and six other subjects ingested 50 μCi of D00079 dissolved in a food-grade vegetable oil in a hard gelatin capsule. Each dose was followed by eight ounces of vanilla flavoured Instant Breakfast® (Carnation Corp., Los Angeles, CA) mixed in whole (3·8% butter-fat) milk.

Blood samples were collected into EDTA prior to and 0, 2, 4, 8, 12, 24, 48, 72 and 96 hr after ingestion. All urine and stool specimens were collected for 96 hr after ingestion. Each stool was collected separately and the time was recorded. Subjects were instructed to save all toilet paper with stool samples. Urine was collected in the following periods: 0–12, 12–24, 24–48, 48–72 and 72–96 hr.

Radioassay of samples. Each whole blood sample was analysed for radioactivity by oxidizing triplicate 1 ml aliquots in Combustocups and Combustocones (Packard Instrument Co., Chicago, IL) using a Packard Tri-Carb Model 306 Sample Oxidizer. The evolved CO_2 was trapped in 7-ml Carbosorb (Packard Instrument Co.) with 12-ml Permafluor (Packard Instrument Co.) added as the scintillation fluid. Individual faecal samples and toilet paper were homogenized in a Waring Blender at high speeds for 2 min using three volumes of water. Triplicate 0·5-gm homogenate samples were oxidized as for blood. For urine, triplicate 5-ml aliquots were suspended in 10 ml Instagel (Packard) and the radioactivity was counted directly. All samples were counted for 10 min with a Beckman LS 100 C Liquid Scintillation Counter (Beckman Instruments, Inc., Fullerton, CA). The total counts per min (cpm) were corrected for quench using the external standard method to convert to absolute radioactivity (dpm).

RESULTS

No blood sample from any subject contained above background radioactivity counts. The detection limit for blood was 0·1% of the administered dose in the entire blood volume. Total urinary radioactivity accounted for less than 0·002% for D00079 (Table 1).

Faecal recovery averaged 79% for D00478 and 93·7% for D00079 (Table 2). If the results are computed omitting Subjects 2, 3 and 10, the faecal recoveries were 95·8% for D00478 and 97·8% for D00079. Subject 2 claimed to have had only two stools during the study, and he discarded the next stool he had 1 wk after the termination of the study. Subject 3 admitted that he had forgotten to collect one or two stool samples and had no 96-hr sample. Subject 10 and 14 loose stools during the study period and discarded his toilet paper, but denied forgetting any stool samples.

No adverse effect was seen in any subject.

DISCUSSION

Blood and urine radioactivity data were consistent with minimal absorption of both compounds. The amount excreted in the urine cannot be assumed to be the total amount absorbed into the circulation as

Table 1. *Recovery of radioactivity in urine after oral administration of [^{14}C]D00478 and [^{14}C]D00079*

Subject	Time after dosing (hr)...	Urinary radioactivity (% of dose) 12	24	48	72	96	Total
		D00478*					
1-BJB		0·033	0·005	0·009	0·000	0·001	0·048
2-RRW		0·018	0·006	0·004	0·000	0·000	0·028
3-JPS		0·027	0·012	0·013	0·006	0·005	0·063
4-GRB		0·023	0·008	0·006	0·003	0·000	0·040
5-GRK		0·015	0·008	0·007	0·001	0·001	0·032
6-DRD		0·016	0·011	0·004	0·004	0·001	0·036
	Mean	0·022	0·008	0·007	0·002	0·001	0·041
		D00079†					
7-DBD		0·010	0·003	0·000	0·000	0·001	0·014
8-BJD		0·004	0·003	0·000	0·000	0·000	0·007
9-RDS		0·012	0·001	0·001	0·001	0·000	0·015
10-MSK		0·013	0·002	0·000	0·006	0·000	0·021
11-GLL		0·008	0·008	0·001	0·000	0·001	0·018
12-BDB		0·011	0·000	0·000	0·005	0·006	0·022
	Mean	0·010	0·003	0·000	0·002	0·001	0·016

*Subjects 1–6 received approximately 50 μCi [^{14}C]D00478 (specific activity 1·01 μCi/mg).
†Subjects 7–12 received 50 μCi of [^{14}C]D00079 (specific activity 3·6 μCi/mg).

Table 2. *Recovery of radioactivity in faeces after oral administration of [^{14}C]D00478 and [^{14}C]D00079*

Subject	Time after dosing (hr)...	Faecal radioactivity (% of dose) 24	48	72	96	Total
		D00478*				
1-BJB		0·02	74·12	21·52	2·85	98·51
2-RRW		0·03	—†	28·92	—†	28·95
3-JPS		0·09	57·03	4·42	—†	61·50
4-GRB		45·64	43·02	—†	7·83	96·69
5-GRK		0·04	60·73	25·05	10·39	96·21
6-DRD		0·10	57·61	29·76	4·46	91·96
	Mean	9·18	58·50	16·15	5·11	78·97
		D00079*				
7-DBD		—†	67·84	26·21	0·45	96·67
8-BTD		42·47	21·42	33·80	3·34	101·03
9-RDS		0·11	53·99	21·72	21·46	97·28
10-MSK		41·33	30·25	0·96	0·40	72·94
11-GLL		0·08	81·80	12·82	4·43	99·13
12-BDB		3·39	51·87	35·01	4·64	94·91
	Mean	17·48	51·20	21·75	5·79	93·66

*Subjects 1–6 received D00478 and Subjects 7–12 received D00079. See Table 1 for the doses.

†Dashes indicate that no faecal sample was obtained during the particular collection period.

biliary excretion could not be measured (Halladay, Enderlin, Parkinson & Honohan, 1978). However, the blood detection limit (0·10%) probably represents a maximal absorption value. This absorption may be due to radiolabelled low-molecular-weight species present in the samples. In experimental animals absorbed radioactivity from oral doses of D00079 could be accounted for by absorption of dimers (Parkinson *et al.* 1978).

Faecal recoveries were excellent except for three subjects (2, 3 and 10), all of whom were first time volunteers who were not familiar with continuous stool collection. These low recoveries were probably due to incomplete faecal recoveries rather than to absorption. If absorption of the unrecovered amount had occurred in these subjects, both the urinary excretion and the blood levels would have been expected to be much higher than those of the other subjects. In addition, the daily excretion values for subjects 3 and 10 were consistent with other subjects until day 3 when the ^{14}C-excretion significantly decreased, suggesting incomplete collection.

Each subject excreted some radioactivity in his last faecal sample (Table 2), and in one case as much as 10% of the administered dose (Subject 5). A longer collection period would probably have increased faecal recovery. This was unexpected from previous animal results which showed essentially total excretion within 48 hr in rats, mice, guinea-pigs and rabbits.

These data from humans confirm previous results with experimental animals and suggest that minimum exposure of internal organs should result from ingestion of these polymeric additives in foods.

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RESULTS OF A TWO-YEAR CHRONIC TOXICITY AND ONCOGENIC STUDY OF RATS INGESTING DIETS CONTAINING 2,4,5-TRICHLOROPHENOXYACETIC ACID (2,4,5-T)

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Abstract—Groups of Sprague–Dawley rats (50 males and 50 females) were maintained on diets supplying 3, 10 or 30 mg 2,4,5-T/kg body weight/day for up to 2 yr, with an interim autopsy (on an additional ten males and ten females per group) after 118–119 days. The highest dose level was associated with some degree of toxicity, including a decrease in body-weight gain and increases in relative kidney weight, in the volume of urine excreted and in the urinary excretion of coproporphyrin and uroporphyrin, plus slight morphological changes in the kidney, liver and lungs. The kidney changes involved, primarily, the presence of mineralized deposits in the renal pelvis. Parameters not adversely affected by this dose level included death rate, food consumption, the occurrence of palpable masses, haematological indices (red-cell count, haemoglobin, packed cell volume, total and differential white-cell counts, thrombocytes and reticulocytes), the results of routine urine analyses, urinary excretion of creatinine and δ-aminolaevulinic acid, serum-chemistry values (urea nitrogen, glutamic–pyruvic-transaminase and alkaline-phosphatase activities, bilirubin, total protein, albumin and globulin), weights of organs other than the kidneys, tumour incidence and gross and microscopic morphology of all the organ systems examined, with the exception of those mentioned above. At the intermediate dose level (10 mg/kg/day) only minimal effects were noted, primarily an increased incidence of mineralized deposits in the renal pelvis and, in the males and only during the early phase of the study, an increase in urinary excretion of coproporphyrin. At the lower dose level (3 mg/kg/day) there were no changes that were considered to be related to treatment throughout the 2-yr period. Thus, this study revealed no oncogenic response in rats, even when the duration of 2,4,5-T administration extended over most of their lifespan at a dosage high enough to induce toxicity.

INTRODUCTION

2,4,5-Trichlorophenoxyacetic acid (2,4,5-T) has been used about 25 yr as a selective herbicide in the management of certain crops, pasture, rangeland and forests. In the production of 2,4,5-T, the starting material for the aromatic portion of the compound is tetrachlorobenzene, which is subsequently hydrolysed to 2,4,5-trichlorophenol. In the production of the trichlorophenol, several impurities may be formed under unfavourable conditions. One of these impurities is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) which may be found at low levels in the trichlorophenol. Over the past decade, a concerted effort has been made to minimize the level of TCDD in 2,4,5-T, and commercial 2,4,5-T currently contains no more than 0·1 ppm TCDD.

Various toxicological studies have been conducted with 2,4,5-T over the past 25 yr. The single oral dose LD_{50} of 2,4,5-T has been reported to be 389 mg/kg in mice, 381 mg/kg in guinea-pigs, 500 mg/kg in rats and 310 mg/kg in chicks (Rowe & Hymas, 1954). The LD_{50} in dogs of 100 mg/kg, reported by Drill & Hiratzka (1953), is lower than in other species, most probably because of their slower rate of urinary elimination of organic acids such as 2,4,5-T (Piper, Rose, Leng & Gehring, 1973).

The compound 2,4,5-T is readily absorbed and rapidly excreted by mammals, including man. The half-life values for the clearance of ^{14}C activity from the plasma of rats given single oral doses of labelled 2,4,5-T at doses of 5, 50, 100 and 200 mg/kg were 4·7, 4·2, 19·4 and 25·2 hr, respectively (Piper *et al.* 1973). The half-lives for elimination from the body of these rats were 13·6, 13·1, 19·3 and 28·9 hr, respectively. In dogs given ^{14}C-labelled 2,4,5-T at 5 mg/kg, half-life values for clearance from the plasma and clearance from the body were 77·0 and 86·6 hr, respectively (Piper *et al.* 1973). This lower rate of clearance probably explains the greater toxicity of 2,4,5-T in dogs than in rats. When five human volunteers ingested 2,4,5-T, containing less than 0·05 ppm TCDD, in a dose of 5 mg/kg (Gehring, Kramer, Schwetz, Rose & Rowe, 1973), essentially all the 2,4,5-T was absorbed and excreted unchanged in the urine, with a half-life of 23·1 hr. This rate of excretion resembles more closely that observed in the rat than that in the dog.

In an unpublished subacute toxicity study conducted previously by this laboratory, no adverse effects were observed in rats fed 2,4,5-T at dose levels of 3 or 10 mg/kg/day for 90 days. At higher dose levels (30 or 100 mg/kg/day), there were increases in liver and kidney weights, elevations in serum enzymes

and other indications of slight toxicity. Drill & Hiratzka (1953) found no adverse effects in dogs given 2·5 or 10 mg 2,4,5-T/kg for 5 days/wk for 3 months; four dogs could not tolerate a higher dose level of 20 mg/kg and died during the experiment.

Highman, Gaines & Schumacher (1976) reported results of short-term toxicity studies in mice using samples of 2,4,5-T with varying contents of TCDD. These studies, which were conducted in conjunction with teratology studies, demonstrated multiple toxic effects from treatment with doses of 120 or 60 mg 2,4,5-T/kg; lower dose levels were not studied.

Smith, Schwetz, Murray, Crawford, John, Kociba & Humiston (1978) recently reported a three-generation reproduction study in rats ingesting 2,4,5-T. No adverse effects were seen at a dose level of 3 mg/kg/day. Higher dose levels of 2,4,5-T which were sufficient to cause signs of toxicity had no effect on the reproductive capacity of the rats, except for a tendency toward a reduction in neonatal survival at dose levels of 10 and 30 mg/kg/day.

Several studies have been conducted in mice over the past 10 yr to assess the potential oncogenicity of 2,4,5-T. Innes, Ulland, Valerio, Petrucelli, Fishbein, Hart, Palotta, Bates, Falk, Gart, Klein, Mitchell & Peters (1969) reported no increase in the incidence of tumours in two strains of mice given the maximum tolerated dose (21·5 mg/kg/day) of 2,4,5-T (containing approximately 30 ppm TCDD) by stomach tube from day 7 to day 28 of age. After the mice were weaned at 28 days of age, 2,4,5-T was mixed directly in the diet at a concentration of 60 ppm for approximately 18 months. In a study conducted for the National Cancer Institute by the Bionetics Research Laboratories (1968) but never published, weanling mice (two strains) were given a single dose of 215 mg 2,4,5-T/kg by sc injection and were observed for the following 18 months. The authors concluded that there was no increase due to 2,4,5-T in the incidence of tumours.

In an initial publication, Muranyi-Kovacs, Rudali & Imbert (1976) reported results of their study of two inbred strains of mice (C3Hf and XVIIG) given 2,4,5-T (100 mg/litre) in the drinking-water for 2 months from 6 wk of age. The mice were subsequently fed diets containing 80 ppm 2,4,5-T (approximately 12 mg/kg/day) for the rest of the lifetime study. On the basis of their method of combining tumour types, these authors reported a statistically significant increase in tumour incidence in C3Hf mice following 2,4,5-T treatment, but no significant differences in tumour incidence in the mice of the XVIIG strain. In a subsequent communication, Muranyi-Kovacs, Rudali & Imbert (1977) reported that no increase in tumours was noted in other studies in which they gave the same two strains of mice a series of neonatal sc injections of 2,4,5-T. Muranyi-Kovacs *et al.* (1976) stated, however, that the mouse may not be the best experimental model for testing the carcinogenicity of 2,4,5-T and recommended further testing in greater numbers of animals and in other species, such as the rat.

In order to evaluate the long-term toxicity and oncogenic potential of 2,4,5-T in rats, the study reported here was conducted. It involved the dietary administration of 3, 10 or 30 mg 2,4,5-T/kg/day to rats for up to 2 yr.

EXPERIMENTAL

Groups of 100 rats (50 males and 50 females) were maintained for up to 2 yr on diets supplying 3, 10 or 30 mg 2,4,5-T/kg body weight/day. The control group for this part of the study consisted of 86 males and 86 females. An additional ten rats of each sex were included for each treatment and control group for the interim kill on days 118 and 119.

Test material. The purified sample of 2,4,5-T was supplied by The Dow Chemical Company, Midland, MI. The identity of the 2,4,5-T was verified, prior to the initiation of the study, by elemental analysis, gas chromatography, solid-probe mass spectrometry, gas chromatography–mass spectrometry, nuclear magnetic resonance spectrometry and infra-red spectrometry. Within the limits of precision of each of these techniques, the material was essentially 2,4,5-trichlorophenoxyacetic acid. Assay by standard procedures indicated approximately 99% 2,4,5-trichlorophenoxyacetic acid. Other phenoxy acid impurities amounted to 1·3% (w/w) by gas chromatography. Using gas chromatography–mass spectrometry, TCDD, hexachlorodibenzo-*p*-dioxin, heptachlorodibenzo-*p*-dioxin and octachlorodibenzo-*p*-dioxin were not detected, the limits of detection being 0·33, 0·12, 0·40 and 0·40 ppb (μg/kg), respectively. The recrystallization solvent tetrachloroethylene, was present at a level of 0·22%.

Diet preparation. The test diets were prepared by dissolving an appropriate amount of 2,4,5-T in reagent-grade acetone and mixing the solution thoroughly with ground laboratory chow to give a 1% premix. This premix, after drying, was mixed with rat 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 of each sex per 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 collected during months 5, 7, 12, 19 and 23 and analysed for 2,4,5-T.

Experimental design. Male and female Sprague–Dawley rats, 7–8 wk old (from Spartan Research Animals, Haslett, MI) were randomly placed (using a table of random numbers) in suspended wire-bottomed cages (two rats/cage) for a 118/119-day and a 2-yr chronic toxicity study. Individual rats were identified by metal ear tags and dose levels were also identified by toe-clipping. Food (Purina Laboratory Chow, Ralson-Purina Company, St. Louis, MO) and water were available *ad lib.*

Clinical observations. Rats were observed for their general state of health and for possible toxicological responses during the study, observations being recorded when the rats were weighed. From month 6, observations were also recorded at each monthly examination for the detection of palpable masses. Body weights were recorded at approximately weekly intervals for all the rats scheduled for the interim kill at 118/119 days and also for 20 rats of each sex in the control and test groups during the first 3 months of the 2-yr part of the study. Thereafter, body weights of the latter rats were recorded at approximately monthly intervals. Food consumption was recorded

approximately twice weekly for all the rats scheduled for the interim kill and during the first 4 months of the 2-yr study for 20 rats of each sex from each group. For the rest of the 2-yr study food consumption was recorded for up to 20 rats of each sex per group for 1 wk/month.

Blood samples for haematological determinations were collected from the tail veins of eight rats of each sex and group, on days 82/83 for the rats scheduled for the interim kill and on days 89/90, 364/365 and 726/727 for those in the 2-yr study (when group sizes permitted). The total erythrocyte count, total and differential leucocyte counts, thrombocyte and reticulocyte counts, packed cell volumes and haemoglobin concentrations were determined using automated techniques (Coulter Counter Model ZBI, from Coulter Electronics, Hialeah, FL) or manual procedures.

Urine samples were collected from the same numbers of animals and at the same times as the blood samples. The specific gravity and pH of the urine and the presence or absence of glucose, protein, ketones, bilirubin and occult blood were determined (Ames Bililabstix or Multistix, from Ames Company, Elkhart, IN; TS Meter, from AO Optical, Buffalo, NY) at each of these times, and urinary urobilinogen was determined on days 364/365 and 726/727 of treatment.

Urinary levels of creatinine, coproporphyrin and uroporphyrin with or without δ-aminolaevulinic acid (δ-ALA) were determined by Bio-Science Laboratories, Van Nuys, CA. Rats were housed individually in metabolism cages for collection of 24- or 48-hr urine samples, which were transported in glass jars wrapped in aluminium foil and packed in dry ice. Urine samples were collected from five rats of each sex and group on three occasions before day 106 of treatment and once just prior to the interim kill in the subsidiary study, and from four or five rats of each sex and group after 95–97, 188–190, 361–363, 564–566 and 698–700 days of the 2-yr treatment. Urine volumes were recorded for each individual rat.

At both the interim and terminal autopsies, serum samples were collected from a maximum of ten rats of each sex and group for the determination of urea nitrogen (BUN), activities of glutamic–pyruvic transaminase (SGPT) and alkaline phosphatase (SAP), total, direct and indirect bilirubin, and total protein, albumin and globulin. Automated procedures were used for these determinations (Technicon Auto-Analyzer, Technicon Corporation, Rye, NY).

Post mortem examination. Rats scheduled for the interim autopsy were deprived of food overnight after treatment for 118 or 119 days and then killed by decapitation. The eyes of all rats were examined, by means of a glass slide pressed gently against the cornea under bright fluorescent illumination. The eyes from a maximum of five rats of each sex and group were preserved in Zenker's fixative and those from the remaining rats were fixed in 10% buffered formalin. A complete gross pathological examination was performed on all the rats by a veterinary pathologist. Representative sections of all major organs and tissues, including liver, kidneys, heart, pancreas, spleen, brain (cerebrum, cerebellum and brain stem), spinal cord, peripheral (sciatic) nerve, pituitary gland, stomach, small and large intestines, mesenteric lymph node(s), skeletal (thigh) muscle, salivary gland, testes, epididymis, accessory male sex glands, urinary bladder, uterus, ovary, trachea, oesophagus, aorta, thoracic lymph node(s), thymus, lungs, bronchi, integument, thyroid gland, parathyroid glands, adipose tissue, eyes, adrenal gland(s), and any grossly observed lesion, were preserved in formalin fixative. The weights of the liver, kidneys, brain, heart, thymus, spleen and testes or ovaries/uterus (as a unit) were recorded for all the rats.

All rats dying or killed during the course of the 2-yr study were also subjected to a gross pathological examination. Representative parts of the skull (including nasal turbinates and ear canal) along with any gross lesions suggestive of a significant pathological process of tumour formation were collected from each rat and preserved in formalin fixative.

A terminal autopsy was conducted on all rats surviving the 2-yr period of treatment. The organs and tissues weighed and preserved and the procedures used for these animals and for those autopsied during the 2-yr period were as described for the interim autopsies. At the terminal autopsy, smears of peripheral blood and femoral bone marrow were prepared for most rats, and filed for future reference if needed. Portions of fat, liver and kidney for possible 2,4,5-T analysis were saved from a maximum of five rats of each sex and group at both the interim and terminal autopsies.

Histological examination of tissues. Paraffin-embedded sections of tissues were stained with haematoxylin and eosin. Sections of the preserved tissues from the control and high-dose groups killed on days 118/119 and from all treated and control rats dying or killed during the 2-yr study or killed at termination were subjected to histological examination, but those taken from the lower-dose groups at the interim kill were not examined, because of a lack of any definite treatment-related effects in the high-dose group. Additional sections of liver and kidney from most control and top-dose rats killed after 118/119 days were stained for lipid content using Oil Red O. Additional sections of kidneys taken from five to seven female rats from the control and high-dose groups at the terminal kill were stained with Oil Red O, by Mallory's reaction for iron, with Ziehl Neelsen stain for acid fastness, by Dahl's method for calcium and by the periodic-acid–Schiff reaction (Luna, 1968).

Statistical evaluation of data. Haematological, urinary and clinical chemistry parameters, body weights, organ weights and organ/body weight ratios were analysed statistically by a one-way analysis of variance followed by Dunnett's Test, with $P < 0.05$ (Steel & Torrie, 1960). Data on mortality, palpable masses, gross pathology, histopathology and tumour incidence in the rats of the 2-yr study were analysed using Fisher's Exact Probability, $P < 0.05$, one-sided test (Siegel, 1956). For gross pathological observations, statistical evaluation of the cumulative findings for the entire 2-yr study involved comparison of the data of each of the treatment groups against those of the control group of that sex. The data were inspected and those suggestive of a difference from the control were analysed statistically. For histopathological observations and tumour incidences, the cumulative

Table 1. *Summary of body-weights, clinical observations and organ weights in rats fed diets containing 2,4,5-T for up to 2 yr*†

Dose of 2,4,5-T (mg/kg/day)	No. of rats/ group‡	Body weight (g) on day				Urinary excretion					Kidney weights: 118/119-day study (g/100 g body weight)
						Termination of 118/119-day study			Termination of 2-yr study		
		26	53	95	314	Total 48-hr urine vol. (ml)	Copro porphyrin (μg/48 hr)	Uro porphyrin (μg/48 hr)	Copro porphyrin (μg/48 hr)	Uro porphyrin (μg/48 hr)	
						Males					
0	86 + 10	385 ± 21	444 ± 30	498 ± 36	627 ± 53	38 ± 8	17·5 ± 4·9	4·1 ± 1·8	14·6 ± 6·0	4·7 ± 1·8	0·70 ± 0·09
3	50 + 10	385 ± 25	447 ± 34	503 ± 41	631 ± 59	40 ± 10	23·1 ± 4·2	4·5 ± 2·0	15·2 ± 3·6	3·0 ± 0·9	0·72 ± 0·05
10	50 + 10	386 ± 25	449 ± 36	508 ± 43	635 ± 59	36 ± 14	24·6 ± 9·6*	5·0 ± 3·1	9·7 ± 4·4	3·0 ± 1·3	0·73 ± 0·05
30	50 + 10	375 ± 21	437 ± 30	492 ± 38	616 ± 50	49 ± 11*	43·6 ± 9·3*	7·7 ± 4·7*	22·8 ± 13·0	4·0 ± 1·5	0·79 ± 0·06*
						Females					
0	86 + 10	253 ± 14	278 ± 16	301 ± 17	361 ± 28		9·6 ± 1·9		5·8 ± 1·9		
3	50 + 10	254 ± 12	279 ± 15	306 ± 17	363 ± 26	NE	10·3 ± 3·5	NE	6·5 ± 2·3	NE	NE
10	50 + 10	253 ± 15	273 ± 15	299 ± 17	365 ± 28	NE	9·2 ± 0·8	NE	9·8 ± 2·3	NE	NE
30	50 + 10	244 ± 12*	266 ± 15*	288 ± 17*	347 ± 27*	NE	16·4 ± 7·0*	NE	8·0 ± 9·8	NE	NE

NE = No treatment-related effect

†Selected to depict the effects considered to be related to treatment.

‡In 2-yr + 118/119-day studies.

Values are means ± SD and those marked with an asterisk differ significantly (by analysis of variance and Dunnett's Test) from control values: $*P < 0.05$.

data of each dose group were compared with those of the control group of that sex. The exact number of tissues examined was used as the total group size in each analysis performed. The data were inspected and those suggestive of a statistical difference from the control were analysed.

RESULTS

Dietary content of 2,4,5-T

The results of analyses of feed samples on five occasions indicated that the dosage levels of 3, 10 and 30 mg 2,4,5-T/kg/day equated with approximately 85, 230 and 677 ppm of 2,4,5-T in the diet. Repeated analyses of the feed samples indicated generally good agreement between the intended and the analytically measured concentrations of 2,4,5-T. On one occasion, analysis of the diet for the group of rats given 3 mg/kg/day indicated a somewhat higher than expected 2,4,5-T content, but this was considered to have a negligible impact on the outcome of the study.

Body weights

There were no statistically significant differences from control means in the mean body weights of any of the treated male and female rats in the 118/119 day study. Mean body weights of females given 30 mg 2,4,5-T/kg/day showed a statistically significant decrease four times during the course of the 2-yr study (Table 1), and those of the males on this dose showed a trend towards slightly lower values during most of the 2-yr period. Males and females given 3 or 10 mg 2,4,5-T/kg/day did not differ statistically from the control body weights, except at one period when the mean body weights of males given 10 mg 2,4,5-T/kg/day were statistically increased. This isolated observation was considered of no toxicological significance.

Food consumption data

There were no consistent deviations in the rate of the food consumption of males or females given any of the three dose levels during the course of either the 118/119-day or the 2-yr study. The few sporadic cases in which there was a statistical increase or decrease in food consumption between the control and various treatment groups followed no consistent trend, and were considered of no toxicological significance.

Mortality

The incidence of deaths in the group of male rats given 30 mg 2,4,5-T/kg/day showed a statistically significant decrease during the latter part of the 2-yr study, a finding considered to be secondary to the less severe chronic renal disease in these rats. The mortality in the females given this high dose level showed no difference from the control pattern. Four or five times during the study there were statistically significant increases in deaths in males and females given 10 mg 2,4,5-T/kg/day, while males given 3 mg/kg/day showed statistically significant decreases at four intervals. Females on this low dose level showed no differences from control values. These differences in mortality patterns in rats given 3 or 10 mg 2,4,5-T/kg/day showed no dose-related effects and were considered to be of no toxicological significance.

Palpable masses and animal observations

The first palpable mass was noted in a male rat of the control group. There were no statistically significant differences between control and treated groups of male rats except during months 13 and 14, when the group given 10 mg 2,4,5-T/kg/day had an increased incidence of palpable masses. This was considered of no toxicological significance, because of its isolated occurrence and lack of a dose- or temporal-response relationship. There were no differences in the incidence rates of palpable masses in groups of treated females except for statistically significant increases during months 9 and 10 in the group receiving 30 mg/kg/day and during month 14 in that group receiving 10 mg/kg/day. These observations were considered of no toxicological significance, since they were isolated occurrences and did not occur during the latter phases of the study. Examination of the rats during the course of the 2-yr study revealed no overt clinical evidence of toxicity attributable to these three levels of treatment with 2,4,5-T.

Haematology and urine analyses

Repetitive evaluation of all haematological parameters revealed no statistical differences from control values at any dose level at any time during the course of the subchronic or chronic parts of the study. Routine urine analyses also revealed no changes considered to be related to ingestion of 2,4,5-T. On one occasion there was a statistical decrease in the specific gravity of the urine of male rats given 10 mg 2,4,5-T/kg/day, but because of its isolated occurrence and lack of a dose response this was considered to be of no toxicological significance.

Repetitive evaluation of urinary porphyrins did reveal observations that were considered to be related to the higher dose levels of treatment (Table 1). In males receiving 30 mg 2,4,5-T/kg/day, there were statistically significant increases in total urine volume and in the excretion of coproporphyrin and uroporphyrin. In males receiving 10 mg/kg/day, there was a significant increase in coproporphyrin excretion, but only in the early phase of the study. In females receiving 30 mg 2,4,5-T/kg/day, excretion of coproporphyrin was increased at most of the sampling times; excretion of uroporphyrin showed a statistically significant decrease after 119 days but an increase after 566 days. No significant differences in total urinary volume or in the excretion of creatinine, coproporphyrin, uroporphyrin or δ-ALA were recorded for male rats on 3 mg/kg/day or for females on 3 or 10 mg/kg/day at any time during the study.

Clinical chemistry

Terminal analyses of serum samples for BUN, SGPT, SAP, bilirubin (total, direct and indirect), total protein, albumin and globulin after 118/119 days or 2 yr showed no alterations considered to be related to 2,4,5-T treatment at any dose level SGPT showed a statistically significant increase in the group of females given 10 mg/kg/day for 119 days, but this was considered to be of no toxicological significance, in view of its isolated nature, the lack of dose response and the absence of any similar finding at the end of the 2-yr study.

Table 2. *Summary of major gross and histopathological observations in rats maintained on diets containing 2,4,5-T for 2 yr*†

Lesion / Dose (mg/kg/day)...	No. of rats affected/no. examined							
	Males				Females			
	0	3	10	30	0	3	10	30
Kidney								
Mineralized deposits in pelvis		NE	NE	NE	1/86	1/50	5/50*	8/50*
Localized reaction adjacent to mineralized deposits		NE	NE	NE	25/86	19/50	22/50	37/50*
Moderate to severe chronic renal disease	56/86	28/50	30/50	22/50*	6/86	6/50	2/50	1/50
Increased pigment in renal tubular cytoplasm		NE	NE	NE	0/86	0/50	0/50	11/50*
Mineralization secondary to renal disease seen in:								
pulmonary alveoli	13/86	8/50	4/50	0/50*		NE	NE	NE
myocardium	5/86	3/50	2/50	0/50		NE	NE	NE
myocardial blood vessels	12/86	6/50	5/50	0/50*		NE	NE	NE
gastric mucosa and muscularis	25/85	13/49	9/49	1/50*		NE	NE	NE
Liver								
Enlargement	11/86	3/50	7/50	1/50*		NE	NE	NE
Focal biliary hyperplasia	9/86	6/50	8/50	14/50*		NE	NE	NE
Periportal inflammation	29/86	7/50*	9/50	28/50*		NE	NE	NE
Focal aggregates of reticulo-endothelial cells adjacent to degenerate or necrotic hepatocytes		NE	NE	NE	36/36	17/50	21/50	34/50*
Multiple foci of hepatocellular alteration (swollen hepatocytes)		NE	NE	NE	15/86	4/50	6/50	3/50*
Focal hepatocellular cytoplasmic vacuolization	34/86	20/50	12/50*	8/50*		NE	NE	NE
Lungs								
Focal interstitial inflammation	21/86	5/50*	5/50*	27/50*		NE	NE	NE
Focal accumulations of alveolar macrophages in alveoli	28/86	16/50	13/50	29/50*		NE	NE	NE
Focal cholesterol clefts	6/86	4/50	5/50	9/50*		NE	NE	NE
Focal accumulations of secretory material in alveoli		NE	NE	NE	0/86	2/50	2/50	4/50*
Cardiovascular system								
Dilated/flaccid ventricle	28/86	15/50	7/50	9/50	0/86	1/50	2/50	3/50*
Periarteritis	20/86	12/50	8/50	8/50		NE	NE	NE
Left atrial thrombosis	12/86	6/50	4/50	3/50		NE	NE	NE

NE = No treatment-related effect

†Selected from the results of examination of all organ systems, to depict the effects considered to be related to treatment. The tumour incidence in all the organ systems was considered to be unaffected by any level of treatment.

Values marked with an asterisk differ significantly (by Fisher's Exact Probability Test) from the corresponding control incidence: $*P < 0.05$.

Organ weights

A statistically significant increase in the relative weight of the kidneys of male rats killed after being given 30 mg 2,4,5-T/kg/day for 118 days was considered to be related to treatment (Table 1). No other organ-weight changes were noted at the interim autopsies. At the terminal autopsy after the 2-yr treatment, the only statistically significant difference was a decrease in the absolute heart weight of males given 30 mg/kg/day; this was considered to be of highly questionable toxicological significance because of an absence of any discernible gross or histopathological changes in these hearts. No alterations considered to be related to treatment were found in the weights of brain, liver, testes, spleen, thymus or uterus/ovaries at any time or at any dose level.

Gross and histopathological observations

The major pathological observations considered to be related to 2,4,5-T treatment are summarized in Table 2. Full details of the gross and histopathological findings for both the 2-yr and the shorter study are on file with the authors.

The kidneys of female rats receiving 30 mg 2,4,5-T/kg/day showed gross and microscopic evidence of an increased incidence of mineralized deposits in the renal papillae or pelvis (Fig. 1); these mineralized deposits were sometimes accompanied by a localized reaction of the adjacent renal-pelvis epithelium. Other renal effects attributed to this dose of 2,4,5-T included an increased content of pigment (Fig. 2) within the cytoplasm of the proximal convoluted tubular epithelial cells (in females), and a decrease in the severity of the chronic nephropathy (Fig. 3) that normally occurs spontaneously, especially in the males, in this strain of rat. The special stains applied to sections of kidneys of the control and high-dose groups indicated similar staining reactions for both groups,

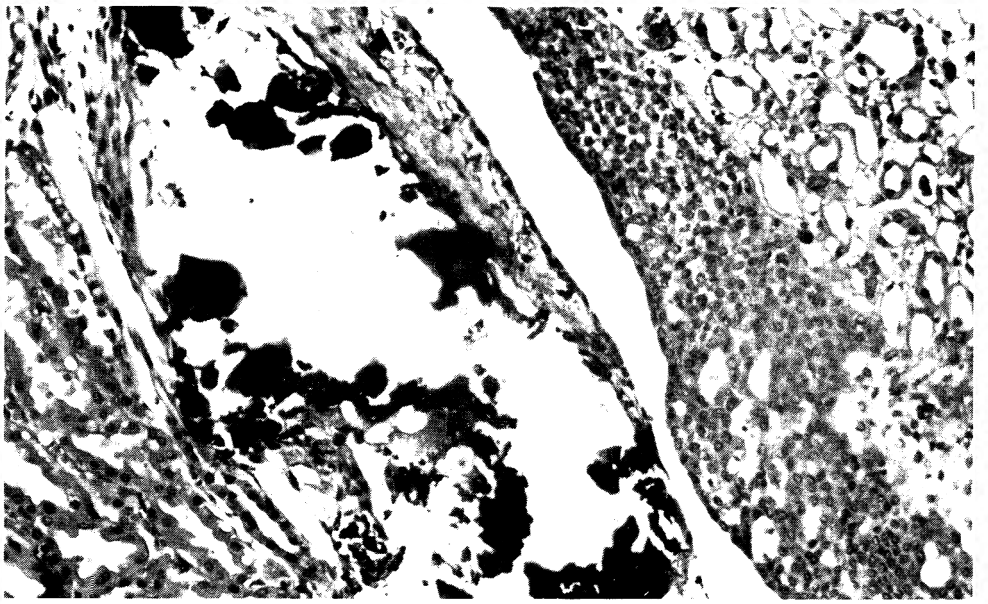

Fig. 1. Mineralized deposit in the renal papilla adjacent to the renal pelvis of the kidney of a rat given 30 mg 2,4,5-T/kg/day for 2 yr. Haematoxylin and eosin ×100.

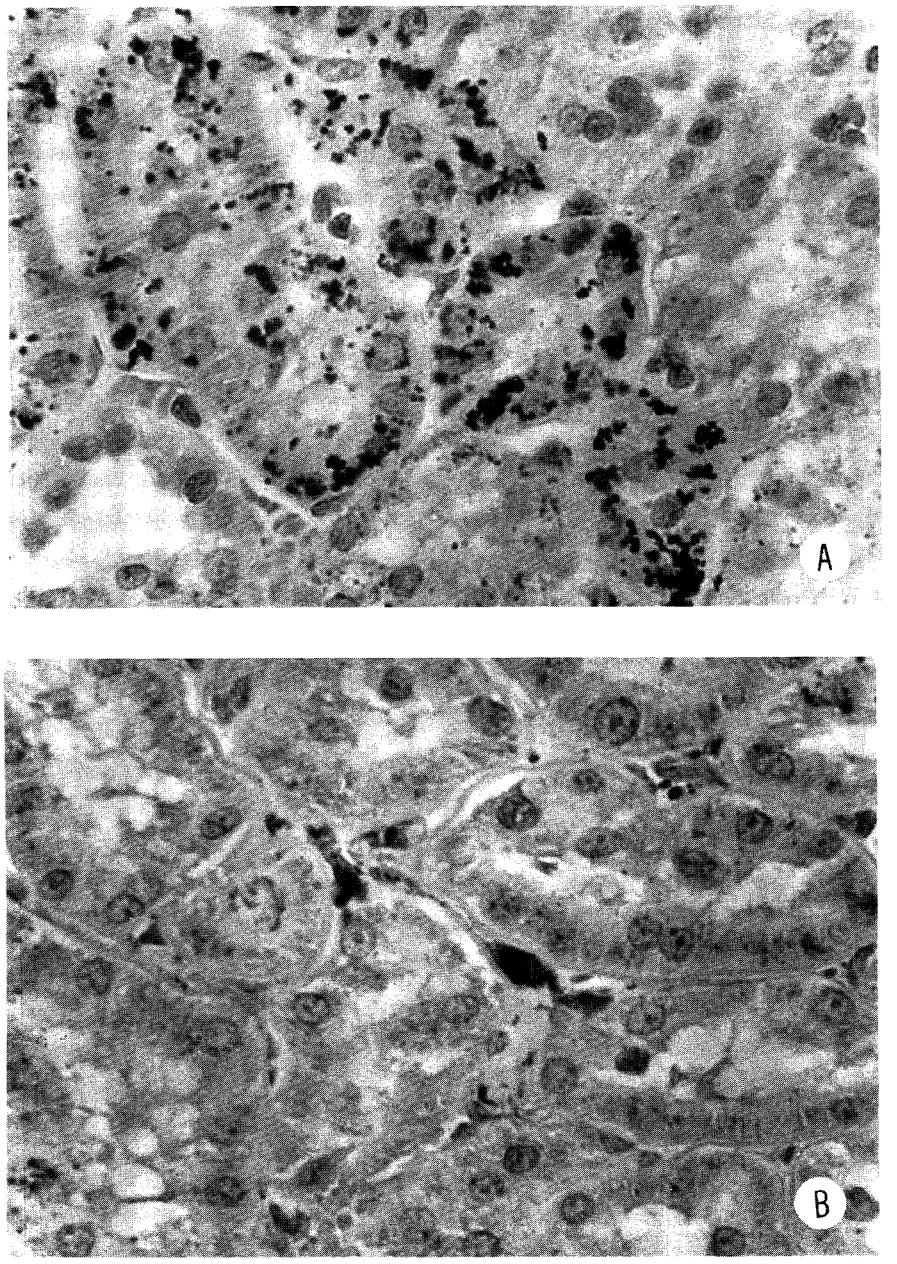

Fig. 2. (a) Increased content of pigment, staining positive for iron, in the renal tubular epithelial cells of a female rat given 30 mg 2,4,5-T/kg/day for 2 yr, compared with that from a control rat (b). Mallory's stain ×400.

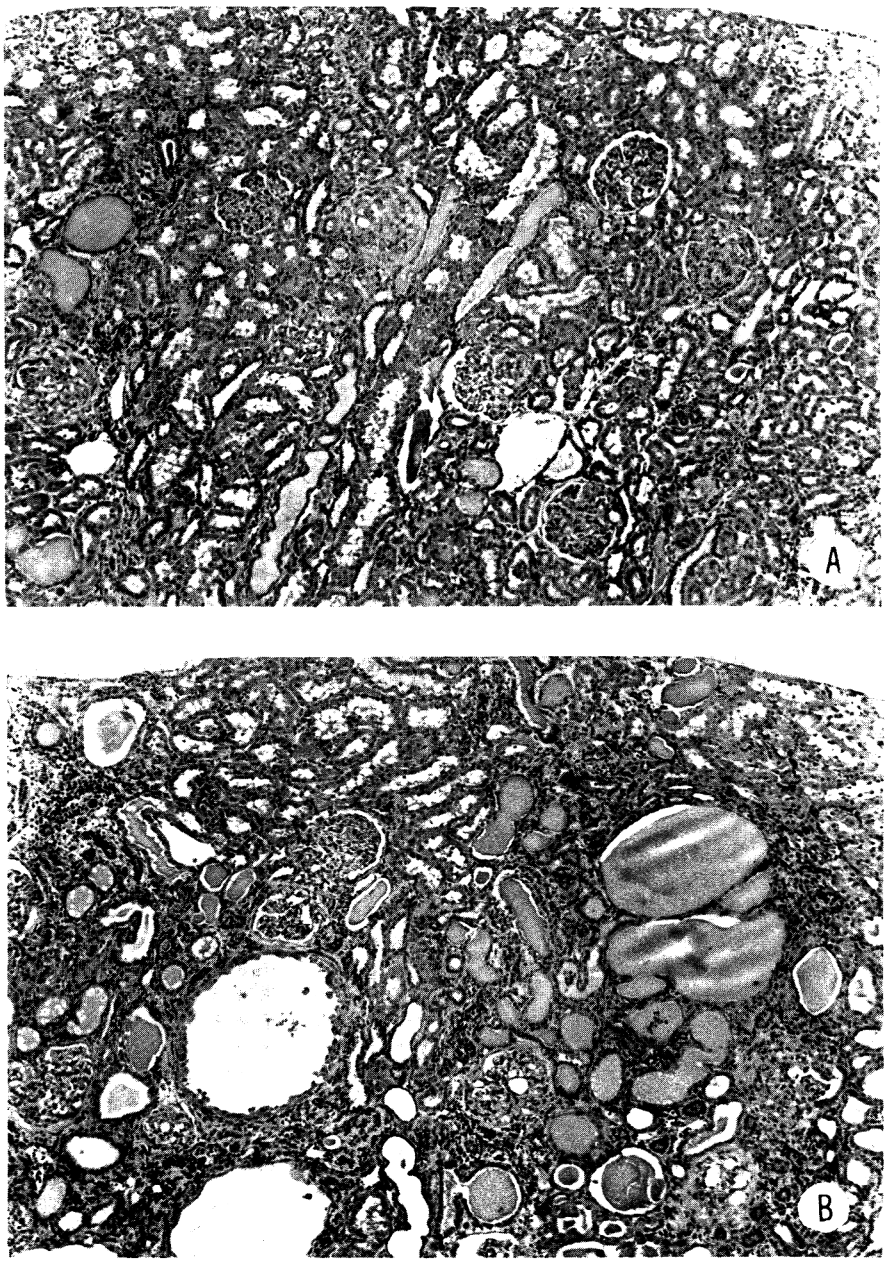

Fig. 3. Chronic nephropathic lesion in the kidney of a rat given 30 mg 2,4,5-T/kg/day (a) compared with the more severe lesion in a control rat (b). Haematoxylin and eosin × 40.

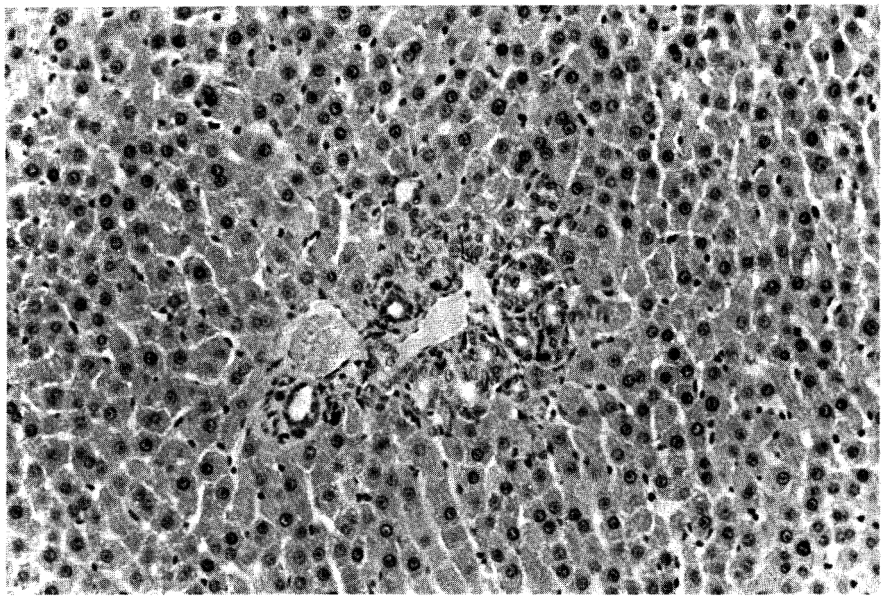

Fig. 4. Maximal response in the liver of a rat given 30 mg 2,4,5-T/kg/day, comprising a slight degree of focal biliary hyperplasia and periportal inflammation. Haematoxylin and eosin × 100.

except that the increased pigment noted in the renal tubular cells of the high-dose group gave an increased positive reaction with Mallory's stain for iron (see Fig. 2). At the intermediate dose level (10 mg/kg/day), the females showed an increased incidence of mineralized deposits in the renal pelvis of female rats and statistically significant increases in the incidence of minimal degenerative and inflammatory changes, including focal renal tubular atrophy and renal aggregations of lymphoid cells. The latter increases, however, were considered to be a reflection of an overall decrease in the severity of the geriatric chronic renal disease noted in this group. A statistically significant decrease in the incidence of mineralized deposits in the renal pelvis of male rats given 10 mg 2,4,5-T/kg/day may or may not have been the result of treatment. In the males and females on 3 mg/kg/day there were no renal lesions of any type that were considered to be related to treatment.

The decrease in the severity of the spontaneous chronic nephropathy noted in male rats receiving 30 mg 2,4,5-T/kg/day (Fig. 3) was accompanied by a secondary decrease in the incidence of mineralization of the pulmonary alveoli, myocardium, myocardial blood vessels, and gastric mucosa and muscularis.

Although the kidney was the primary target organ, the liver was also affected slightly by lifetime treatment with 30 mg 2,4,5-T/kg/day, the males showing a decreased incidence of liver enlargement and an increased incidence of focal biliary hyperplasia and periportal inflammation (Fig. 4), and the females an increased incidence of focal aggregation of reticuloendothelial cells adjacent to degenerate or necrotic hepatocytes and a decreased incidence of multiple foci of hepatocellular change (swollen hepatocytes). The incidence of focal hepatocellular cytoplasmic vacuolization was decreased in the livers of males given 10 or 30 mg/kg/day. Although the incidence of grossly visible liver nodules was higher in the control group of females than in the treatment groups, this control incidence was also somewhat higher than that generally encountered in our controls. Livers of rats given 3 mg 2,4,5-T/kg/day showed no changes attributable to treatment.

The lungs showed some changes that, directly or indirectly, may have been the result of treatment with 30 mg 2,4,5-T/kg/day; the males had an increased incidence of focal pulmonary interstitial inflammation, focal accumulations of alveolar macrophages and cholesterol clefts, while a few females showed focal accumulations of secreted material in the alveoli. At the intermediate and lower dose levels there were no pulmonary changes attributable to treatment, with the possible exception of an increased incidence of focal interstitial fibrosis in the lungs of females on the intermediate dose level. As this change was not seen at the higher dose level, it was of highly questionable toxicological significance.

Examination of the tissues of the cardiovascular system revealed no lesions that could definitely be attributed to the ingestion of 2,4,5-T. In the group given 30 mg/kg/day, the females showed a statistically significant increase in the incidence of hearts with a dilated, flaccid ventricle, but the opposite trend occurred in the males. Males given either 10 or 30 mg/kg/day showed an apparent trend toward a decrease in the incidence of both periarteritis and left atrial thrombosis, but these observations may not have been the result of 2,4,5-T treatment.

No other gross or histopathological changes considered to be attributable to 2,4,5-T ingestion were detected in any organs.

Tumour incidence

The incidence of tumours in male and female rats is detailed in Tables 3 and 4 and summarized in Table 5. The same spectrum of tumours observed historically in rats of this strain were noted in the liver, nasal turbinates/hard palate, lungs, pancreas, kidney, urinary bladder, testes, ovary, uterus, musculo-skeletal tissue, oral cavity, tongue, salivary glands, stomach, small intestine, large intestine, subcutaneous tissues, integument, mammary gland, ear canal, brain, peripheral nerves, pituitary gland, cranial cavity, adrenal glands, eye, lymph nodes, thymus, spleen, mesentery, thyroid and parathyroid glands. Statistical analyses of these data showed that the incidence of each type of tumour in any of the 2,4,5-T-treated groups was comparable to that in the control group, with the exception of the incidence of interfollicular C-cell adenoma of the thyroid, which was increased significantly in females given 3 mg/kg/day. This observation was not considered to be related to treatment, since there was no dose response and the female control group had an unusually low incidence of thyroid adenomas compared to the historical control data (up to 17%). Thus, none of the tumours listed in Tables 3 and 4 were considered to be related to treatment with any of the dose levels of 2,4,5-T. Neither were the total numbers of tumours per group, the average numbers per rat or the times of observation of tumours affected by any of the levels of treatment, these data in each case being comparable to those of the control groups (Table 5).

DISCUSSION

During the course of this 2-yr study, the 2,4,5-T treatment had no adverse effects on the onset of mortality, food consumption, the development of palpable masses, haematology, routine urine analyses, urinary excretion of creatinine or δ-ALA, serum clinical chemistry, organ weights (except the relative weight of the kidney at the interim kill in rats given 30 mg/kg/day), tumour incidence and gross and microscopic morphology of all organ systems of the body except the kidney, liver and possibly lung. Nevertheless, the highest dose level (30 mg 2,4,5-T/kg/day) predictably caused some degree of toxicity. This included a decrease in body-weight gain, increases in total urine volume, urinary coproporphyrin and uroporphyrin, an increase in relative kidney weights and morphological alterations in the kidney, liver and possibly lung.

In the kidney, these changes consisted mainly of mineralized deposits in the renal papillae or pelvis, sometimes accompanied by a resultant localized reaction of the adjacent epithelium of the renal pelvis. The chronic nephropathy normally encountered in aged rats was decreased in rats given 30 mg 2,4,5-T/kg/day. Morphological alterations in the liver attributed to treatment with 30 mg/kg/day included an

Table 3. *Tumour incidence in male rats maintained on diets containing 2,4,5-T for up to 2 yr*

Tumours or tumour-like lesions	Dose (mg/kg/day)... / No. of rats examined...	No. of rats found to be affected* in: Months 13–18				Month 19–terminal kill				Whole period of study (total)†			
		0 / 14	3 / 12	10 / 22	30 / 9	0 / 69	3 / 37	10 / 27	30 / 41	0 / 86	3 / 50	10 / 50	30 / 50
Hepatocellular carcinoma		0	0	0	0	3	1	0	1	3	1	0	1
Keratoacanthoma of nasal turbinates/hard palate		1	0	0	0	0	0	0	0	1	0	0	0
Squamous cell carcinoma of hard palate		0	0	0	0	1	1	1	0	1	1	1	0
Mixed adenoma of hard palate		0	0	0	0	1	0	0	0	1	0	0	0
Pulmonary adenoma		0	1	0	0	0	0	0	0	0	1	0	0
Pancreatic acinar adenoma		2	2	2	3	23	15	7	17	26	17	9	20
Pancreatic acinar adenocarcinoma		0	0	0	0	2	0	0	0	2	0	0	0
Pancreatic islet-cell adenoma		0	2	1	0	12	8	6	7	13	10	7	7
Pancreatic islet-cell adenocarcinoma		1	0	0	0	0	0	1	0	1	0	1	0
Renal pelvis transitional-cell adenoma		0	0	0	0	0	0	1	0	0	0	1	0
Renal tubular adenoma		0	0	0	0	0	0	0	1	0	0	0	1
Papillary adenoma of renal pelvis		0	0	0	0	1	0	0	0	1	0	0	0
Renal carcinoma		0	0	0	0	1	0	0	0	1	0	0	0
Transitional-cell carcinoma of urinary bladder with metastasis		0	0	0	0	1	0	0	0	1	0	0	0
Interstitial-cell adenoma of testis		0	0	0	1	4	1	2	2	4	1	2	3
Mesothelioma of testis		0	0	0	0	1	0	0	0	1	0	0	0
Sertoli cell tumour of testis		1	0	0	0	0	0	0	0	1	0	0	0
Chondrosarcoma of rib		0	0	0	0	1	0	0	0	1	0	0	0
Oral papilloma		0	0	0	0	1	0	0	0	1	0	0	0
Squamous-cell carcinoma of tongue		0	0	0	1	1	1	0	3	1	1	0	4
Salivary-gland adenoma		0	0	0	0	1	0	0	0	1	0	0	0
Squamous papilloma of stomach mucosa		0	0	0	0	3	1	0	0	3	1	0	0
Mucocystadenocarcinoma or adenocarcinoma of small intestine		0	0	0	0	2	2	0	0	2	2	0	0
Fibrosarcoma of small intestine		0	0	0	0	0	0	0	1	0	0	0	1
Lymphosarcoma of small intestine		0	0	1	0	1	0	0	0	1	0	1	0
Undifferentiated sarcoma of small intestine		0	0	0	1	0	0	0	0	0	0	0	1
Lymphosarcoma of small or large intestine with metastasis		0	0	0	0	1	0	0	1	1	0	0	1
Papillary adenoma or mycocystadenoma of small intestine		0	0	0	0	1	1	0	0	1	1	0	0
Subcutaneous fibroma		1	1	0	0	3	7/5	1	3	4	8/6	2	3
Subcutaneous adenocarcinoma		0	0	0	1	0	0	0	0	0	0	0	1
Malignant fibrous histiocytoma with metastasis		0	0	0	0	0	1	0	0	0	1	0	0
Malignant fibrous histiocytoma without metastasis		0	0	0	0	1	0	0	0	1	0	0	0
Subcutaneous liposarcoma		0	0	0	0	0	0	0	1	0	0	0	1
Subcutaneous myxoma		0	0	0	0	0	1	0	0	0	1	0	0

Subcutaneous carcinoma	0	0	0	0	0	0	0	1	0	0	0	1
Mammary-gland fibroadenoma/adenofibroma	0	0	1	0	1	2	0	1	1	2	1	1
Mammary-gland adenocarcinoma without metastasis	0	0	0	0	1	0	0	0	1	0	0	0
Mammary-gland adenocarcinoma with metastasis	0	0	0	0	1	0	0	0	1	0	0	0
Mammary-gland carcinosarcoma	0	0	0	0	1	0	0	0	1	0	0	0
Squamous papilloma of integument	0	0	0	0	1	0	1	1	1	0	1	1
Cutaneous adnexal carcinoma with metastasis	0	0	1	0	0	0	0	0	0	0	1	0
Fibrosarcoma adjacent to ear tag	0	0	1	0	0	0	0	0	0	0	1	0
Cutaneous adnexal cystadenoma	0	0	0	0	1	0	0	0	1	0	0	0
Zymbal-gland carcinoma of ear canal	1	0	0	0	3	0	1	1	4	0	1	1
Subauricular undifferentiated sarcoma with metastasis	0	0	0	0	1	0	0	0	1	0	0	0
Astrocytoma or mixed glial neoplasm of brain	0	1	1	0	1	0	0	1	1	1	1	1
Granular cell myoblastoma of brain	0	0	0	0	0	1	1	1	0	1	1	1
Mediastinal malignant schwannoma or malignant schwannoma of cranial nerve	0	0	0	0	1	1	0	0	1	1	0	0
Schwannoma of cranial nerve	0	0	0	0	0	0	1	0	0	0	1	0
Pituitary adenoma formation(s)	2	1	3	0	20	9	10	10	22	10	13	10
Pituitary adenocarcinoma without invasion of brain	0	0	1	0	4	2	1	3	4	2	2	3
Pituitary adenocarcinoma with invasion of brain	0	0	0	1	1	0	0	0	1	0	0	1
Neurofibroma in cranium	0	0	0	0	1	0	0	0	1	0	0	0
Adenoma of adrenal cortex	0	0	1	0	1	3	1	3	1	3	2	3
Adenocarcinoma of adrenal cortex	0	0	0	0	1	1	0	0	1	1	0	0
Adrenal phaeochromocytoma—unilateral or bilateral	3	2	4	1	33	10	14	19	36	12	18	20
Adrenal phaeochromocytoma—malignant	0	0	0	0	7	4	1	1	8	4	1	1
Adrenal ganglioneuroma	0	0	0	0	0	0	0	1	0	0	0	1
Retro-ocular squamous-cell carcinoma	0	0	0	0	0	0	0	1	0	0	0	1
Intraorbital undifferentiated sarcoma	0	0	0	0	0	0	1	0	0	0	1	0
Mesenteric lipoma	0	0	0	0	1	0	0	0	1	0	0	0
Generalized lymphosarcoma	0	1	0	0	1	0	0	0	2	1	0	0
Thymic lymphosarcoma	0	0	0	0	0	1	0	0	0	1	0	0
Splenic lymphosarcoma	0	0	0	0	1	0	0	0	1	0	0	0
Thyroid interfollicular C-cell adenoma	0	0	0	1	6	3	2	6	6	3	2	7
Thyroid interfollicular C-cell adenocarcinoma without metastasis	0	0	0	0	1	1	0	0	1	1	0	0

*There were no statistical differences (by Fisher's Exact Probability Test, $P < 0.05$) between control values and those for any treated groups. Where multiple tumours of the same classification occurred in individual rats, the data are listed as the total no. of tumours of that type/no. of rats bearing them.

†During months 1–6 there was only one tumour noted (a generalized lymphosarcoma) in one control rat. Tumours occurring during months 7–12 included one pancreatic acinar adenoma (control), one pancreatic islet-cell adenoma (control), one subcutaneous fibroma (10 mg/kg/day) and one adrenal phaeochromocytoma, malignant (control). All of these tumours are included in the total tabulation.

Table 4. *Tumour incidence in female rats maintained on diets containing 2,4,5-T for up to 2 yr*

Tumour or tumour-like lesions	Dose (mg/kg/day)... / No. of rats examined...	No. of rats found to be affected† in: Months 13–18				Month 19–terminal kill				Whole period of study (total)‡			
		0 / 17	3 / 11	10 / 11	30 / 9	0 / 67	3 / 37	10 / 36	30 / 39	0 / 86	3 / 50	10 / 50	30 / 50
Hepatocellular hyperplastic nodule(s)		1	0	1	0	3	2	2	1	4	2	3	1
Hepatocelluluar carcinoma(s)		1	0	0	0	1	0	0	0	2	0	0	0
Pancreatic islet-cell adenoma		1	0	0	0	7	1	3	2	8	1	3	2
Pancreatic islet-cell adenocarcinoma		0	0	0	0	0	0	0	1	0	0	0	1
Pancreatic acinar adenoma		0	0	0	0	0	0	1	0	0	0	1	0
Renal haemangioma		0	0	0	0	0	0	0	1	0	0	0	1
Granulosal-cell neoplasm of ovary		0	0	0	0	4	1	1	0	4	1	1	0
Stromal polyp of uterus		1	0	2/1	1	13	4	8/7	11/10	14	4	11/9	12/11
Malignant schwannoma—uterus (metastasis), or vagina		0	0	0	0	1	0	0	1	1	0	0	1
Adenocarcinoma of uterus		0	0	0	0	4	0	1	0	4	0	1	0
Myxosarcoma of uterus		0	0	1	0	0	0	0	0	0	0	1	0
Schwannoma of uterus		0	1	0	0	0	0	1	0	0	1	1	0
Leiomyosarcoma of uterus with metastasis		0	0	0	0	0	0	1	0	0	0	1	0
Fibroma of uterus/vagina		0	0	0	0	1	1	0	0	1	1	0	0
Paravertebral rhabdomyosarcoma with metastasis		0	0	0	1	0	0	0	0	0	0	0	1
Fibroma of gingiva		0	0	0	0	0	0	0	1	0	0	0	1
Squamous-cell carcinoma of tongue		0	0	0	0	0	0	0	1	0	0	0	1
Adenoma of tongue		0	0	0	0	1	0	0	0	1	0	0	0
Squamous polyp or papilloma of stomach		0	0	0	0	3	1	0	0	3	1	0	0
Mucocystadenocarcinoma or adenocarcinoma of small intestine		0	0	0	0	0	1	0	1	1	1	0	0
Lymphosarcoma of small intestine		0	0	0	0	0	0	0	1	0	0	0	1
Leiomyoma or leiomyosarcoma of small intestine		0	0	0	0	1	0	1	0	1	0	1	0
Subcutaneous lipoma		0	0	0	0	0	1	0	0	0	1	0	0
Subcutaneous fibroma		0	0	0	1	0	0	0	0	0	0	0	1
Subcutaneous fibrosarcoma		0	0	0	0	0	0	0	1	0	0	0	1
Subcutaneous carcinosarcoma		0	0	0	0	0	1	0	0	0	1	0	0
Subcutaneous myxosarcoma		0	0	0	0	0	1	0	0	0	1	0	0
Mammary-gland fibroadenoma/adenofibroma		25/13	9/7	12/9	11/7	113/52	38/24	49/28	43/25	138/65	47/31	62/38	55/33
Mammary-gland adenoma		3/2	1	0	1	12/8	6/5	4/3	8/7	15/10	7/6	4/3	9/8
Mammary-gland adenocarcinoma without metastasis		3/2	0	0	1	4	4	1	5	7/6	4	1	6
Mammary-gland adenocarcinoma with metastasis		0	1	0	1	1	1	0	2	1	2	0	4
Mammary-gland cystadenoma		0	1	0	0	2	1	6/4	3	2	2	6/4	3
Mammary-gland fibroma		1	0	0	0	3	1	3	3/2	4	1	3	3/2
Mammary-gland cystfibroadenoma/cystadenofibroma		0	0	0	0	13/11	10/7	9/7	5	13/11	10/7	9/7	5
Mammary-gland unclassified malignant neoplasm (carcinoma)		0	0	0	0	0	0	0	0	1	0	0	0

Mammary-gland cystadenocarcinoma	0	0	0	0	0	0	0	1	0	0	0	1
Squamous papilloma or keratoacanthoma of integument	0	0	0	0	2	0	0	0	2	0	0	0
Zymbal-gland carcinoma of ear canal	0	0	0	0	1	0	0	0	1	0	0	0
Adenoma of Zymbal gland	0	0	0	0	0	1	0	0	0	1	0	0
Pituitary adenoma formation(s)	8	3	1	3	34	20	19	16	42	23	20	19
Pituitary adenocarcinoma without extension or invasion of brain	2	0	0	0	4	6	3	5	6	6	3	5
Pituitary adenocarcinoma with extension of brain	1	0	0	0	4	2	2	2	5	2	2	2
Pituitary adenoma arising from pars intermedia	0	0	0	0	1	0	0	0	1	0	0	0
Neurofibrosarcoma in cranium	0	0	0	0	0	0	1	0	0	0	1	0
Adenoma of adrenal cortex	2	0	0	2	4	2	4	1	6	2	4	3
Adrenal phaeochromocytoma, unilateral	1	0	0	0	5	3	2	2	6	3	2	2
Adrenal phaeochromocytoma, malignant	0	0	0	0	1	0	0	0	1	0	0	0
Mesenteric lipoma	0	0	0	0	1	0	0	0	1	0	0	0
Abdominal haemangiosarcoma	0	0	1	0	0	0	0	0	0	0	1	0
Granulocytic leukaemia	0	0	0	0	0	0	1	0	0	0	1	0
Generalized monocytic leukaemia	0	0	0	0	0	1	0	0	0	1	0	0
Generalized lymphosarcoma	1	0	1	0	1	0	0	1	2	1	1	1
Intra-abdominal lymphosarcoma or carcinosarcoma with metastasis	0	0	0	0	1	0	0	0	1	0	0	0
Lymphangioma of mesenteric lymph node	0	0	0	0	1	0	0	0	1	0	0	0
Thymic lymphosarcoma	0	0	0	0	0	0	0	1	0	0	0	1
Splenic myeloid leukaemia	0	0	0	0	0	1	0	0	0	1	0	0
Thyroid interfollicular C-cell adenoma	0	1	0	1	3	9	2	5	3	10*	2	6
Thyroid interfollicular C-cell adenocarcinoma without metastasis	0	0	0	0	3	2	0	1	3	2	0	1
Thyroid interfollicular C-cell adenocarcinoma with metastasis	0	0	0	0	1	0	1	0	1	0	1	0
Parathyroid adenoma	0	0	0	0	1	0	0	1	1	0	0	1

†Where multiple tumours of the same classification occurred in individual rats, the data are listed as the total no. of tumours of that type/no. of rats bearing them.

‡During months 1–6, there was only one tumour noted (a generalized lymphosarcoma) in one rat given 3 mg/kg/day. Tumours occurring during months 7–12 included one stromal polyp of the uterus (10 mg/kg/day), one mammary-gland fibroadenoma/adenofibroma in both the 10- and 30-mg/kg/day groups, one mammary-gland adenocarcinoma with metastasis (30 mg/kg/day) and one mammary-gland unclassified malignant neoplasm (carcinoma) (control). All of these tumours are included in the total tabulation.

The value marked with an asterisk differs significantly (by Fisher's Exact Probability Test, $*P < 0{\cdot}05$) from the control value.

Table 5. *Summary of data on total tumour incidence in rats maintained on diets containing 2,4,5-T for up to 2 yr*

Dose of 2,4,5-T (mg/kg/day)	No. of rats/group	Total no. of tumours/ group	Mean no. of tumours/ rat	No. of tumour-bearing rats/rats autopsied					
				During months				At terminal kill	Total*
				1–6	7–12	13–18	19–24		
Males									
0	86	172	2·0	1/1	1/2	8/14	60/63	6/6	76/86 (88%)
3	50	89	1·8	0/0	0/1	9/12	26/28	9/9	44/50 (88%)
10	50	71	1·4	0/0	1/1	15/22	20/23	4/4	40/50 (80%)
30	50	97	1·9	0/0	0/0	6/9	24/25	14/16	44/50 (88%)
Females									
0	86	308	3·6	0/0	1/2	16/17	30/31	36/36	83/86 (97%)
3	50	140	2·8	1/1	0/1	10/11	18/18	19/19	48/50 (96%)
10	50	147	2·9	0/1	1/2	11/11	25/25	11/11	48/50 (96%)
30	50	151	3·0	0/0	2/2	9/9	18/18	21/21	50/50 (100%)

*Values for treated groups showed no statistically significant differences from the control values when analysed by Fisher's Exact Probability Test ($P < 0.05$).

increased incidence of periportal inflammation, focal biliary hyperplasia and inflammatory cell aggregations adjacent to degenerate hepatocytes; these findings were accompanied by a decreased incidence of multiple foci of swollen hepatocytes and focal cytoplasmic vacuolization. Lungs of rats given 30 mg 2,4,5-T/kg/day showed indications of focal pulmonary inflammation, including focal accumulations of alveolar macrophages and cholesterol clefts.

Only minimal toxicity was associated with the intermediate dose level (10 mg/kg/day), primarily an increased incidence of mineralized deposits in the renal pelvis. An increase in the urinary excretion of coproporphyrin was noted in males on this dose level only during the early phase of the study. At the lower dose level (3 mg/kg/day) there were no effects attributable to 2,4,5-T treatment throughout the 2-yr study.

The absence of any tumours considered to be the result of treatment with 2,4,5-T at any of the three dose levels concurs with the results of most of the oncogenic studies on 2,4,5-T previously reported. Innes *et al.* (1969) found no oncogenic response in either of two strains of mice given the maximum tolerated dose of 21·5 mg 2,4,5-T/kg/day, and the Bionetics Research Laboratory (1968) noted no increase in tumours in mice of two strains given a single sc injection of 215 mg 2,4,5-T/kg. The only published oncogenic study reporting an increase in tumours allegedly due to treatment with 2,4,5-T is one of the four studies conducted by Muranyi-Kovacs *et al.* (1976 & 1977). In these studies, mice of the C3Hf strain given a single dose level of 80 ppm (approximately 12 mg/kg/day) of 2,4,5-T had a statistically significant increase of the so-called 'non-incidental' types of tumours, but mice of the XVIIG strain given identical 2,4,5-T treatment showed no increase in tumour incidence. When Muranyi-Kovacs *et al.* (1977) gave neonatal mice of these same two strains a series of sc injections of 2,4,5-T there was no resultant increase in tumour incidence in either strain. It is interesting to note that in the one study in which Muranyi-Kovacs *et al.* reported an oncogenic response to 2,4,5-T, no specific target tissue showed the alleged response. This is contrary to the vast majority of cases, in which a positive oncogenic response typically affects specific target tissue(s), from which the resulting increase in tumours originates.

Overall, therefore, the results of this study concur with those of all but one of the previously reported oncogenic studies on 2,4,5-T. Rats given 10 or 30 mg 2,4,5-T/kg/day for 2 yr showed some dose-related toxicity but no oncogenic response, while in those given 3 mg/kg/day there were no adverse effects related to treatment.

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ANALYSIS OF FAECES FOR BENZO[*a*]PYRENE AFTER CONSUMPTION OF CHARCOAL-BROILED BEEF BY RATS AND HUMANS

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Abstract—The excretion of benzo[*a*]pyrene (B[*a*]P) and its metabolites in faeces were studied in rats after administration by gavage of varying doses of ^{14}C-labelled B[*a*]P and the excretion of unchanged B[*a*]P in rat and human faeces was investigated after feeding charcoal-broiled meat containing B[*a*]P. When rats were given ^{14}C-labelled B[*a*]P in doses of 0·04 μmol (10·2 μg), 0·4 μmol and 4·0 μmol/rat, 74–79% of the dose was excreted in the faeces in the first 48 hr; unchanged B[*a*]P in faeces amounted to 13·0, 7·8 and 5·6%, respectively, for the three doses studied. Faecal metabolites included 3-hydroxyB[*a*]P, 9-hydroxyB[*a*]P, B[*a*]P-3,6-dione, B[*a*]P-1,6-dione and trace amounts of 4,5-dihydro-4,5-dihydroxyB[*a*]P, and 7,8-dihydro-7,8-dihydroxyB[*a*]P. When rats were fed charcoal-broiled hamburger containing 52·7 μg B[*a*]P/kg, 11% (0·06 μg/rat) of the B[*a*]P consumed was excreted unchanged in the faeces. When humans ate meals containing charcoal-broiled meat (24·2 μg B[*a*]P/kg) B[*a*]P was not detected in the faeces, despite the fact that each person consumed 8·6 μg B[*a*]P.

INTRODUCTION

Charcoal-broiled beef contains the carcinogen benzo[*a*]pyrene (B[*a*]P) (up to 50 μg/kg) as well as other polynuclear aromatic hydrocarbons (PAH) (Lijinsky & Ross, 1967; Lijinsky & Shubik, 1964; Panalaks, 1976). Additional mutagens are also formed during broiling of fish and meat and in pyrolysis of amino acids and proteins (Nagao, Honda, Seino, Yahagi, Kawachi & Sugimura, 1977; Nagao, Honda, Seino, Yahagi & Sugimura, 1977; Matsumato, Yoshida, Mizusaki & Okamato, 1977). These compounds and others present in broiled foods may affect human health. For example, consumption of charcoal-broiled beef by humans increases the rate of phenacetin metabolism, presumably due to induction of drug metabolizing enzymes by a component formed during broiling (Pantuck, Hsiao, Conney, Garland, Kappas, Anderson & Alvares, 1976). Metabolic studies have shown that cultured human colon can convert B[*a*]P to the proximate carcinogen 7,8-dihydro-7,8-dihydroxyB[*a*]P, resulting in binding of B[*a*]P to colonic DNA and that human faeces and faecal bacteria can also hydrolyze biliary metabolites of B[*a*]P to dihydrodiols, phenols, and quinones (Autrup, Harris, Trump & Jeffrey, 1978; Renwick & Drasar, 1976).

However, the relationship between exposure to specific components of broiled meat, such as B[*a*]P, and human disease, such as colon cancer, is not known. Bioassay data on the effect of B[*a*]P and other PAH on the colon, which is the major route of excretion for these compounds in rodents, are limited (Weisburger, Reddy & Joftes, 1975) and little is known about the concentrations of B[*a*]P and its metabolites in faeces, after ingestion of food containing B[*a*]P. To understand more fully the possible effects on the colon of B[*a*]P and other PAH in charcoal-broiled beef, methods were developed to analyse B[*a*]P in faeces. These methods were used to investigate rat faeces and human faeces after ingestion of charcoal-broiled beef. This paper describes our initial experiments to assess the exposure of colonic tissue to B[*a*]P and metabolites after ingestion of dietary B[*a*]P.

EXPERIMENTAL

Apparatus. High-pressure liquid chromatography (HPLC) was carried out with a Waters Associates Model ALC/GPC-204 high-performance liquid chromatograph equipped with a model 6000A solvent delivery system, a model 660 solvent programmer, a model U6K septumless injector and a model 440 UV/visible detector (Waters Associates, Milford, MA). HPLC columns were as follows: column A, 6 mm × 30 cm Microbondapak-C_{18} (Waters Associates); column B, 4·6 mm × 30 cm Chromegabond-C_{18}, 5 μ (ES Industries, Marlton, NJ). For specific wavelength detection, a Beckman Model 25 Spectrophotometer (Beckman Instruments, Fullerton, CA) was used and for fluorescence detection, a Varian Fluorichrom fluorescence detector (Varian Associates, Palo Alto, CA) was used. Ultraviolet (UV) spectra were determined on a Cary Model 118 instrument (Varian Associates). A Nuclear-Chicago Isocap 300 scintillation system (Nuclear Chicago Corp., Des Plaines, IL) was used for the liquid scintillation counting. Collection of faeces and urine was carried out using Hoeltge HB-11M cages with HB-17 urine faeces separators and HB-66 Tunnel Feeders (Hoeltge, Inc., Cincinnati, OH). All operations were done in laboratories illuminated with yellow lights (Sylvania, F-40GO). Evaporations were carried out under reduced pressure with water temperatures below 60°C.

Reagents. All organic solvents were spectroquality. B[*a*]P was obtained from Aldrich Chemical Company (Milwaukee, WI) and [7, 10-^{14}C]B[*a*]P from Amersham-Searle (Arlington Heights, IL). Both were

purified before use by column chromatography on silica gel. B[*a*]P metabolite standards were obtained by courtesy of the National Cancer Institute. B[*a*]P was dissolved in Planters peanut oil before administration to rats by gavage.

Excretion of B[a]P and metabolites in rat faeces. Male F-344 rats (250–300 g) were housed in metabolism cages, one rat per cage and maintained at 20 ± 2°C, 50 ± 10% relative humidity, with a 12/12-hr light/dark cycle. Animals were given Purina Lab Chow, except when broiled meat was fed, and water *ad lib.*

To determine the effect of dose on excretion of unchanged B[*a*]P, three groups of ten animals were used. Each animal in groups 1, 2 and 3 respectively received 0·04 μmol (4·3 × 10^6 dpm), 0·4 μmol (3·8 × 10^6 dpm) or 4·0 μmol (4·2 × 10^6 dpm) ^{14}C-labelled B[*a*]P in 0·5 ml peanut oil, administered by gavage. Faeces were collected every 6 hr for 168 hr and frozen immediately at −76°C.

Each faecal sample was homogenized in ethanol, air-dried, and weighed. Duplicate aliquots were taken for combustion analysis (New England Nuclear Corp., Boston, MA). The combined 48-hr faeces from each group were analysed for B[*a*]P. Excretion of B[*a*]P and metabolites in urine was determined by counting aliquots.

For detection of B[*a*]P metabolites in faeces, ten male F-344 rats were each given a dose of 36 μmol (2·1 × 10^6 dpm) ^{14}C-labelled B[*a*]P in 0·5 ml peanut oil by gavage. Faeces were collected three times daily and kept at −76°C after each collection. The combined faeces were homogenized and analysed.

For detection of B[*a*]P in faeces after consumption of charcoal-broiled meat, a group of ten male F-344 rats (270–340 g) was used. Each rat received 10 g ground charcoal-broiled hamburger. Faeces were collected and frozen three times daily until 48 hr after consumption of the meat. The combined faeces were homogenized and analysed.

Analysis of B[a]P in rat faeces. Two different analytical methods were used. In the first method (Method A) dried, homogenized faeces (75–150 g) were extracted with benzene-methanol (4:1, v/v) for 48 hr in a Soxhlet extractor. The solvent was removed and the residue dissolved in 150 ml cyclohexane. The cyclohexane was extracted four times with 150 ml DMSO. The DMSO layers were combined, added to an equal volume of H_2O, and extracted four times with 1 litre cyclohexane (Schmeltz, Tosk & Hoffmann, 1976). All the cyclohexane layers were combined, dried (Na_2SO_4) and the solvent removed. The residue was chromatographed on 40 g Silicar CC-7 (Mallinckrodt, Inc., St. Louis, MO) with elution by hexane–benzene (1:1, v/v). B[*a*]P eluted in 150–200 ml. The solvent was removed and the residue was redissolved in methanol and aliquots injected on column A with elution by 85% methanol in H_2O at a flow-rate of 2·0 ml/min and a system pressure of 2000–4000 psi. Specific wavelength detection (385 nm) was used for doses of 0·04–4 μmol B[*a*]P/rat; fluorescence detection (325–440 nm) was used when charcoal-broiled meat was fed to rats.

The second method (Method B) followed a slightly different pattern. The benzene–methanol extract of faeces was dissolved in 500 ml methanol–H_2O (4:1, v/v) and extracted three times with 500 ml cyclohexane. The cyclohexane layers were combined, concentrated to 500 ml and extracted three times with 500 ml nitromethane (Hoffmann & Wynder, 1960). The nitromethane was removed and the residue was redissolved in hexane and dried (Na_2SO_4). Silica-gel chromatography and HPLC analysis were carried out as in the first method.

Detection of B[a]P metabolites in rat faeces. The extraction and partition steps were done as described in Method A above. After extraction of the DMSO–H_2O layers with cyclohexane, they were extracted four times with equal volumes of ethyl acetate. The ethyl acetate layers were washed with H_2O to remove residual DMSO, and concentrated. The cyclohexane and ethyl acetate extracts were each chromatographed on silica gel with elution by hexane, benzene, methylene chloride, ether and methanol. Fractions were collected and radioactivity determined. Radioactive fractions were combined and analysed by HPLC on column A using a linear gradient from 30% methanol in H_2O to 70% methanol in H_2O in 33 min at a flow rate of 2·5 ml/min with UV detection at 254 nm. Peaks having retention volumes identical to standards were collected and their UV-spectra determined.

Preparation of meat. Ground beef (chuck) was made into 1/2 in. thick hamburgers and broiled over an electrically-ignited charcoal fire. The meat was cooked at a distance of 6 in. from the coals for approximately 4 min per side. Control meat was cooked the same way except that aluminium foil was placed between the charcoal fire and the meat.

Analysis of B[a]P in charcoal-broiled hamburger. Meat (300–400 g) was ground in a blender and extracted for 40 hr with benzene–methanol (4:1, v/v) in a Soxhlet extractor. To the extract was added 2·2 × 10^5 dpm (0·5 μg) ^{14}C-labelled B[*a*]P as an internal standard. The benzene–methanol was removed and the residue was brought to a volume of 1 litre with methanol–H_2O (4:1, v/v) and extracted three times with 1 litre portions of hexane. The combined hexane layers were concentrated to 500 ml, dried (Na_2SO_4) and extracted with three 500 ml portions of nitromethane. The nitromethane fractions were combined and evaporated to dryness. The residue was chromatographed on 12 g silica gel with elution by hexane, hexane–benzene (1:1, v/v), and hexane–benzene (7:1, v/v). The radioactive fractions were combined, concentrated, and redissolved in methanol. Aliquots were injected on column A with gradient elution from 70% to 85% methanol in H_2O at a flow rate of 2·0 ml/min and a system pressure of 2000–4000 psi. Quantitation was done with specific wavelength detection at 385 nm. Aliquots were counted to determine recovery.

Human studies. Eight male volunteers were recruited. All were in good health and were non-smokers. Volunteers did not consume other broiled foods during the experiment. The experiments were conducted on 2 days. On day 1, the volunteers ate lunch and dinner in the dining facility of the Institute. Lunch consisted of salad, juice and broiled homogenized beef in a mushroom sauce. Dinner was the same broiled beef with onion and tomato sauce, a baked tomato, broccoli, and juice. The amount of meat con-

Table 1. *Excretion of B[a]P and metabolites in faeces after administration of ^{14}C-labelled B[a]P to rats*

B[a]P administered/rat (μmol)	% of [^{14}C] dose excreted* after... 12 hr	24 hr	48 hr	% B[a]P in faeces†
4·0	1	71	79	5·6
0·4	11	65	78	7·8
0·04	39	67	74	13·0

*Determined by combustion analysis.
†Isolated amounts.

sumed by each volunteer was measured and aliquots were analysed for B[a]P. Faeces were collected for 72 hr with immediate cooling to −76°F. The combined 72-hr sample for each volunteer was analysed. On day 2, a week later, the volunteer consumed the same meals, except with control meat (prepared by placing aluminium foil between the beef and the flame). Faeces were collected for 72 hr and analysed.

Analysis of B[a]P in human faeces. Frozen faeces (300–700 g) were extracted in a 1500 ml Soxhlet extractor for 20 hr with 2500 ml benzene–methanol (4:1, v/v). To the extract was added ^{14}C-labelled B[a]P ($7·3 \times 10^4$ dpm, 0·1 μg) before concentration to approximately 100 ml. The volume was readjusted to 500 ml by addition of methanol–H_2O (4:1, v/v). The resulting mixture was extracted four times with 300 ml portions of hexane. The hexane layers were combined, concentrated to 500 ml, dried (Na_2SO_4), and then extracted with three 200 ml portions of nitromethane. The nitromethane was removed and the residue was chromatographed on 40 g silica gel with elution by hexane, 10% benzene in hexane, and 15% benzene in hexane. The radioactive fractions were combined, concentrated, and redissolved in 1 ml methanol. A portion of the sample, approximately 5%, was injected (50 μl/injection) on column A with a solvent program of 65% methanol in H_2O to 85% methanol in H_2O in 30 min at a flow rate of 2 ml/min. The region corresponding in retention volume to B[a]P was collected with absolute ethanol and carefully concentrated to dryness. The residue was redissolved in methanol; an aliquot was counted and another aliquot was injected for quantitation on column B with elution by 90% methanol in H_2O at a flow rate of 1 ml/min.

RESULTS

When groups of ten rats were dosed by gavage with [^{14}C]B[a]P at levels of 4·0, 0·4 and 0·04 μmol/rat most of the dose was excreted in the faeces in the first 24–48 hr (Table 1). Total excretion of label in faeces after 168 hr averaged 85%; in each case 1–3% of the dose was excreted in urine. For analysis of B[a]P in the above samples, faeces were extracted with benzene–methanol (4:1, v/v). This solvent system was efficient for extraction of unchanged B[a]P from faeces. However, in all three cases, only 58–60% of the radioactivity present in faeces was extracted. B[a]P was enriched by solvent partitions and silica gel chromatography before quantitation by HPLC with UV detection at 385 nm. The results are summarized in Table 1. Excretion of unchanged B[a]P in faeces ranged from 5·6–13% of the dose.

For characterization of faecal metabolites of B[a]P, a dose of 36 μmol/rat was given. The metabolites identified and the isolated amounts of each were 3-hydroxyB[a]P (3% of the dose), 9-hydroxyB[a]P (< 1%), B[a]P-3,6-dione (3%), B[a]P-1,6-dione (6%), 4,5-dihydro-4,5-dihydroxyB[a]P (< 1%), and 7,8-dihydro-7,8-dihydroxyB[a]P (< 1%). Quantitation of metabolites other than the diones at lower doses was not feasible without further enrichment steps before HPLC analysis.

With the patterns of excretion and analytical methodology for faeces established, the fate of ingested B[a]P in charcoal-broiled meat was investigated. The concentration of B[a]P in a typical sample of broiled beef was 52·7 ± 6·8 μg/kg*. Unchanged B[a]P in the faeces of ten rats fed a total of 0·1 kg beef containing 5·3 μg B[a]P amounted to 0·6 μg, or 11% of the total dose. The detection limit for this method was approximately 0·05 μg B[a]P/total faecal sample. The results for man are summarized in Table 2. B[a]P was not detected in any of the sixteen faecal samples, although recoveries of internal standard ^{14}C-labelled B[a]P averaged 65%. In each case, a peak was observed corresponding to the added internal standard.

DISCUSSION

The analytical methodology described here can be used to determine trace amounts of B[a]P in rat faeces, human faeces, and broiled meats. In most cases, enrichment of B[a]P by solvent partitions and silica gel chromatography provided adequate purification before quantitation of B[a]P by reverse-phase HPLC. However, for very low levels of B[a]P, an additional purification using reverse-phase HPLC was necessary. For determination of B[a]P in meat (0·02–0·05 μg/g), or in rat faeces (1–100 μg/g dry weight faeces) after the doses used in the model studies, specific wavelength detection at 385 nm was suitable. For measurement of B[a]P in faeces (1–10 ng/g) after feeding charcoal-broiled meat, fluorescence detection was necessary.

When the dose of B[a]P, administered by gavage to rats, was varied 100-fold, the proportion of radioactivity in faeces after 12 hr was greatest at the lowest dose (Table 1). In each case, most of the dose was excreted in the faeces in the first 24–48 hr. These results are in agreement with previous studies of

* Mean ± SD for three analyses of one batch of broiled meat, determined by the isotope dilution method.

Table 2. *Analysis of B[a]P in faeces after consumption of charcoal-broiled beef and control beef by humans*

Parameter	Charcoal-broiled beef	Control beef*
B[*a*]P content of beef (μg/kg)	24·2†	<0·1
Beef consumed/person‡ (g)	357	419
B[*a*]P consumed/person (μg)	8·6	<0·04
B[*a*]P detected in faeces/person† (μg)	<0·1	<0·1

*Prepared by placing aluminium foil between the beef and the flame.
†Calculated by the isotope dilution method.
‡Mean of eight values for total beef in lunch and dinner.

excretion of ^{14}C-labelled B[*a*]P in rats and mice (Heidelberger & Weiss, 1951; Kotin, Falk & Brusser, 1959). The amount of unchanged B[*a*]P excreted varied from 5·6–13·0%; in previous studies, approximately 1% unchanged B[*a*]P was detected in faeces of rats and mice (Berenblum & Schoental, 1942; Chalmers & Kirby, 1940; Kotin *et al.* 1959).

In all cases, only about 60% of the radioactivity present in the faeces could be extracted by benzene–methanol (4:1, v/v). Soxhlet extraction with ethyl acetate, methanol, or water did not significantly increase extraction efficiency. The nature of the unextracted material is not known.

The metabolites of B[*a*]P detected in rat faeces have also been observed in previous studies of biliary and faecal B[*a*]P metabolites and in experiments *in vitro* with rat liver homogenates (Berenblum, Crowfoot, Holiday & Schoental, 1943; Falk, Kotin, Lee & Nathan, 1962; Kinoshita, Shears & Gelboin, 1973). Only trace amounts of 7,8-dihydro-7,8-dihydroxy-B[*a*]P, which is a proximate carcinogen of B[*a*]P on mouse skin, were detected. Unchanged B[*a*]P and the metabolites identified in faeces accounted for approximately 20–25% of the dose. The remaining 55–60% of the dose appearing in rat faeces consisted of unidentified metabolites, some of which were not extracted from the faeces, as discussed above. These products may result from further metabolism by faecal microflora. Their chemical and biological properties remain to be investigated.

The B[*a*]P content of the charcoal-broiled hamburgers ranged from 24–52 μg/kg. These results are in good agreement with previous studies (Lijinsky & Shubik, 1964; Lijinsky & Ross, 1967; Panalaks, 1976). Formation of B[*a*]P was prevented by broiling the meat with a piece of aluminium foil inserted between the meat and the flame, as observed previously (Lijinsky & Ross, 1967). When meat containing 52·7 μg/kg B[*a*]P was fed to rats, the faecal excretion of unmetabolized B[*a*]P was 11% of the dose, which agrees well with the model studies in which B[*a*]P was given by gavage.

In the studies with humans, each volunteer consumed about 9 μg B[*a*]P. Based on the results with rats, excretion of approximately 0·9 μg unchanged B[*a*]P/person might have been expected. Instead, B[*a*]P levels in faeces were less than 0·1 μg/person. This lower level of B[*a*]P in the faeces of humans than rats could be due to differences in metabolism of B[*a*]P in the liver or by faecal microflora, to differences in dose, or to other factors. Further studies are necessary to define the nature of these differences and to assess the level in human faeces of 7,8-dihydro-7,8-dihydroxyB[*a*]P.

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SHORT PAPERS

ABSENCE OF MUTAGENIC ACTIVITY IN *SALMONELLA TYPHIMURIUM* OF SOME IMPURITIES FOUND IN SACCHARIN

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Abstract—*o*-Sulphobenzoic acid, *o*-toluenesulphonamide and ammonium *o*-sulphobenzoate were assayed for their possible mutagenicity in several *Salmonella typhimurium* strains. No mutagenic activity was detected towards any strain, at any of the levels tested, either in the absence or presence of fortified rat liver post-mitochondrial fractions.

Introduction

The equivocal results of various mutagenicity assays on commercial saccharin suggest that the effects detected may be mainly if not entirely due to some impurities (Kramers, 1975, 1977). The artificial sweetener is synthesized by two major processes which result in different impurities in the final preparations. A typical commercial grade of saccharin as prepared by the Remsen-Fahlberg procedure (Remsen & Fahlberg, 1879) contains variable amounts of the following impurities: *o*- and *p*-toluenesulphonamide, *o*- and *p*-sulphamoylbenzoic acid and *o*- and *p*-sulphobenzoic acid. A much smaller total amount of contaminants, namely anthranilic acid, *o*-sulphamoylbenzoic acid, *o*-sulphobenzoic acid and *o*-toluenesulphonamide is present in saccharin prepared by the Maumee procedure (Subcommittee on Non nutritive Sweeteners, 1974).

Commercial saccharin preparations have been shown to contain compounds responsible for the induction of mutations in *Salmonella typhimurium* TA98 and TA100. Mutagenic activity was also demonstrable in the urines of mice fed the compound as well as in a host-mediated assay. However, highly-purified saccharin did not exhibit any mutagenic effect in the direct assay but when administered orally to mice, mutagenic activity towards strain TA100 was found in the urines of these animals (Batzinger, Suh-Yun & Bueding, 1977). Moreover, the impurities soluble in organic solvents, extracted from some lots of commercially-produced saccharin exhibited mutagenic activity towards strains TA100 and TA98 (Stolz, Stavric, Klassen, Bendall, Craig, 1977). The recent experimental demonstration of foetal resorption and ocular anomalies caused in rat foetuses by three saccharin contaminants, namely: *o*-sulphobenzoic acid, ammonium *o*-sulphobenzoate and *o*-toluenesulphonamide (Lederer, 1977) prompted us to investigate their possible mutagenicity.

Experimental

o-Sulphobenzoic acid, *o*-toluenesulphonamide and ammonium *o*-sulphobenzoate were a gift from the Laboratory of General and Organic Chemistry (Pro-

Table 1. *Numbers of His⁺ revertants/plate observed after incubation in the absence of liver post-mitochondrial fractions*

	No. of His⁺ revertants/plate								
	o-Toluene sulphonamide			Ammonium *o*-sulphobenzoate			*o*-Sulphobenzoic acid		
Strain	0	10^{-2} M	10^{-1} M	0	10^{-2} M	10^{-1} M	0	10^{-2} M	10^{-1} M
TA1530	21	16	16	19	32	20	23	35	23
TA1535	15	12	6	16	27	35	15	13	10
TA1537	5	16	10	9	6	17	9	19	6
TA1538				20	22	17			
TA100	98	147	104	130	157	159	130	156	158
TA98	27	19	18	29	21	28	20	17	10

Table 2. *Numbers of His⁺ revertants/plate calculated after incubation in the presence of fortified liver post-mitochondrial fractions*

Strain	Concn. of test material...	No. of His⁺ revertants/plate										
		S9 mix (Arochlor 1254 pretreatment)							S9 mix (Phenobarbital pretreatment)			
		0	10^{-5} M	10^{-4} M	10^{-3} M	10^{-2} M	10^{-1} M	M	0	10^{-2} M	10^{-1} M	M
							***o*-Toluenesulphonamide**					
TA1530		19	23	14	9	13	20	9	23	30	20	3
TA1535		13	13	11	14	14	16	11	12	21	16	23
TA1537		11	15	11	7				14	14	22	21
TA1538		21	28	41	23				24	26	21	14
TA100		98				93	104	95				
TA98		17				21	26	18	17	18	19	5
							Ammonium *o*-sulphobenzoate					
TA1530		16				24	25		19	28	15	29
TA1535		15				15	20		15	27	28	21
TA1537		11				11	11		12	14	8	11
TA1538		24				22	20		22	25	32	16
TA100		125				101	90					
TA98		33				29	36	29	28	32	9	11
							***o*-Sulphobenzoic acid**					
TA1530		16	22	13	18	14	22	37	19	19	21	
TA1535		15	11	9	9	13	15	11	12	20	17	
TA1537		10	12	7	17	9	10		13	13	12	
TA1538		21	16	27	16				19	18	11	
TA100		138	179	153	181	115	108	98				
TA98		34	21	27	32	20	25	29	19	20	18	

fessor P. Van Brandt, Faculty of Sciences, University of Louvain, 1348 Louvain-la-Neuve, Belgium). All the other products were of the purest grade commercially available. Dilutions of the three compounds were made in dimethylsulphoxide (DMSO). They were kept in the dark at 4°C.

Adult male Wistar rats (200–250 g) were fed an RAL diet. The animals were either injected ip with 500 mg Arochlor 1254/kg diluted in corn-oil (200 mg/ml) 5 days before they were killed or injected ip with 75 mg phenobarbital/kg diluted in water 24 hr and 48 hr before the preparation of the liver fractions (Ames, McCann, Yamasaki, 1975). *S. typhimurium* strains TA1530, TA1535, TA1537, TA1538, TA100 and TA98 were kindly provided by Professor B. N. Ames. The post-mitochondrial (S9) fractions were obtained from three pooled rat livers, from the treated rats, by centrifuging the homogenate (3 ml of 0·15 M-KCl/g wet liver; Ames, McCann, Yamasaki, 1975). The S9 mix was prepared by adding $MgCl_2$ (8 μmol/ml mix), KCl (33 μmol/ml mix), sodium phosphate (100 μmol/ml mix), glucose-6-phosphate (5 μmol/ml mix), $NADP^+$ (4 μmol/ml mix) and 100 μl S9(25 mg wet liver)/ml mix.

Plate incorporation tests were performed in duplicate by mixing dilutions of test materials (0·1 ml/plate), $2–8 \times 10^7$ bacterial cells from an overnight culture in nutrient broth (Difco)/plate, and S9 mix (0·5 ml/plate) in histidine-biotin (0·05 mM) supplemented top agar which was layered on minimal glucose agar. The plates were incubated for 48 hr at 37°C in the dark and the numbers of macroscopic colonies were calculated. The cytotoxicity was evaluated by determining the bacterial survival with a lower bacterial inoculum (10^4-fold dilution) and plates of nutrient agar (Difco).

Results

As shown in Table 1, no direct mutagenic effect was detected towards the experimental strains of *S. typhimurium* with any of the three compounds. In the presence of fortified liver post-mitochondrial fractions from rats pretreated with Arochlor 1254 or phenobarbital (Table 2), the numbers of His^+ revertants/plate were never significantly higher than those corresponding to the spontaneous reversion rates at doses of test material ranging from 4×10^{-7} mol up to 4×10^{-2} mol/plate. Moreover in those experimental conditions, no cytotoxic effects were observed with any of the tested strains.

Conclusions

The ortho forms of the most frequent impurities present in commercial saccharin did not increase the mutation frequency of the usual strains of *S. typhimurium* either in the absence or in the presence of fortified rat-liver fractions. More attention should be paid to the potential mutagenic effects of the para forms of these impurities.

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Fd Cosmet. Toxicol. Vol. 17, pp. 233 to 236

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TOXICITY OF *DIPLODIA MACROSPORA* TO LABORATORY ANIMALS

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Abstract—The toxicity of *Diplodia macrospora* isolated from Zambian white maize to ducklings and rats is described. Pure cultures of *D. macrospora* on autoclaved maize were acutely toxic and caused the death of four out of four rats within 7 days and four out of four ducklings within 5 days. In a short-term toxicity trial dietary levels of 8, 16 and 32% mouldy meal in the diet caused the death of one, four and four male and nought, three and four female rats out of four in the respective treatment groups. Dietary levels as low as 2% mouldy meal caused significant reductions in weight gain of male and female rats compared to the controls. Pulmonary haemorrhage, alveolar, septal and perivascular oedema of the lung, mild cholangitis and a mild renal tubular nephrosis were the most important histological changes. Systemic shock, possibly related to a direct endothelial toxicity, appeared to be the main cause of death.

Introduction

Diplodia macrospora Earle causes a leaf blight of maize (*Zea mays* L.) as well as a dry rot of stalks and ears very similar to the disease caused by the closely related *Diplodia maydis* (Berk.) Sacc. (Earle, 1897; Eddins, 1930; Latterell, 1976; Marasas & Van der Westhuizen, 1978). The two fungi are very similar morphologically and *D. macrospora* is distinguished mainly by its much larger conidia (Earle, 1897; Eddins, 1930; Sutton, 1964; Sutton & Waterston, 1966). *D. macrospora* has been reported from tropical and subtropical areas of the United States of America, Central and South America, Asia, Australia and Africa (Commonwealth Mycological Institute, 1958; Sutton, 1964; Sutton & Waterston, 1966). In the south-eastern United States, *D. maydis* is much more common than *D. macrospora* on maize stalks as well as kernels (Larsh, 1938; Stevens, 1943). In certain other warm, humid areas such as Zambia, *D. macrospora* is, however, the more prevalent species (Sutton & Waterston, 1966; Logan, 1974; Marasas, Kriek, Van Rensburg, Steyn & Van Schalkwyk, 1978).

Maize naturally infected by *D. maydis* causes a mycotoxicosis (diplodiosis) of cattle and sheep grazing in harvested maize fields in southern Africa (Marasas, 1977). Diplodiosis has been reproduced experimentally in cattle and sheep with pure cultures of southern African isolates of *D. maydis* (Mitchell, 1918; Theiler, 1927; Shone & Drummond, 1965). *D. maydis* has also been shown to be toxic to laboratory animals (Rabie, Van Rensburg, Kriek & Lübben, 1977) and a mycotoxin, diplodiatoxin, has been isolated from a toxic South African strain (Steyn, Wessels, Holzapfel, Potgieter & Louw, 1972). Diplodiatoxin accounted for only 10% of the toxicity of this strain and it is not known whether this compound plays any role in the aetiology of diplodiosis or not.

Zambian maize visibly infected by *Diplodia* spp. was previously found to be acutely toxic to ducklings and to cause neonatal mortality in rats (MacDonald & Raemakers, 1974). The maize used in these experiments was presumably naturally infected by *D. macrospora* as well as *D. maydis* (Marasas *et al.* 1978). No information on the toxicity of pure cultures of *D. macrospora* to experimental animals could be found in the literature. A new mycotoxin, diplosporin, was recently isolated from the toxic *D. macrospora* culture material used in the experiments reported on here (Chalmers, Gorst-Allman, Kriek, Marasas, Steyn and Vleggaar, 1978). This paper reports on the isolation of *D. macrospora* from Zambian maize and on the toxicity of pure cultures of this fungus to ducklings and rats.

Experimental

Materials. Samples of 1974/75 Zambian white maize containing different levels of visibly diseased kernels were obtained from Mount Makulu Research Station, Chilanga, Zambia (Marasas *et al.* 1978). Subsamples of kernels (100 g) from each of the Zambian maize samples were surface-sterilized in a commercial 5% aqueous solution of sodium hypochlorite for 1 min, rinsed twice with sterile distilled water, and 100 kernels/sample placed on 1·5% malt extract agar containing 100 mg/litre sodium novobiocin, five kernels/plate. The plates were incubated at 25°C in the dark for 5 days and thereafter plates containing colonies of *Diplodia* spp. were incubated at 20°C under near ultra-violet (UV) radiation (12 hr/day) for 21 to 28 days when sporulating colonies of *D. macrospora* and *D. maydis* could be positively identified. Single conidial isolations of *D. macrospora* were made from conidia produced in pycnidia on infected kernels. Stock cultures of two single-conidial isolates (MRC 143 and MRC 144) were maintained on potato dextrose agar slants and on autoclaved moistened maize kernels in 250-ml Erlenmeyer flasks.

Yellow maize kernels (400 g in 400 ml water) in 2 litre glass jars were autoclaved at 121°C for 1 hr on each of two consecutive days and inoculated with conidial suspensions prepared from sporulating stock cultures. The cultures were incubated at 25°C in the dark for 6 to 8 wk when the contents of the jars were harvested, dried at 50°C for 24 hr, ground in a Wiley mill and the mouldy meal was stored at 5°C until used.

Toxicity trials in ducklings and rats. The acute toxicity of culture material of the *D. macrospora* isolates was assayed by feeding diets containing mouldy meal and either commercial chicken or rat mash (1:1, w/w) *ad lib* for 14 days to groups of four 1-day old Pekin ducklings (c. 50 g) and four weanling male Wistar rats (c. 70 g). Control diets contained 50% maize meal prepared from autoclaved non-inoculated yellow maize kernels and 50% chicken or rat mash.

A short-term toxicity trial in rats was also performed to determine the toxic effects of different levels of mouldy meal. Culture material of isolate MRC 143 was mixed with commercial rat mash to obtain diets containing either 2, 4, 8, 16 or 32% (w/w) mouldy meal and fed for 102 days to groups of four male and four female weanling BD IX inbred black rats initially weighing *c.* 60 and 56 g, respectively. Control diets contained either 2% or 32% maize meal prepared from autoclaved uninoculated yellow maize kernels mixed with commercial rat mash. Rats were weighed individually at the beginning of the experiment and at weekly intervals thereafter. At the termination of the experiment all the rats were killed by decapitation and given a *post mortem* examination. Organs and tissues including the liver, kidney, lung, heart, brain, oesophagus, stomach, small and large intestine, adrenal and genitals were preserved in 10% buffered formalin. Conventionally processed paraffin sections stained with haemotoxylin and eosin were examined by light microscopy.

Results

Mycology

Diplodia spp. were isolated from 8 to 31% of the kernels in four different Zambian maize samples. Subsequent identification of sporulating cultures revealed that most of these isolates were *D. macrospora* and only a few were *D. maydis*. Single-conidial cultures of *D. macrospora* grew rapidly on 1·5% malt extract agar at 25°C and the densely floccose, white aerial mycelium covered the surface of 9 cm petri dishes within 5 days. Few or no pycnidia developed in these cultures incubated in the dark, but large black immersed and superficial pycnidia were present after 28 days in cultures on 1·5% malt extract agar or autoclaved maize kernels incubated at 20°C under near UV radiation. Conidia extruded from the ostioles of these pycnidia were cylindrical with a rounded apex and truncate base, straight or curved, brown with 0–3 septa and measured 37·5–93·7 × 6·5–8·7 μm. Dried sporulating cultures of isolate MRC 143 have been deposited in the Mycological Herbarium of the Plant Protection Research Institute, Private Bag X134, Pretoria, under accession number PREM 45082.

Toxicity trials in ducklings and rats

Both isolates of *D. macrospora* proved to be acutely toxic to ducklings as well as to rats (Table 1). Isolate MRC 143 appeared to be slightly more toxic than MRC 144 and culture material of the former was used in a short-term toxicity trial in rats to determine the toxic effects of different levels of mouldy meal in the diet.

Short-term toxicity trial in rats

The effect of the different dietary levels of mouldy meal on male and female rats are summarized in Table 2. Only slight differences in the toxicity of the mouldy material to either males or females were observed. At the end of the experiment, the weight gains of surviving male as well as female rats in all groups were significantly less ($P < 0.001$) than those of both control groups.

No remarkable gross lesions were observed in any of the organs of the experimental or control rats. In those that died during the experiment a generalized congestion was evident and muscle wasting and depletion of fat reserves corresponded to the decreased weight gain (Table 2).

Histopathological changes

The changes in all the animals of the different groups that died were similar although they were more severe in animals on the higher dietary levels. Vascular changes including a slight vasculitis, fibrin clots, fibrinoid changes and haemorrhage occurred particularly in the lung but also in the stomach, brain, heart, liver and pancreas. The lung was the organ

Table 1. *Acute toxicity of* Diplodia macrospora *culture material to ducklings and rats*

Mouldy diets*	No. of deaths†		Mean time of death (days‡)	
	Ducklings	Rats	Ducklings	Rats
D. macrospora MRC 143	4	4	5·0	7·0
D. macrospora MRC 144	4	4	6·7	7·5
Control	0	0	—	—

**D. macrospora* culture material mixed 50:50 (w/w) with commercial rat mash or chicken mash, and fed *ad lib* for 14 days. Control diet consisted of ground autoclaved maize meal mixed 50:50 (w/w) with rat or chicken mash.

†In groups consisting initially of either four 1-day old Pekin ducklings or four weanling male Wistar rats.

‡From the start of feeding.

Table 2. *Short-term toxicity of* D. macrospora *culture material to rats*

Dietary concn. of mouldy meal*	No. of deaths†		Mean time of death and range (days‡)		Mean terminal weight (g)§	
	Males	Females	Males	Females	Males	Females
2%	0	0	—	—	181	143
4%	0	1	—	13	143	143
8%	1	0	23	—	135	114
16%	4	3	18(8–25)	12(8–15)	—	82
32%	4	4	6·75(6–7)	7·5(6–10)	—	—
2% Control	0	0	—	—	239	189
32% Control	0	0	—	—	289	189

*Culture material of *D. macrospora* MRC 143 incorporated on a percentage basis (w/w) into commercial rat mash and fed *ad lib* to groups of four male and four female rats at the various levels for 102 days. Control diets consisted of ground autoclaved maize meal incorporated on a percentage basis (w/w) into commercial rat mash.

†In groups consisting initially of four males or four females.

‡From the start of feeding.

§ANOVA: All treatment groups, males as well as females < corresponding control group ($P < 0.001$).

most severely affected and showed a marked congestion in all and alveolar haemorrhage in 50% of cases. The alveolar walls were moderately thickened in all cases and a perivascular and septal oedema occurred in 50% of cases. A marked alveolar oedema and fibrin exudation occurred in the most severely affected cases on the 32% dietary level. The vascular changes in the lung occurred in some animals at all the dietary levels in which rats died. Constant but slight cholangitis and pericholangitis characterized by a mixed round cell and neutrophil infiltrate in association with focal degenerative changes and necrosis of the bile-duct epithelium were seen in the smaller portal tracts. The changes in the other organs were non-specific and slight and included a perivascular round cell infiltrate, mild degenerative changes and incipient necrosis of the myocardium, tubular nephrosis particularly of the medullary and distal convoluted tubules in the kidney, focal necrosis and haemorrhage in the glandular stomach of a few cases and scattered single or groups of necrotic cells in the lower third of the crypts in the small intestine. Small ring haemorrhages, particularly common in the rats on the highest dietary level, were noticed in the brain stem.

A mild chronic cholangitis was the most constant lesion observed in the survivors. Nuclear polymorphism was marked in the affected ducts and low papillary projections were present in the large bile ducts in one case. A very mild tubular nephrosis was the only other noteworthy lesion.

The changes observed in the controls, i.e. a mild pneumonitis and peribronchitis in the lung and a slight mesangial hyperplasia in the renal glomeruli were also constant findings in the experimental groups and are considered to be non-specific.

Discussion

The toxicity of *D. macrospora* culture material to ducklings and rats has been established in these experiments. Apart from causing mortalities it also resulted in a marked reduction in weight gain. The lesions observed at autopsy were not very marked and the cause of death may be attributed to a shock syndrome induced by the toxin. This may be ascribed to a direct endothelial toxicity, a mechanism of action which is also reflected by its irritating effect on the bile ducts where it induced a low-grade cholangitis. The liver lesions described do not resemble those caused by *D. maydis* in laboratory animals (Rabie *et al.* 1977) and it seems unlikely that *D. macrospora* can be incriminated as an additional or sole aetiological factor in the causation of diplodiosis of cattle and sheep (Mitchell, 1918, 1919; Theiler, 1927; Shone & Drummond, 1965). There is, however, a similarity as far as the hepatic, and to a certain extent, the renal lesions are concerned to the changes described for various members of the *Aspergillus ochraceus* group (Zimmermann, Carlton, Tuite & Fennell, 1977) and *Penicillium viridicatum* (Carlton, Tuite & Mislivec, 1968; Budiarso, Carlton & Tuite, 1971). However, even at the highest dietary level, the severity of the lesions caused by *D. macrospora* never reached that of the Aspergilli or of *P. viridicatum*, nor was hepatocellular necrosis observed. It is furthermore not considered to be the primary or most important lesion. The importance of this common seedborne fungus in maize as the possible cause of disease in man will depend, amongst other things, on the natural occurrence of significant levels of the *D. macrospora* toxin in dietary constituents.

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IN VITRO INHIBITION OF RAT INTESTINAL HISTAMINE-METABOLIZING ENZYMES*

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Abstract—When used at 10 mM concentrations in assays *in vitro*, seven of 37 chemicals strongly inhibited (>75% inhibition) rat jejunal mucosa histamine-*N*-methyltransferase (HMT), while eight of 37 chemicals strongly inhibited rat jejunal mucosa diamine oxidase (DAO). The HMT inhibitors included tyramine, β-phenylethylamine, tryptamine, octopamine, agmatine, aminoguanidine and nicotine. The most potent DAO inhibitors were aminoguanidine, anserine, carnosine, histamine, agmatine, thiamine, cadaverine and tyramine. Since many of these inhibitors occur together with histamine in spoiled tuna, the inhibition of the two major intestinal histamine-catabolizing enzymes, HMT and DAO, may play a vital role in the chemical potentiation of histamine toxicity.

Introduction

Histamine has been implicated as the causative agent in certain food-poisoning episodes, particularly in allergy-like reactions due to consumption of spoiled tuna or mackerel (Merson, Baine, Gangarosa & Swanson, 1974). The involvement of histamine in this type of food poisoning is strongly supported by the similarity of the symptoms to those of iv histamine administration, the efficacy of antihistamine therapy, and the consistent association between high histamine levels in implicated foods and the development of toxic reactions. However, a rather remarkable lack of toxic response occurs after oral dosing of histamine to humans or various laboratory animals (Douglas, 1970; Geiger, 1955; Granerus, 1968). This relative lack of oral histamine toxicity is possibly due to the presence of histamine-catabolizing enzymes in the intestines. Two primary intestinal histamine-metabolizing enzymes, histamine-*N*-methyltransferase (HMT) and diamine oxidase (DAO), have been identified in several species (Kim, Backus, Harris & Rourke, 1969; Taylor & Lieber, 1978). The paradox between the apparent toxicity of histamine when consumed with spoiled tuna and the lack of toxicity of pure histamine remains unexplained. One possible explanation would be the chemical potentiation of histamine toxicity by the coincident existence of inhibitors of the intestinal histamine-catabolizing enzymes in the spoiled tuna. This study was undertaken to assess the potential inhibitory effect on the intestinal histamine-catabolizing enzymes of various chemicals likely to be consumed with tuna.

Experimental

Enzyme preparation. Male Sprague–Dawley rats weighing approximately 250–350 g were maintained on a chow diet *ad lib.* and then killed by cervical dislocation. The jejunal segment of the small intestine was removed and placed in cold 0·9% NaCl. After expression of the remaining intestinal contents, the jejunal segments were sliced open and rinsed with cold 0·9% NaCl. The mucosal layer was removed by scraping. All further preparatory procedures were carried out at 4°C. The jejunal mucosa was weighed and homogenized on 0·1 M-potassium phosphate buffer (pH 7·4), by four strokes of a glass-Teflon homogenizer set at 400 rpm. The homogenate was filtered through two layers of cheesecloth, and made to a final volume of 10 ml/g mucosa with 0·1 M-potassium phosphate buffer (pH 7·4). The filtered homogenate was centrifuged at 100,000 g for 60 min. The resultant supernatant or soluble fraction was used as the enzyme source for all experiments.

Enzyme and protein analysis. HMT was assayed by a modification of the method of Axelrod (1971). The 1-ml reaction mixtures contained 0·2 ml of 0·15 mM-histamine in 0·5 M-potassium phosphate buffer, pH 7·6, 0·2 ml of 0·5 mM-[^{14}C]*S*-adenosylmethionine with a specific activity of $1{\cdot}45 \times 10^3$ cpm/nmol, 0·5 ml of enzyme preparation, and 0·1 ml of water or inhibitor solution. Unless otherwise specified (in Table 1), chemicals that were tested as inhibitors were prepared as 100 mM-solutions in water, and the final concentration of the inhibitors in the reaction mixture was 10 mM. The inhibitor (or deionized water), enzyme and *S*-adenosylmethionine were pre-incubated together for 5 min at 37°C before the reaction was initiated by the addition of histamine. Reaction mixtures were incubated for 20 min before stopping by addition of 0·5 ml of 5 N-NaOH. After stopping the reaction, 6 ml of toluene—isoamyl alcohol (1:1, v/v) was added and mixed vigorously. The organic layer was removed and filtered through anhydrous sodium sulphate, and a known portion of filtered organic solution was placed in scintillation vials containing

*The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

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10 ml of ACS™ solution (Amersham Corp., Arlington Heights). The counting efficiency was 95%. Radioactivity measurements were done in a liquid scintillation counter. Corrections were made for the radioactivity of an enzyme–substrate blank prepared by adding histamine after the 5 N-NaOH.

A modification of the procedure of Okuyama & Kobayashi (1961) was used in the assay of DAO. The 1-ml reaction mixtures contained 0·4 ml of 0·5 M-potassium phosphate buffer (pH 7·2), 0·1 ml of 28 mM-putrescine with a specific activity of 87·2 cpm/nmol, 0·3 ml of enzyme preparation, 0·1 ml of water and 0·1 ml of water or inhibitor solution. Inhibitor solutions and concentrations were identical to those used in the HMT assays. A 5-min pre-incubation period at 37°C for the inhibitor and enzyme was used. The reaction was initiated by addition of putrescine. After 60 min incubation at 37°C, the reaction was stopped by addition of 0·2 ml of 10% $HClO_4$. One ml of alkaline $NaHCO_3$ (Okuyama & Kobayashi, 1961) was added, mixed and 6·0 ml of toluene was added. After mixing, the organic layer was removed and filtered through anhydrous sodium sulphate. A known portion of the filtered toluene phase was placed in scintillation vials containing 10 ml of ACS™ solution and radioactivity measured as before. Corrections were made for the radioactivity of enzyme-substrate blanks prepared by adding the $HClO_4$ before putrescine.

The protein concentration of the enzyme preparation was measured by the modified Folin phenol procedure (Lowry, Rosebrough, Farr & Randall, 1951; Miller, 1959). The protein concentration of the soluble fraction used as the enzyme source for these studies was 4·32 mg/ml.

Results

The effects of 38 chemicals on the *in vitro* activity of rat jejunal mucosal HMT and DAO are presented in Table 1. The most potent inhibitors of HMT activity were tyramine (99% inhibition), β-phenylethylamine (99% inhibition), tryptamine (98% inhibition), octopamine (94% inhibition), agmatine (87% inhibition), aminoguanidine (81% inhibition) and nicotine (78% inhibition), when tested at 10 mM. The most potent inhibitors of DAO activity were aminoguanidine (100% inhibition), anserine (100% inhibition), carnosine (100% inhibition), histamine (99% inhibition), agmatine (97% inhibition), thiamine (92% inhibition), cadaverine (87% inhibition) and tyramine (77% inhibition), when tested at 10 mM. Several chemicals gave intermediate levels of inhibition including cadaverine, indole, tartrazine, theophylline, thiamine and trimethylamine with HMT and caffeine, hypoxanthine, indole, 1-methylhistidine, 3-methylhistidine, nicotine, octopamine, β-phenylethylamine, piperazine, spermidine, spermine, synephrine, theobromine, theophylline, tryptamine and xanthine with DAO. Many chemicals such as the amino acids including arginine, glycine, histidine, lysine, ornithine and tryptophan, had essentially no effect on HMT and/or DAO. A few chemicals appeared to activate the enzymes. For example, 144% of the original HMT activity was observed after inclusion of 10 mM-anserine in the reaction mixtures. Ethanol and glycine activated DAO substantially, while anserine, caffeine, carnosine, hypoxanthine, spermidine, theobromine, uric acid and xanthine activated HMT to at least 120% of its original activity.

Table 1. *Effect of various chemicals on the* in vitro *activity of rat intestinal histamine-*N*-methyltransferase (HMT) and diamine oxidase (DAO)*

Potential inhibitor	Final inhibitor concn (mM)	Percentage of original activity*	
		HMT	DAO
Agmatine	10	13	2·6
Aminoguanidine	10	19	0
Anserine	10	144	0
Arginine	10	111	109
Cadaverine	10	65	13
Caffeine	10	123	51
Carnosine	10	125	0
Ethanol	10	111	123
Glucosamine	10	112	107
Glycine	10	104	121
Histamine	10	–	1·0
Histidine	10	98	116
Hypoxanthine†	5	140	55
Indole	10	61	78
Lysine	10	102	105
1-Methylhistidine	10	111	71
3-Methylhistidine	10	98	75
Niacinamide	10	93	115
Nicotine	10	22	75
Octopamine	10	5·9	59
Ornithine	10	98	103
β-Phenylethylamine	10	1·0	58
Piperazine	10	110	73
Putrescine	10	89	–
Spermidine	10	120	76
Spermine	10	116	71
Synephrine†	5	98	56
Tartrazine	10	68	101
Theobromine†	5	136	64
Theophylline	10	60	74
Thiamine	10	51	7·5
Trimethylamine	10	46	104
Tryptamine	10	2·3	56
Tryptophan	10	91	100
Tyramine	10	0·6	23
Urea	10	96	84
Uric acid†	5	138	85
Xanthine†	5	120	58

*Average of two assays.
†These chemicals were dissolved in 0·05 N-NaOH. while all other chemicals listed were dissolved in water.

Discussion

Many inhibitors of HMT and DAO have been identified previously. HMT is known to be inhibited by *S*-adenosyl-L-methionine analogues such as *S*-adenosyl-L-homocysteine (Borchardt & Wu, 1974; Borchardt, Huber & Wu, 1974). HMT can also be inhibited by a variety of antihistaminic drugs (Hanna & Borchardt, 1974; Taylor & Snyder, 1972). Additionally, HMT is subject to substrate inhibition by high concentrations of histamine (Taylor & Leiber, 1978; Taylor & Snyder, 1972). DAO is known to be sensitive to inhibition by numerous substances, particularly bases, such as amidines and guanidines, car-

bonyl reagents, substituted hydrazines, and chelating agents (Buffoni, 1966; Zeller, 1963). Many of these known DAO inhibitors belong to a class of drugs referred to as monoamine oxidase inhibitors (Crabbe & Bardsley, 1974). DAO is also subject to substrate inhibition, when certain diamines are used as substrates (Beavan & Shaff, 1975; Taylor & Lieber, 1978).

Despite the rather lengthy list of known inhibitors for both HMT and DAO, very few chemicals commonly found in foods were known to be inhibitors of the enzymes. Thiamine and certain aliphatic diamines, which are listed as DAO inhibitors (Buffoni, 1966), were the only previously identified food-borne inhibitors of either enzyme. However, as shown in Table 1, many chemicals often found in foods can inhibit HMT and/or DAO *in vitro*. Most of the chemicals selected for testing in this study were nitrogen-containing bases, since similar substances had been shown to be inhibitors of these enzymes. The most potent inhibitors were monoamines, diamines or guanidines, although correlations between chemical structure and inhibitory activity were difficult to define.

Many of the identified inhibitory chemicals might be found in tuna along with histamine. For example, among the most potent inhibitors of HMT and DAO were a number of amines including tyramine, β-phenylethylamine, tryptamine, cadaverine, putrescine and agmatine, which are decarboxylation products of tyrosine, phenylalanine, tryptophan, lysine, ornithine and arginine, respectively. Histamine is a decarboxylation product of histidine, which is formed in tuna by the action of microorganisms possessing the requisite enzyme, histidine decarboxylase. Many microorganisms also possess the necessary enzymes for the decarboxylation of tyrosine, phenylalanine, tryptophan, lysine, ornithine and arginine (Gale, 1946). Consequently, tuna exposed to microbial spoilage might well contain histamine and a variety of other amines. Spoiled tuna has rather high levels of cadaverine and putrescine (Mietz & Karmas, 1977). Also, spoiled tuna extracts contain numerous ninhydrin-reactive components (Lieber & Taylor, 1978). Additionally, anserine and carnosine, which were inhibitors of histamine catabolism (Table 1), are present in tuna meat (Lukton & Olcott, 1958).

The ability of these amines to inhibit intestinal histamine catabolism could magnify the oral toxicity of histamine and provide an explanation for the apparently greater toxicity of histamine consumed with spoiled tuna. The idea of chemical potentiation of histamine toxicity is not new. Kawabata, Ishizaka & Miura (1955), discussed the existence of an unidentified potentiator, termed 'saurine'. Foo (1976) recently determined that 'saurine' was actually histamine phosphate, an artifact of extraction. The critical involvement of enzyme inhibition in amine toxicity has been demonstrated by the synergism of monoamine oxidase inhibitors in precipitating toxic reactions to tyramine in foods (Blackwell & Marley, 1966). While the hypothesis of chemical potentiation of histamine poisoning seems viable and is supported by these preliminary experiments, further work is needed, aimed at the isolation of inhibitors from spoiled tuna and demonstration of *in vivo* inhibition of intestinal histamine catabolism.

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MONOGRAPHS

Monographs on Fragrance Raw Materials*

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5-ACETYL-1,1,2,3,3,6-HEXAMETHYLINDAN (SUPPLEMENT)†

Biological data

Phototoxicity. Undiluted 5-acetyl-1,1,2,3,3,6-hexamethylindan produced phototoxic effects on the skin of the hairless mouse (Forbes, Urbach & Davies, 1978). Various concentrations in methanol were also tested on the skin of the hairless mouse for phototoxic effects, and the no-effect level was found to be approximately 1%, equal to approximately 0·04 mg/cm^2 of skin (Forbes, 1978).

5-Acetyl-1,1,2,3,3,6-hexamethylindan was found to be phototoxic in the guinea-pig and rabbit when tested in concentrations as low as 5% in ethanol (S. Ohta, Shiseido Laboratories, personal communication, 26 August 1978).

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*The most recent of the previous sets of these monographs appeared in *Food and Cosmetics Toxicology* 1977, **15**, no. 6 (pp. 611–638) and the 1978 Supplement (pp. 637–884).

†See original monograph: *Food and Cosmetics Toxicology* 1975, **13**, 693.

ANISOLE

Synonyms: Methoxybenzene; methyl phenyl ether.
Structure: $C_6H_5 \cdot O \cdot CH_3$.
Description and physical properties: *Merck Index* (1976).
Occurrence: Reported to be found in the oil of *Artemisia dracunculus* var. *turkestanica* (*Fenaroli's Handbook of Flavor Ingredients*, 1975).
Preparation: From phenol in mild aqueous alkali with dimethyl sulphate (Arctander, 1969).
Uses: In public use since the 1930s. Use in fragrances in the USA amounts to approximately 1000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·005	0·1
Maximum	0·1	0·01	0·03	0·4

Analytical data: Gas chromatogram, RIFM no. 74–164; infra-red curve, RIFM no. 74–164.

Status

Anisole was given GRAS status by FEMA (1965), is approved by the FDA for food use (21 CFR 172.515) and was included by the Council of Europe (1974) in the list of artificial flavouring substances that may be added temporarily to foodstuffs without hazard to public health. The *Food Chemicals Codex* (1972) has a monograph on anisole. CAS Registry No. 100–66–3.

Biological data

Acute toxicity. The acute oral LD_{50} was reported as 2800 mg/kg for the mouse (National Institute for Occupational Safety and Health, 1976) and as 3700 mg/kg for the rat (Jenner, Hagan, Taylor, Cook & Fitzhugh, 1964; Taylor, Jenner & Jones, 1964). Four daily oral doses of 1230 mg anisole/kg given to six rats caused the death of one but produced no macroscopic liver lesions (Taylor *et al.* 1964). In rats, the sc LD was reported as 3500–4000 mg/kg (Hake & Rowe, 1963), the ip LD as 100–900 mg/kg (Hake & Rowe, 1963) and the ip LD_{50} as 1950 mg/kg (Grübner, Klinger & Ankermann, 1972). The acute dermal LD_{50} in rabbits exceeded 5 g/kg (Moreno, 1976). A 10% solution of anisole was the highest non-toxic concentration applied to one third of the body surface of a mouse; whole-body application to a calf was also non-toxic (Lebedev, 1969).

Irritation. Anisole applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was moderately irritating (Moreno, 1976). Tested at 4% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Epstein, 1976).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 32 volunteers. The material (RIFM no. 74–164) was tested at a concentration of 4% in petrolatum and produced no sensitization reactions (Epstein, 1976).

Metabolism. Gastro-intestinal absorption of anisole in the rat proceeded at higher rates than that of any of three other benzene derivatives tested, 64% of the anisole introduced into the stomach and 76% of that introduced into the intestine being absorbed within 120 min (Fritsch, de Saint Blanquat & Derache, 1975). It has been shown that anisole administered to the rabbit is excreted as *p*-hydroxyphenyl methyl ether, 2% as the free compound, 48% as the glucuronate and 29% as the ethereal sulphate (Hake & Rowe, 1963; Williams, 1959).

Pharmacology. No variation in the histology of the intestinal mucosa nor in the absorption of glucose, methionine, butyric acid or water by rat intestine during *in situ* perfusion was found when anisole was added to the perfusate at a concentration of 2 mg/kg (Fritsch, Lamboeuf, de Saint Blanquet & Canal, 1975). Administration of 195 mg anisole/kg ip to rats decreased hexobarbital sleeping time and markedly stimulated the excretion of ascorbic acid in the urine and the *N*-demethylation of aminopyrine by the liver, changes that indicated the induction of liver microsomal enzymes (Grübner *et al.* 1972). Anisole antagonized hexenal sleep, prolonged apomorphine stereotypy and prevented apomorphine hypothermia in rats and mice (Lupandin & Mar'yanovskii, 1972). The activity of frog olfactory receptors in response to several aromatic vapours, including anisole, has been studied (Duchamp, Revial, Holley & MacLeod, 1974; Getchell & Gesteland, 1972). Anisole has been examined as an *in vitro* inhibitor of the bovine α-chymotrypsin-catalysed hydrolysis of acetyl-L-valine methyl ester (Wallace, Kurtz & Niemann, 1963).

Tumour-promoting activity. When a 20% solution of anisole in acetone was applied twice weekly to the skin of female mice following a single application of the carcinogen, dimethylbenz[*a*]anthracene, 34 of the 36 treated mice survived the 12-wk test period but 9% of the survivors had papillomas and 3% had carcinomas (Boutwell & Bosch, 1959).

Cytotoxicity. Ascites sarcoma BP8 cells were 8% inhibited by 1 mM anisole but 0·1 mM produced no inhibition (Pilotti, Ancker, Arrhenius & Enzell, 1975).

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BUTYL BUTYROLACTATE

Synonyms: Butyl butyryl lactate; lactic acid, butyl ester, butyrate.
Structure: $CH_3 \cdot (CH_3 \cdot [CH_2]_2 \cdot OCO)CH \cdot OCO \cdot [CH_2]_3 \cdot CH_3$.
Description and physical properties: *Food Chemicals Codex* (1972).
Occurrence: Has apparently not been reported to occur in nature.
Preparation: By esterifying butyl lactate with butyric anhydride with or without the use of catalysts.
Uses: Use in fragrances in the USA amounts to less than 1000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	—	—	0·01	0·1
Maximum	—	—	0·04	0·4

Analytical data: Gas chromatogram, RIFM no. 76–28; infra-red curve, RIFM no. 76–28.

Status

Butyl butyrolactate was given GRAS status by FEMA (1965), is approved by the FDA for food use (21 CFR 172.515) and was included by the Council of Europe (1974) in the list of artificial flavouring substances that may be added temporarily to foodstuffs without hazard to public health. The *Food Chemicals Codex* (1972) has a monograph on butyl butyrolactate. CAS Registry No. 7492–70–8.

Biological data

Acute toxicity. Both the acute oral LD_{50} in rats and the acute dermal LD_{50} in rabbits exceeded 5 g/kg (Levenstein, 1976).

Irritation. Butyl butyrolactate applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was irritating (Levenstein, 1976). Tested at 4% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Epstein, 1976).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material (RIFM no. 76–28) was tested at a concentration of 4% in petrolatum and produced no sensitization reactions (Epstein, 1976).

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Levenstein, I. (1976). Report to RIFM, 18 May.

BUTYL OLEATE

Synonym: *n*-Butyl oleate.
Structure: $CH_3 \cdot [CH_2]_3 \cdot OCO \cdot [CH_2]_7 \cdot CH{:}CH \cdot [CH_2]_7 \cdot CH_3$.
Description and physical properties: A pale-yellow liquid.
Occurrence: Has apparently not been reported to occur in nature.
Preparation: From *n*-butanol and oleic acid by heating to 100–150°C with sulphuric acid as a catalyst (Arctander, 1969).
Uses: In public use since the 1930s. Use in fragrances in the USA amounts to less than 1000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·005	0·1
Maximum	0·1	0·01	0·03	1·0

Status

The Council of Europe (1974) included butyl oleate in the list of artificial flavouring substances not fully evaluated. CAS Registry No. 142-77-8.

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, 1975).

Irritation. Butyl oleate applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was moderately irritating (Moreno, 1975). Tested at 10% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1975).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material (RIFM no. 75-6) was tested at a concentration of 10% in petrolatum and produced no sensitization reactions (Kligman, 1975).

Pharmacology. Butyl oleate inhibited the activity of isolated frog heart and decreased the arterial pressure of anaesthetized cats (Myasoedova, 1968). The ester had no effect on human red blood cells or on blood grouping anti-serum, but when incorporated into finished plastics sheets and added to red blood cells and antisera for extended periods, haemolysis of the red blood cells and impairment of the antisera were noted (Haberman, Guess, Rowan, Bowman & Bower, 1967).

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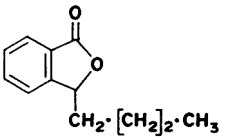
3-BUTYL PHTHALIDE

Synonym: 3-*n*-Butyl phthalide.
Structure:



Description and physical properties: A colourless oily liquid.
Occurrence: Found in *Levisticum officinale* and *L. acutilobum* and also in celery oil (*Fenaroli's Handbook of Flavor Ingredients*, 1975).
Preparation: From phthalide via bromation and reaction of the phthalaldehydic acid with *n*-butyl magnesium bromide (Arctander, 1969).
Use: In perfumes, in a maximum concentration of 0·2%.

Status

3-Butyl phthalide was given GRAS status by FEMA (1973). CAS Registry No. 6066–49–5.

Biological data

Acute toxicity. The acute oral LD_{50} in rats was reported as 2·45 g/kg (1·83–3·28 g/kg) and the acute dermal LD_{50} in rabbits exceeded 5 g/kg (Moreno, 1976).

Irritation. 3-Butyl phthalide applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was moderately irritating (Moreno, 1976). Tested at 2% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Epstein, 1976).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 30 volunteers. The material (RIFM no. 76–31) was tested at a concentration of 2% in petrolatum and produced no sensitization reactions (Epstein, 1976).

Pharmacology. The compound may possess some antispasmodic activity (Ko, Wang, Wang, Wang, Liu & Lin, 1974).

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Moreno, O. M. (1976). Report to RIFM, 2 August.

CINNAMIC ALDEHYDE

Synonyms: β-Phenylacrolein; 3-phenylpropenal; cinnamaldehyde; cinnamal.
Structure: $C_6H_5 \cdot CH{:}CH \cdot CHO$.
Description and physical properties: EOA Spec. no. 204.
Occurrence: Has been identified in the essential oils of Ceylon and Madagascar cinnamon leaves, Ceylon, Seychelles, and Japanese (*Cinnamomum laureirii*) cinnamon bark, and in other cinnamon species in varying amounts (0·1–76%), and is also found in the essential oils of hyacinth, myrrh, Bulgarian rose, patchouli and others (*Fenaroli's Handbook of Flavor Ingredients*, 1975).
Preparation: By condensation of acetaldehyde with benzaldehyde.
Uses: In public use before 1900. Use in fragrances in the USA amounts to approximately 100,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·003	0·1
Maximum	0·2	0·02	0·02	0·8

Analytical data: Gas chromatogram, RIFM nos 73-13, 73-68; infra-red curve, RIFM nos 73-13, 73-68.

Status

Cinnamic aldehyde was given GRAS status by FEMA (1965), is approved by the FDA for food use (GRAS) and was listed by the Council of Europe (1974) with an ADI of 1·25 mg/kg. The *Food Chemicals Codex* (1972) has a monograph on cinnamic aldehyde and the Joint FAO/WHO Expert Committee on Food Additives (1967) published a monograph and specifications, giving a conditional ADI of 0–1·25 mg/kg. CAS Registry No. 104-55-2.

Biological data

Acute toxicity. The LD_{50} values for cinnamic aldehyde administered by oral intubation to rats and guinea-pigs were 2·22 and 1·16 g/kg, respectively (Jenner, Hagan, Taylor, Cook & Fitzhugh, 1964). The rats developed depression, diarrhoea and a scrawny appearance and died within 2–3 hr, while in the guinea-pigs, coma was followed by death within 2 hr to 4 days. Sporn, Dinu & Stanciu (1965) reported an oral LD_{50} in rats of 3·35 g/kg. When cinnamic aldehyde was administered orally to groups of white rats, white mongrel mice and guinea-pigs, an LD_{50} of 3·4 g/kg (25·8 mmol/kg) was established for all three species and no sex differences were observed (Zaitsev & Rakhmanina, 1974).

The LD_{50} of cinnamic aldehyde in mice by the ip route was reported as 0·2 g/kg by Fassett (1963), but as 2·318 g/kg by Sporn *et al.* (1965). The low lethal parenteral dose in mice has been reported as 0·2 g/kg (National Institute for Occupational Safety and Health, 1976). The acute dermal LD_{50} in rabbits was reported as 0·59 ml/kg (0·42–0·84 ml/kg) by Shelanski (1973).

Subacute toxicity. The maximum tolerated dose (MTD), defined as the maximum single dose tolerated by all of a group of five mice after receiving six ip injections over a 2-wk period, was found to be 0·25 g/kg for cinnamic aldehyde (Stoner, Shimkin, Kniazeff, Weisburger, Weisburger & Gori, 1973).

When cinnamic aldehyde was fed to ten male and ten female rats in a concentration of 10,000 ppm in the diet over a 16-wk period, slight swelling of the hepatic cells and slight hyperkeratosis of the squamous portion of the stomach lining were observed (Hagan, Hansen, Fitzhugh, Jenner, Jones, Taylor, Long, Nelson & Brouwer, 1967). No macroscopic effects were observed at levels of 2500 or 1000 ppm.

At total doses equivalent to the $LD_{50} \times 5$ given over 25 days, no cumulative effects were observed in white rats, and when cinnamic aldehyde was administered to rats orally at 0·02 LD_{50}/day for 4 months, no significant effects on the blood serum were found at 40 and 140 days (Zaitsev & Rakhmanina, 1974).

Cinnamic aldehyde given in doses of 10 or 50 mg on alternate days to animals on normal or low-protein diets did not affect growth, liver weight and ascorbic acid content, or the activity of aspartic–glutamic transaminase (Sporn *et al.* 1965). Succinic dehydrogenase activity tended to decrease and liver aldolase activity increased. When Sporn *et al.* also administered 2 mg cinnamic aldehyde on alternate days to two generations of rats for 223 and 210 days, respectively, they found that body-weight gain, reproductive ability, development and viability of young, and the protein content and aldolase activity of the liver were not modified but that the lipid content of the liver was increased by 20% in the first generation and 22% in the second.

Inhalation. W. R. Troy has reviewed the respiratory irritation potential of 14 fragrance raw materials, including cinnamic aldehyde (personal communication to RIFM, 1977).

Irritation. Cinnamic aldehyde tested at 3% in petrolatum produced no irritation after a 48-hr

closed-patch test on human subjects (Kligman, 1974). Tested at 8% in petrolatum in a 48-hr closed-patch test on human subjects, it proved to be severely irritating and the concentration had to be reduced to 2% for the test to be completed (Kligman, 1973). In guinea-pigs, the highest non-irritating concentrations of cinnamic aldehyde were 0·5% in vaseline and 1% in acetone (Majeti & Suskind, 1976/1977).

Sensitization. Using the method of Buehler (1965), sensitization reactions were produced in guinea-pigs by challenge with 0·5% cinnamic aldehyde (Majeti & Suskind, 1966/1977; Suskind & Majeti, 1976). Cross-sensitization reactions in cinnamic aldehyde-sensitized guinea-pigs were produced by challenge with α-methyl- and other relatively reactive α-alkylcinnamic aldehydes (Majeti & Suskind, 1976/1977). Existing evidence supported the view that Schiff-base ligands of cinnamic aldehyde on protein side chains were probably responsible for initiating the allergenic response (Majeti & Suskind, 1977). Also using the Buehler method in guinea-pigs, Briggs (1974) produced several sensitization reactions with cinnamic aldehyde at 2%, while a guinea-pig patch test using the method of Maguire (1973) with cinnamic aldehyde at 2% produced sensitization reactions in all six guinea-pigs tested (Prince, 1974).

A review of the literature indicates that cinnamic aldehyde is a skin irritant and a strong sensitizer. In a Danish firm, almost all workers exposed to high concentrations during the manufacture of cinnamon spice substitutes developed sensitivity to cinnamic aldehyde (Collins & Mitchell, 1975). *trans*-Cinnamic aldehyde was identified in 11 of 138 investigational and complaint samples of cosmetic and perfume products (Collins & Mitchell, 1975). In closed-patch tests, cinnamic aldehyde (2% in petrolatum) produced urticarial reactions in patients sensitive to balsam of Peru (Forsbeck & Slog, 1977). Positive skin patch tests to cinnamic aldehyde (1% in petrolatum) were produced in patients with contact dermatitis following the use of an ointment containing oil of cinnamon (Calnan, 1976). In other closed-patch tests, cinnamic aldehyde produced erythema in some subjects with normal skin, at 2 or 5% in vaseline or ointment, and in a few subjects with dermatoses, at 0·2% in ethanol or a cream base (Fujii, Furukawa & Suzuki, 1972).

Hjorth (1961) found that cinnamic aldehyde (5 or 2% in vaseline) frequently produced positive patch-test reactions. At 5% in vaseline, the compound was found to be a primary irritant—primary irritant and true allergic reactions being difficult to distinguish. Positive reactions were far more frequent in patients sensitive to balsam of Peru, and cross-sensitization reactions between cinnamic aldehyde and related materials were observed (Hjorth, 1961).

Development of an immediate but temporary local reaction (erythema, wheal formation) was demonstrated in normal and allergic subjects, by means of uncovered patch tests with 3 or 10% *trans*-cinnamic aldehyde (Nater, de Jong, Baar & Bleumink, 1977).

Cinnamic aldehyde (2% in white petrolatum) produced positive skin patch tests in 3% of 34 male patients and 9% of 55 female patients with contact dermatitis of unknown aetiology (Schorr, 1975a). Studies carried out by the North American Contact Dermatitis Research Group indicated that cinnamic aldehyde may be a frequent cause of allergic reactions to perfumes (Schorr, 1975b). This group reported positive reactions to cinnamic aldehyde in 3·4% of 202 patients tested (Rudner, 1977).

Cinnamic aldehyde at a 5% concentration in a closed-patch test on 82 patients with contact dermatitis produced 24 sensitization reactions (M. Ishihara, personal communication to RIFM, 1977).

Allergic contact dermatitis and allergic contact stomatitis have been attributed to the presence of cinnamic aldehyde in toothpaste (Drake & Maibach, 1976; Kirton & Wilkinson, 1973; Magnusson & Wilkinson, 1975; Millard, 1973). Human sensitization to samples of cinnamic aldehyde dimethyl acetal have been attributed to the presence of 0·8% free cinnamic aldehyde in the sample (Opdyke, 1975).

A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material (RIFM no. 72–247) was tested at a concentration, in petrolatum, of 2% (which was 25% of its original test concentration) and produced eleven sensitization reactions (Kligman, 1973). The test was repeated on 25 additional volunteers using a different sample of cinnamic aldehyde (RIFM no. CA-BG) at a concentration of 3% in petrolatum and three sensitization reactions were produced (Kligman, 1974). To determine whether use of a lower concentration would avoid sensitization, cinnamic aldehyde was reduced from a concentration of 8% to a concentration of 0·5% for testing purposes. The maximization procedure (Kligman, 1966; Kligman & Epstein, 1975) was used, but the number of exposures to cinnamic aldehyde was increased, the same set of subjects being exposed to cinnamic aldehyde for four test periods. The results (Kligman, 1977) were as follows: at the first exposure (5 × 48 hr), one sensitization reaction was produced; at the second (10 × 48 hr) and third (15 × 48 hr) exposures, no additional reactions were produced; at the fourth exposure (20 × 48 hr), one additional sensitization reaction was produced.

Quenching studies: In the course of maximization testing in human subjects, three instances arose in which an individual aldehyde (phenylacetaldehyde, citral or cinnamic aldehyde), occurring widely in nature, proved to be a skin sensitizer. However, the essential oil in which the aldehyde occurred naturally did not induce sensitization reactions, although the aldehyde was present in concentrations as high as 85%. It appeared that some other component(s) of the natural oil inhibited the induction or expression of sensitization. As a test of this hypothesis, several terpenes and alcohols, found

Table 1. *Results of quenching tests on mixtures of cinnamic aldehyde with other essential-oil components*

Second test material	Relative proportions*	Results of sensitization test
Dipropylene glycol	1:1	+
Phenylethyl alcohol	1:1	+
Eugenol	1:1	−
	1:1†	−
	2·5:1†	+
Cinnamic alcohol	1:1	+
Benzyl salicylate	1:1	+
d-Limonene	1:1	−

*Ratio (w/w) of cinnamic aldehyde to second test material. Each mixture was tested, at an overall concentration of 6% in petrolatum, by the maximization procedure (Kligman, 1966; Kligman & Epstein, 1975).
†Duplicate tests

along with the particular aldehyde in the natural composition, were combined with each of the aldehydes in question. It appears now to be a consistent finding that each of these aldehydes, although producing sensitization reactions when applied alone, produces no sensitization reactions in selected simple mixtures with other compounds (Opdyke, 1976). The results of quenching tests on cinnamic aldehyde using the maximization procedure are summarized in Table 1.

In the guinea-pig, sensitization to cinnamic aldehyde was not inhibited by the presence of eugenol. Animals treated with a cinnamic aldehyde–eugenol mixture showed, upon challenge, skin reactions to cinnamic aldehyde as well as to the combination of cinnamic aldehyde and eugenol (Majeti & Suskind, 1976/1977; Suskind & Majeti, 1976).

Although cinnamic aldehyde is a common sensitizer, a patient sensitive to this compound did not react to a perfume in which the aldehyde was known to be present. It was suggested that the aldehyde might have reacted chemically in the perfume to produce a non-sensitizing compound (Fisher & Dooms-Goossens, 1976).

Percutaneous absorption. Cinnamic aldehyde was not absorbed into the intact shaved abdominal skin of the mouse within 2 hr of application (Meyer & Meyer, 1959).

Effects on the eye. Cinnamic aldehyde was inactive in tests of its ability to affect the transparency of the rat lens (phacotoxic activity) (Moro, Sparatore & Piccinini, 1969).

Metabolism in intact animals. After rats had received 0·23 or 0·30 mmol cinnamic aldehyde ip, 29·3% of the compound was detected in the urine as hippuric acid (Teuchy, Quatacker, Wolf & Van Sumere, 1971). Cinnamic aldehyde is oxidized to cinnamic acid which is then degraded to benzoic acid (Williams, 1959).

Pharmacology. The sedative effects reported for cinnamic aldehyde include a decrease in spontaneous motor activity, antagonism to methamphetamine-induced hyperactivity, and prolongation of hexobarbital-induced anaesthesia; hypothermic and antipyretic effects in mice have also been noted (Harada & Ozaki, 1972). The spontaneous activity of mice was not affected by doses of 100, 31·6 or 10 mg cinnamic aldehyde/kg, but strangely enough was depressed by doses of 3·16 and 1 mg/kg (Wagner & Sprinkmeyer, 1973). Cinnamic aldehyde moderately inhibited stomach movement in the rat and intestinal propulsion in the mouse, prevented gastric erosions in stressed mice when administered orally, and increased biliary excretion in rats (Harada & Yano, 1975).

Its effects on EEG patterns were studied in rabbits by Harada, Fujii & Kamiya (1976). It converted resting patterns in the EEG to arousal patterns in gallamine-paralysed preparations with intact brains, and also inhibited recruiting and augmenting responses and produced a centrally originating EEG activation through a direct or indirect excitatory action on the brainstem reticular structure.

Cinnamic aldehyde had a hypotensive effect in anaesthetized dogs and guinea-pigs, apparently by causing peripheral vasodilation (Harada & Yano, 1975). The vasodilation was related to a papaverine-like musculotropic effect which was also demonstrated on isolated ileum from the guinea-pig and mouse. Cinnamaldehyde exerted positive inotropic and chronotropic effects in isolated guinea-pig heart preparations, as well as an increase in cardiac contractile force and beating rate, but repeated applications resulted in cardiac inhibition, although coronary circulation was increased (Harada & Yano, 1975).

Cinnamaldehyde was effective as a repellent against dogs and coyotes when tested on sheep (Lehner, Krumm & Cringan, 1976) and against coyotes when tested on rabbits (Linhart, Dasch, Roberts & Savarie, 1977).

Carcinogenicity. Cinnamic aldehyde painted on female 50-day-old mice for three consecutive days in concentrations of 5, 1 or 0·1% in acetone did not suppress non-specific esterase activity in the sebaceous glands of the skin (Barry, Chasseaud, Hunter & Robinson, 1972). Suppression of this function has been correlated with carcinogenicity. In male and female mice of the A strain given

16 ip injections of cinnamic aldehyde in impure tricaprylin in a total dose of 4·0 or 0·8 g/kg, the incidence of primary lung tumours was no higher than that in control mice treated with the vehicle alone (Stoner *et al.* 1973).

Mutagenicity. In tests using late embryonic and young larvae of *Drosophila melanogaster*, cinnamic aldehyde was shown to be weakly mutagenic, with a tendency to produce nondisjunction (Venkatasetty, 1971).

Cytotoxicity. Growth of ascites sarcoma BP8 cells, cultured in suspension, was completely inhibited by cinnamic aldehyde concentrations of 1 and 0·1 mM, but was only slightly inhibited by a concentration of 0·01 mM (Pilotti, Ancker, Arrhenius & Enzell, 1975).

Effects on isolated tissues. Cinnamic aldehyde displayed a spasmolytic effect against carbachol-, histamine- and barium chloride-induced contractions of isolated guinea-pig ileum (Wagner & Sprinkmeyer. 1973) and in limited studies was found to be an effective liberator of histamine from human leucocytes (Nater *et al.* 1977). It had no effect on the shrinkage temperature of goatskins or of human, bovine and canine aortae (Milch, 1965).

Vertebrate enzymes and proteins. Cinnamic aldehyde was not a substrate for certain aldehyde-oxidase enzyme systems isolated from rat-liver mitochondria (Smith & Packer, 1972), but Boyland & Chasseaud (1968) found that enzymes isolated from rat liver and kidney and other sources catalysed a reaction between glutathione and *trans*-cinnamic aldehyde. Cinnamic aldehyde interacted with ovalbumin, human serum albumin, human γ-globulin, and guinea-pig albumin at pH 9·0 to form conjugates indicated to be Schiff bases (Majeti & Suskind, 1966/1977) and in studies using human serum albumin, it caused dehelicization of the albumin and altered peptide maps in both alcoholic and aqueous media (Bagdasar'yan & Troitskii, 1971).

Micro-organisms. Cinnamic aldehyde was a substrate for an aryl alcohol NADP oxidoreductase purified from the mycelia of *Neurospora crassa* (Gross & Zenk, 1969) and stimulated the germination of uredospores of *Puccinia helianthi* (French, Gale, Graham, Latterell, Schmitt, Marchetti & Rines, 1975). Like certain other products of the oxidative breakdown of lignin, it was found to display fungicidal properties (Telysheva, Sergeeva & Gavare, 1968), and it was identified as the component chiefly responsible for the fungicidal activity of cinnamon oil toward *Trichophyton mentagrophytes* (Korbely & Florian, 1971).

Cinnamic aldehyde in concentrations of 0·1–2% killed cultures of nine bacteria within 2·5–120 min (Beilfuss, 1976).

Invertebrates. Cinnamic aldehyde was found to be strongly lethal towards Anisakis type 1 larvae obtained from cod and Alaskan pollack liver, Terranova type A larvae from the muscle of the same fish, and Contracaecum type A larvae from the caecal pylorus of the fish (Oishi, Mori & Nishiura, 1974). Isolated from the leaves of *Pogostemon heyneanus*, it showed insecticidal activity against stored grain weevils (*Sitophilus oryzae*) and beetles (*Stegobium paniceum*, *Tribolium castaneum* and *Bruchus chinensis*) (Deshpande, Adhikary & Tipnis, 1974). *trans*-Cinnamic aldehyde was a substrate for certain glutathione *S*-transferases isolated from the fat bodies of the cockroach *Periplaneta americana* (Usui, Fukami & Shishido, 1977).

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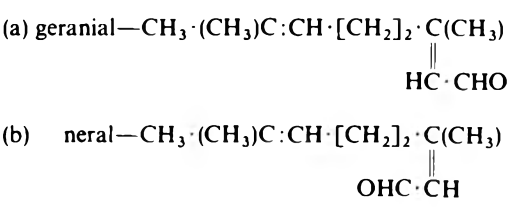
CITRAL

Structure: A mixture of two geometric isomers:



Description and physical properties: EOA Spec. no. 15. A new procedure for determining the neral and geranial content in essential oils has been reported by Jones, Neale & Ridlington (1977), who reviewed earlier analyses and compared them with the new method, in which the isomeric mixture is first reduced with $NaBH_4$ to nerol and geraniol, respectively, and then resolved by gas–liquid chromatography. These authors noted the instability of citral. Nuclear magnetic resonance has also been used to evaluate mixtures of geranial and neral (Joseph-Nathan & Manjarrez, 1967) and high-speed liquid chromatography of citral has been reported (Rabinowitz, Sibeud & Lefar, 1974). Padula, Collura, Rondina, Mizrahi, Coussio & Juarez (1977) have described analytical techniques that can be used to determine the percentage of neral and geranial in lemongrass oil.

Occurrence: Originally reported to be found in lemongrass oil (*Cymbopogon flexuosus* (Nees.) Stapf.) in amounts up to 75%, citral has also been identified in *Litsea citrata* (approx. 90%), *L. cubeba* Blume. (approx. 70%), *Lindera citriodora* (approx. 65%), *Backhousia citriodora* (approx. 95–97%), *Calypranthes parriculata* (approx. 62%), *Leptospermum liversidgei* var. A. leaves (approx. 70–80%) and *Ocimum gratissimum* (approx. 66·5%). It is also present in lemon (2–5%), lime (6–9%) and *Citrus aurantifolia* leaves (petitgrain, approx. 36%) (*Fenaroli's Handbook of Flavor Ingredients*, 1975).

Preparation: By isolation from citral-containing oils or by chemical synthesis (*Fenaroli's Handbook of Flavor Ingredients*, 1975).

Uses: In public use since the 1900s. Use in fragrances in the USA amounts to approximately 75,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·02	0·002	0·005	0·2
Maximum	0·2	0·02	0·02	0·8

Analytical data: Gas chromatogram, RIFM nos 70–64, 71–1, 71–2, 71–3; infra-red curve, RIFM nos 70–64, 71–1, 71–2, 71–3.

Status

Citral was given GRAS status by FEMA (1965), is approved by the FDA for food use (GRAS) and was listed by the Council of Europe (1974) with an ADI of 5 mg/kg. The *Food Chemicals Codex* (1972) has a monograph on citral, and the Joint FAO/WHO Expert Committee on Food Additives (1967) published a monograph and specifications on this material, giving it a conditional ADI of 0–1 mg/kg. CAS Registry No. 5392-40-5.

Biological data

Acute toxicity. The oral LD_{50} value for rats was reported as 4·96 g/kg, with depression followed by death within 4 hr to 4 days (Jenner, Hagan, Taylor, Cook & Fitzhugh, 1964). The maximum non-lethal dose in mice was reported as 900 mg/kg orally and as 250 mg/kg ip (Le Bourhis & Soenen, 1973). The dermal LD_{50} value in rabbits was reported as 2·25 g/kg (1·39–3·11 g/kg) (Moreno, 1974).

Subacute and chronic toxicity. Citral administered to rats by ip injection or by admixture with their food for 3 days resulted in 25% increases in the activities of biphenyl 4-hydroxylase, glucuronyl transferase and 4-nitrobenzoate reductase and in cytochrome *P*-450 in liver homogenates (Parke & Rahman, 1969). Fed in the diet to rats for 13 wk, citral produced no macroscopic effects at 10,000, 2500 or 1000 ppm (Hagan, Hansen, Fitzhugh, Jenner, Jones, Taylor, Long, Nelson & Brouwer, 1967). As reported by Boyd & Sheppard (1970), the FAO/WHO has reviewed evidence indicating that the rabbit was more susceptible than the rat to the toxic effects of citral.

Inhalation. In tests on rabbits anaesthetized with urethane, citral dissolved in ethyl alcohol (1 ml/kg body weight) in concentrations of 0·5, 0·75, 1·0, 1·5, 3, 9, 27, 81 and 243 mg/ml was administered by steam inhalation in such a manner that the rabbits absorbed some 1% of the inhalant (Boyd & Sheppard, 1970). Citral produced a dose-dependent increase in the volume of respiratory-tract

fluid as well as a decrease in the fluid's specific gravity, but the expectorant action became progressively less marked when more than 1·5 mg citral/kg body weight was placed in the vaporizer.

Irritation. Citral applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was moderately to markedly irritating (Moreno, 1974). In a cumulative irritation study carried out on eight volunteers using citral concentrations of 1, 4 and 8% in petrolatum, patches were placed on the back daily, removed at 24 hr and read and then replaced with a fresh patch, over a period of 21 days (Maibach, 1971). The 8% concentration was found to be a marginal irritant. Numerous samples of citral tested at various concentrations from 1 to 8% produced no irritation after 48-hr closed-patch tests on twelve different panels of human subjects (Epstein, 1974; Kligman, 1971, 1972 & 1974).

During an investigation of an outbreak of dermatitis following the introduction of a lemon-scented detergent, citral was shown by patch tests to be a strong primary irritant if applied in association with heat (Rothenborg, Menne & Sjolin, 1977). At 23°C, synthetic citral and two other "pure" citral samples, all at 10% in alcohol, produced numerous slight responses at 20 min, but these disappeared within 24–48 hr. At 43°C, these samples produced a large number of pronounced reactions after 20 min, and the reactions persisted after 24–48 hr. The toxic nature of the response could be detected in biopsies taken as late as 48 hr after exposure. A sample of "essences et fractiones citral" produced no reactions after 20 min–48 hr at 23 or 43°C (Rothenborg *et al.* 1977).

Sensitization. In guinea-pig tests using the method of Buehler (1965), sensitization was induced by 1% citral in vaseline; the highest non-irritating concentration of citral for guinea-pig skin was found to be 1% in vaseline or 2% in acetone (Suskind & Majeti, 1976; Majeti & Suskind, 1976/1977). A guinea-pig patch test on 8% citral using the Maguire method was carriec out on five guinea-pigs and produced sensitization reactions in four of them (H. N. Prince, personal communication to RIFM, 1972).

Maximization tests (Kligman, 1966; Kligman & Epstein, 1975) on human volunteers (in panels averaging 25 subjects each) were used to test various samples of citral from different sources and made by different manufacturing methods. The samples included citral ex lemongrass, citral synthetic, citral natural, citral refined and citral pure, and the following results were obtained: citral samples no. 253169 at 8%, no. 71–3 at 4%, no. 71–2 at 4% and no. 71–1 at 4% produced eight, nine, four and five sensitization reactions, respectively, per test group (Kligman, 1971), samples no. DL–06R, no. DL–10R and no. 72–72, all at 4%, produced three, five and three sensitization reactions, respectively (Kligman, 1972), samples no. 23–45A, no. 23–45B, no. 23–450 and no. 23–45N, all at 5%, produced 16, 14, 8 and 12 sensitization reactions (Kligman, 1974), sample no. 23–45D at 5% produced ten sensitization reactions (Epstein, 1974) and sample no. 25–8–3169 at 1, 4 and 8% produced six sensitization reactions (Maibach, 1971).

Citral was also tested by the repeated-insult patch procedure. With 8% (which had to be reduced to 4% because of irritation) and eleven 24-hr exposures (Draize, 1959), 48% of a panel of 40 human subjects were sensitized (Majors, 1971). A rechallenge patch test was then carried out on 36 of the original 40 panelists and produced six reactions in subjects who had previously reacted and two additional reactions in those who had shown little or no reaction in the original study (Majors, 1971); also with 8% and eleven 24-hr exposures (Draize, 1959), six reactions were produced in 56 human subjects (Maibach, 1971). This was confirmed by a 'use test' in which 1% citral in petrolatum was applied to the cheeks and forearm of five subjects, two of whom developed a dermatitis at the test site (Maibach, 1971). A concentration of 8% citral, in a test involving eleven 48-hr exposures on 105 human subjects, produced a number of reactions by the third exposure and the concentration had to be reduced to 4%, at which level no sensitization reactions were produced (Blau & Kanof, 1971). Two different samples of citral, tested at 4% using eleven 24-hr exposures (Draize, 1959), produced, in the one case, five sensitization reactions in ten subjects (Majors, 1972) and in the other, no reactions in a group of 50 (Shelanski, 1971).

To test the theory that contact allergenicity can be prevented by lowering the concentration, citral was tested by the maximization procedure (Kligman, 1966; Kligman & Epstein, 1975) in a series of tests using reduced concentrations: at 2%, citral produced two sensitization reactions in 24 subjects after five exposures, at 0·5%, two sensitization reactions were produced in 25 new volunteers after ten exposures, and at 0·1%, one sensitization reaction was produced in 25 new volunteers after 15 exposures. Another course of 5–48-hr exposures given to these same subjects produced two additional sensitization reactions after the 20 exposures (Kligman, 1972).

Quenching studies: In the course of maximization testing in human subjects, three instances arose in which an individual aldehyde (phenylacetaldehyde, citral or cinnamic aldehyde), occurring widely in nature, proved to be a skin sensitizer. However, the essential oil in which the aldehyde occurred naturally did not induce sensitization reactions, although the aldehyde was present in concentrations as high as 85%. It appeared that some other component(s) of the natural oil inhibited the induction or expression of sensitization. As a test of this hypothesis, several terpenes and alcohols, found along with the particular aldehyde in the natural composition, were combined with each of the aldehydes in question. It appears now to be a consistent finding that each of these aldehydes, although producing sensitization reactions when applied alone, produces no sensitization reactions in selected simple mixtures with other compounds (Opdyke, 1976). The results of quenching tests

Table 1. *Results of quenching tests on mixtures of citral with other essential-oil components*

Second test material	Relative proportions*	Overall concentration (%)	Results of sensitization test
Lemongrass, East Indian (80% citral)	4:1	4	–
West Indian	4:1	4	–
Mixed citrus terpenes	4:1	5	–
Myrcene	4:1	5	+
Lemongrass	1:4	5	+
α-Pinene	4:1	5	–
d-Limonene	4:1	5	–
	4:1†	5	–
Eugenol	1:1	8	+

*Ratio (w/w) of citral to second test material. Each mixture was tested by the maximization procedure (Kligman, 1966; Kligman & Epstein, 1975).
†Duplicate test.

on citral using the maximization procedure are summarized in Table 1. In guinea-pig tests using the Buehler method, the sensitization reaction was not inhibited in the presence of *d*-limonene Majeti & Suskind, 1976/1977; Suskind & Majeti, 1976).

Metabolism in intact animals. Studies of the absorption, distribution and excretion of [^{14}C]citral in the rat and the mouse demonstrated rapid excretion, with no evidence of long-term storage of citral in the body (Phillips, Kingsnorth, Gangolli & Gaunt, 1976). In rats, single oral doses of 5, 770 and 960 mg/kg were largely excreted within 72 hr, chiefly in the urine (<0·5% as unchanged citral), but also to a significant extent in the faeces and via the lungs; retention of radioactivity was more prolonged in the liver (1·5–2% at 24 hr) than in the rest of the body, apart from the gastro-intestinal tract. Autoradiographic studies of mice following a single dose of 100 mg/kg indicated rapid absorption and fairly uniform distribution throughout the body by 12 hr, most radioactivity being excreted within 120 hr, chiefly in the urine. The studies suggested that biliary excretion may be involved in the metabolism of citral. Williams (1959) reported that citral is metabolized to 2,6-dimethyl-2,6-octadiendioic acid and 2,6-dimethyl-2-octendioic acid.

Pharmacology. Citral (10^{-3} M) evoked both excitatory and inhibitory responses in olfactory neurones when applied to the olfactory epithelium of the burbot, *Lota lota* (Döving, 1966). Orally administered in a dose of 1–100 mg/kg, citral had a depressant effect on the spontaneous activity of mice (Wagner & Sprinkmeyer, 1973), the effect being most pronounced at lower doses (1 and 3·16 mg/kg). When citral was administered as an aqueous solution containing 1% Tween 20 to mice, behavioral effects gave ED_{50} values of 25–100 mg/kg ip and 400 mg/kg orally, and the anticonvulsant ED_{50} values were 100 mg/kg ip and 800 mg/kg orally (Le Bourhis & Soenen, 1973). Wood (1962) reported that citral did not prevent diet-induced hypercholesteraemia in chicks, and subsequently Herzmann (1966) found that citral administered orally at 100 mg/day for 1 wk and then at 200 mg/day for 7 wk to chicks fed an atherogenic diet produced an increase in the aortic concentration of cholesterol greater than that in animals fed only the atherogenic diet.

In preliminary studies, ocular tension was increased in rabbits given a single sc dose of 5 μg citral/kg and in monkeys receiving oral doses of 0·7–1·7 μg/kg/day for 2 wk; damage to the vascular endothelia was also reported (Leach & Lloyd, 1956a). Citral was considered to act as a vitamin A competitor in endothelial cells, and a possible role of citral in glaucoma and cardiovascular diseases in man was suggested (Leach & Lloyd, 1956b). The results were not confirmed, however, by Laughton, Skakum & Levi (1962).

Chemotherapy. Citral has been used for prophylaxis and therapy of postnatal infectious diseases. It can be demonstrated in the blood and urine of patients following intra-uterine application, and when present in blood and urine it has been shown to suppress the growth of *Staphylococcus aureus in vitro* (Osnos, 1964).

Citral offered little or no protection to mice exposed by tail immersion to water containing cercariae of *Schistosoma mansoni* (Gilbert, De Souza, Fascio, Kitagawa, Nascimento, Fortes, Seabra & Pellegrino, 1970). It inhibited the growth of spontaneous carcinomas and, to a lesser extent, of grafted sarcomas when fed to mice in daily doses of 25 mg (Boyland, 1940) but the metabolite, Hildebrandt acid, was less effective. Treatment of tumour-bearing rats and mice with citral produced changes in the nuclear membrane, chromatin and nucleolus of Yoshida ascites sarcoma and Ehrlich ascites hepatoma cells (Osato, Mori & Morita, 1961).

Teratogenic effects. Citral (30 μg/egg) had teratogenic effects on chick embryos (Abramovici, 1972). Injection of 0·02 ml citral solutions (in neutralized olive oil) per egg on day 3 of incubation produced dose-dependent embryotoxic (teratogenic and lethal) effects at concentrations of 0·001–0·1 M. At 0·05 M, various solitary limb and multiple malformations, principally micromelia, phocomelia and

oligodactylia, became apparent after 36 hr in 74 out of 123 embryos. Localization of the malformations near the site of administration suggested a local irritant effect.

Several related studies examined the malformations occurring after injection of citral (30 μg/egg or a 0·05 M concentration) into the suprablastodermic space of chick embryos on day 3 of incubation. On day 6 of incubation, the allantoic fluid of malformed embryos was more acidic than that of controls, while the specific gravity and total solid content of amniotic fluid were increased relative to controls, but the values had returned to normal on day 12 (Abramovici, 1973). Electron microscopy of the striated muscles of localized malformations (phocomelic and amelic legs) on day 12 supported the assumption that citral interfered with myofibrilogenesis of striated muscles during the early gestation period (Abramovici, Liban, Ben-David & Sandbank, 1973). Morphological changes in the ectoderm and mesoderm of micromelic and amelic limbs after 24, 48 and 72 hr were related to the severity of the malformations (Rachmuth, Liban & Abramovici, 1974). Bone differentiation in micromelic limbs was examined by histochemical methods on days 12 and 16 of development (Rachmuth, Liban & Abramovici, 1975) and basic changes in the amino acid composition of muscle protein from malformed limbs were examined on days 6 and 12 (Sporn, Rachmuth & Abramovici, 1975).

No competitive teratogenic effect between citral and vitamin A was detected in chick embryos (Abramovici, 1972 & 1976). Retinyl palmitate (30 or 300 μg/embryo) injected into 3-day-old chick embryos alone or mixed with citral (30 μg/embryo) reduced the teratogenic effect of citral at the lower dose but not at the higher dose. The morphological character of the anomalies found after the mixed injections was always of the citral type (limb and head anomalies), indicating that the mode of action of citral is independent of that of retinyl palmitate.

When two chromatographic fractions prepared from commercial citral were injected into 3-day-old chick embryos at doses of 2·5 μmol, both the purified citral fraction (A) and the heterogeneous fraction (B) were significantly less teratogenic than commercial citral (Rachmuth, Erlanger & Abramovici, 1974). Fraction A appeared to induce more specific malformations than fraction B. It was concluded that commercial citral is a heterogeneous mixture and that its teratogenic activity represents a summation of the activities of its constituents.

Isolated tissues. The possible role of citral as a competitive inhibitor of vitamin A in epithelial differentiation has been studied in chick-embryo organ cultures. Histological examination of chick tracheal explants (Aydelotte, 1963a) showed that citral, at 0·2–1·0 mM, stimulated differentiation of mucous cells and inhibited the differentiation of ciliated cells. At 2·0–3·0 mM, the tracheal epithelium became stratified and occasionally keratinized. When both vitamin A alcohol and citral were present, the effects of vitamin A were inhibited and the effects of citral were reduced, depending on the relative concentrations. Further work (Aydelotte, 1963b) showed that citral (0·2–3·0 mM) and vitamin A, alone or in combination, also altered the pattern of differentiation of chick-embryo oesophageal and corneal epithelium, supporting the view that citral is an inhibitor of vitamin A.

In studies of suckling-hamster trachea in organ culture (Crocker & Sanders, 1970), citral (2 or 4 mM) inhibited ciliary differentiation but did not produce squamous metaplasia. The incidence of squamous metaplasia produced by benzo[*a*]pyrene (BP; 10·5 μg/ml) may have been increased by the addition of citral. These authors speculated that citral produces tissue damage by interfering with vitamin A-associated processes or by peroxidative destruction of vitamin A itself, and they suggested that BP and citral may have an additive effect in producing squamous metaplasia.

In whole-skin slices of guinea-pig ear, vitamin A increased the incorporation of [^{35}S]sulphate in mucopolysaccharides, and citral at a concentration of 10^{-8} M, while having no effect itself on mucopolysaccharides, inhibited the effect of vitamin A (Barker, Cruickshank & Webb, 1964). In human and guinea-pig skin, citral caused a slight increase in sulphate incorporation into dermal polysaccharides but had no effect on this process in the epidermis (Barker *et al.* 1964).

Citral reduced carbachol-, histamine- and $BaCl_2$-induced spasms in isolated guinea-pig ileum (Wagner & Sprinkmeyer, 1973).

Cytotoxicity. Citral (21 μM) inhibited the cell growth of P815Y mast-cell tumours by 50%; neither vitamin A nor its aldehyde inhibited the toxicity of citral to cell growth or to [^{35}S]sulphate- or [^{14}C]glucosamine-incorporation (Thomas & Pasternak, 1969). Citral displayed a markedly cytotoxic effect on HeLa cells (Zolotovich, Nachev, Siljanovska & Stojcev, 1967; Zolotovich, Silyanovska, Stoichev & Nachev, 1969).

Effects on proteins. In studies using human serum albumin, citral was found to cause dehelicization of albumin and to alter peptide maps in both alcoholic and aqueous media (Bagdasar'yan & Troitskii, 1971). Human retinol-binding protein (RBP), a specific protein for vitamin-A transport, was shown to be completely prevented by citral from binding with retinol, because of citral's high affinity for RBP (Hase, Kobashi, Nakai & Onosaka, 1976). Citral was found to react very slowly with glutathione in *in vitro* reactions (Esterbauer, Zollner & Scholz, 1975). The effect of citral on the denaturation and electrophoretic properties of bovine serum and 2% solutions of bovine serum albumin and γ-globulin has been reported (Okulov, 1963).

Micro-organisms. The specific *in vitro* antimicrobial or antifungal activity of citral has been reported for a number of organisms (Beilfuss, 1976; Cole, Blum & Roncadori, 1975; Morris, 1972; Nandi & Fries, 1976; Patakova & Chladek, 1974). The activity of a 98% pure citral against Gram-positive

bacteria, yeasts and moulds was attributed to an impurity, since the separate components, neral and geranial, and their 50:50 mixture were inactive against all bacteria tested and were less active than citral against some yeasts and moulds (Stevens, Jurd, King & Mihara, 1971). A strain of *Pseudomonas conbexa*, LE-X, isolated from soil, oxidized 'citral a' (geranial) to *trans*-geranic acid, and converted 'citral b' (neral) to both *cis*-geranic and *trans*-geranic acids (Hayashi, Takashiba, Ueda & Tatsumi, 1967). *P. aeruginosa* GM2 converted citrals a and b to geranic acid (Hayashi, Takashiba, Ogura, Ueda & Tatsumi, 1968). Geranic acid was also the major metabolite of citral in a bacterium with *P. aeruginosa*-like properties, other metabolites being 6-methyl-5-heptenoic acid, 3-methyl-2-butenoic acid and 1-hydroxy-3,7-dimethyl-6-octen-2-one (Joglekar & Dhavlikar, 1969; Joglekar, Vora, Dhere & Dhavilkar, 1968). In dilutions of 1:500 and 1:1000 citral failed to inhibit the growth of four bacteria found in contaminated cosmetics (Münzing & Schels, 1972 & 1974), but in another study, citral inhibited the growth of *Staphylococcus aureus* in a dilution of 1:1000 (Schweisheimer, 1973).

When mixed with cetylpyridinium bromide, citral, among other terpenes, inhibited the growth of several strains of pathogenic fungi (Gauvreau, 1966). Citral was assayed for antifungal activity *in vitro* against several types of saprophytic and pathogenic fungi (Agafonov, Val'kov & Suvorov, 1968), and markedly inhibited the growth of the fungus *Phellinus tremulae* (Staichenko, 1968).

Citral, like certain other aldehydes, inactivated vesicular stomatitis virus *in vitro* (Kremzner & Harter, 1970).

Insects. Both isomers of citral were found in the secretion from the Nassanoff gland of worker honey bees, and field tests showed that, to honey bees, citral was the most attractive single compound in the secretion (Butler & Calam, 1969; Weaver, Weaver & Law, 1964). The two isomers were also identified in the volatile secretions of both sexes of four species of bee (Prosopis) by Berstrom & Tengo (1973 & 1974), and other studies showed citral as an attractant for the honey bee involved in searching activities (Shearer & Boch, 1966; Waller, 1973; Waller, Loper & Berdel, 1973). Citral was tested as a repellent for honey bees by Atkins, MacDonald, McGovern, Beroza & Greywood-Hale (1975) and as an attractant for the wood wasp *Sirex noctilio* (Simpson, 1976). Head extracts of stingless bees (*Trigona denoiti*) contained *cis*- and *trans*-citral isomers, but synthetic citral elicited no response from the bees (Crewe & Fletcher, 1976).

Both citral isomers were present in the mandibular-gland secretion of the ant *Acanthomyops claviger* (Chadra, Eisner, Monro & Meinwald, 1962). When fed [^{14}C]acetate or mevalonate, these ants produced labelled citral (Happ & Meinwald, 1965 & 1966). Head extracts of *Lestrimelitta limao* contained geranial and neral in a 2:1 ratio (Blum, 1966).

Citral was tested for an electroantennogram response in the American cockroach (Washio & Nishino, 1976). A mixture of citral a and b was inactive as a repellent for a beetle, *Blastophagus piniperda* (Oksanen, Perttunen & Kangas, 1970). Neral and geranial were identified in the rove-beetle pygidial gland (Wheeler, Happ, Araujo & Pasteels, 1972). Geranial was probably present in the defensive secretions of rove beetles (Bellas, Brown & Moore, 1974). Both isomers were present in the androconial secretion of three Pieris (butterfly) species (Bergstrom & Lundgren, 1973). Hirao & Ishikowa (1964) showed citral to be an effective attractant for the larvae of the silkworm (*Bombyx mori*) which could be fed a citral-containing diet (Hamamura, 1963). Citral displayed no juvenile hormone activity in the moths *Antheraea polyphemus* and *Galleria mellonella* (Schneiderman, Krishnakumaran, Kulkarni & Friedman, 1965), and was active as a sex attractant for male codling moths only in laboratory tests (Butt, Beroza, McGovern & Freeman, 1968). Applied in doses of 0·02–0·1 mg/cm^2 to the water surface used by egg-laying mosquitoes, it reduced egg hatching by 70–100% (Saxena & Sharma, 1972).

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CYCLAMEN ALCOHOL

Synonyms: α-Methyl-*p*-isopropylhydrocinnamic alcohol; 2-methyl-3-cumenylpropanol.
Structure: $CH_3 \cdot (CH_3)CH \cdot C_6H_4 \cdot CH_2 \cdot CH(CH_3) \cdot CH_2OH$.
Description and physical properties: A colourless slightly viscous liquid.
Occurrence: Has apparently not been reported to occur in nature.
Preparation: As a by-product in the manufacture of cyclamen aldehyde (Arctander, 1969).
Uses: In public use since before the 1950s.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·02	0·002	0·01	0·25
Maximum	0·15	0·015	0·03	2·0

Analytical data: Gas chromatogram, RIFM no. 74–66; infra-red curve, RIFM no. 74–66.

Status

Cyclamen alcohol is not included in the listings of the FDA, FEMA (1965) or the Council of Europe (1974), or in the *Food Chemicals Codex* (1972).

Biological data

Acute toxicity. Both the acute oral LD_{50} in rats and the acute dermal LD_{50} in rabbits exceeded 5 g/kg (Levenstein, 1974).

Irritation. Cyclamen alcohol applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was not irritating (Levenstein, 1974). Tested at 20% in petrolatum, it produced no irritation after a 48-hr closed-patch test on two different panels of human subjects (Kligman, 1974).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material (RIFM no. 74–66) was tested at a concentration of 20% in petrolatum and produced six sensitization reactions in the 25 subjects tested (Kligman, 1974). When subjected to the same maximization test in 25 new volunteers, again at a concentration of 20% in petrolatum. This material (RIFM no. 74–66) produced sensitization reactions in three subjects (Kligman, 1974). A preparation called 'Cyclamen Aldehyde Special', which contains 50% cyclamen alcohol, has also been tested, at 12%, and was found to sensitize three out of 25 subjects (Kligman, 1976). A sample of cyclamen aldehyde containing 1·5% cyclamen alcohol was tested at 12% and produced no sensitization reactions in 25 subjects (Kligman, 1971).

IFRA data: Cyclamen alcohol at concentrations ranging from 3 to 100% did not sensitize guinea-pigs in the Open Epicutaneous Test (OET) but did produce very strong skin irritation at the 30 and 100% levels (H. Geleick and G. Klecak, unpublished communication 1978). At the lowest concentration (3%), very slight skin irritation was produced after a single as well as after repeated applications.

Five intradermal injections each of 0·1 ml of a 5% emulsion of cyclamen alcohol in Freund's Complete Adjuvant did not sensitize the guinea-pig. Challenges were made topically on day 21 and 35 (H. Geleick and G. Klecak, unpublished communication 1978).

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CYCLAMENALDEHYDE METHYLANTHRANILATE

Synonym: Methyl *N*-(3-*p*-isopropylphenyl)-(2-methylpropylidene)anthranilate.
Structure: $CH_3(CH_3)CH \cdot C_6H_4 \cdot CH_2 \cdot CH(CH_3) \cdot CH{:}N \cdot C_6H_4 \cdot OCO \cdot CH_3$.
Description and physical properties: A yellowish viscous liquid.
Occurrence: Has apparently not been reported to occur in nature.
Preparation: By condensation of cyclamen aldehyde and methyl anthranilate (Arctander, 1969).
Uses: In public use since the 1940s.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·02	0·002	0·005	0·2
Maximum	0·15	0·015	0·03	0·6

Status

Cyclamenaldehyde methylanthranilate is not included in the listings of the FDA, FEMA (1965) or the Council of Europe (1974), or in the *Food Chemicals Codex* (1972). CAS Registry No. 91-50-9.

Biological data

Acute toxicity. Both the acute oral LD_{50} in rats and the acute dermal LD_{50} in rabbits exceeded 5 g/kg (Bailey, 1976).

Irritation. Tested at 6% in petrolatum it produced no irritation after a 48-hr closed-patch test on human subjects (Epstein, 1976).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 33 volunteers. The material (RIFM no. 76–57) was tested at a concentration of 6% in petrolatum and produced no sensitization reactions (Epstein, 1976).

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CYCLOPENTANONE

Synonyms: Ketocyclopentane; adipic ketone.
Structure: $CH_2 \cdot [CH_2]_3 \cdot CO$.
Description and physical properties: *Merck Index* (1976).
Occurrence: Reported to be found in wheat flour and coffee aroma (*Fenaroli's Handbook of Flavor Ingredients*, 1975).
Preparation: By dry distillation of adipic acid in the presence of a barium oxide catalyst.
Uses: Use in fragrances in the USA amounts to less than 1000 lb/yr. The maximum concentration in perfumes (final product) is 1·0%.
Analytical data: Gas chromatogram, RIFM no. 76–58; infra-red curve, RIFM no. 76–58.

Status

Cyclopentanone is not included in the listings of the FDA, FEMA (1965) or the Council of Europe (1974), or in the *Food Chemicals Codex* (1972). CAS Registry No. 120–92–3.

Biological data

Acute toxicity. The oral LD_{50} for mice was reported as 21·66 mmol/kg and the oral LD_{100} as 29·76 mmol/kg (Caujolle & Caujolle, 1965). The acute oral LD_{50} in rats was reported as 1·24 ml/kg (approximately 14 mmol/kg) (Levenstein, 1976). The ip LD_{50} value in mice was reported as 23·2 mmol/kg and the ip LD_{100} as 29·6 mmol/kg, the corresponding figures for rats being 13·93 and 23·80 mmol/kg (Caujolle & Caujolle, 1965). Caujolle, Caujolle & Foulquier (1962) reported that Swiss mice given cyclopentanone in olive oil ip in doses below the LD_{50} (23·2 mmol/kg or 1·95 g/kg) were restless and aggressive and then entered a stage of torpor. Respiration was first accelerated, then slower and arrhythmic, and a drop in body temperature, lasting 5–10 hr, was also noted. The acute dermal LD_{50} in rabbits was reported as >5 g/kg (Levenstein, 1976).

Inhalation. Rats breathing air containing cyclopentanone at a concentration of $6{\cdot}5 \times 10^{-12}$ mol/litre for a period of 4–11 wk showed selective degeneration of the mitral cells of the olfactory bulb (Pinching & Doving, 1974).

Irritation. Cyclopentanone applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was irritating (Levenstein, 1976). Tested at 10% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1976).

Sensitization. A maximization test (Kligman, 1966; Kligman & Epstein, 1975) was carried out on 25 volunteers. The material (RIFM no. 76–58) was tested at a concentration of 10% in petrolatum and produced no sensitization reactions (Kligman, 1976).

Metabolism. Analysis of the urine of rats and rabbits given small doses of cyclopentanone (2·3 mmol/kg) showed the presence of *cis*- and *trans*-2-hydroxycyclopentylmercapturic acid and another unidentified sulphur-containing metabolite (James & Waring, 1971). The rabbit also excreted cyclopentanol as the glucosiduronic acid derivative and as ethereal sulphate. Cyclopentanone caused the level of glutathione in the liver to fall slightly, presumably because glutathione was involved in the metabolism of the cyclopentanone to the corresponding mercapturic acid (James & Waring, 1971).

Pharmacology. Cyclopentanone was assayed for its effectiveness as a contraceptive in rats and mice. It was found to have no effect in males, while in eight females a dose of 50 mg/kg/day for 28 days did not significantly decrease the number of pregnancies or of viable foetuses per litter but greatly increased the number of resorptions per litter (Hall, Carlson, Abernethy & Piantadosi, 1974).

Electrically-measured changes in the olfactory mucosa of the pithed frog exposed to cyclopentanone vapour were found to be characteristic of the compound (Gesteland, 1967). Female rats trained to discriminate the odour of cyclopentanone were tested at concentrations of from 1 to 4 log units below that of the saturated vapour (Phillips & Vallowe, 1975). Cyclic fluctuations in odour discrimination corresponded to the phases of the oestrus cycle; after ovariectomy, no cyclicity in performance was observed. In ovariectomized rats, oestradiol treatment increased performance, while progesterone treatment decreased it.

Cytotoxicity. Cyclopentanone assayed *in vitro* for toxicity to mouse ascites sarcoma BP8 cells was found to inhibit the cell-culture growth rate by 7% at a concentration of 1 mM (Pilotti, Ancker, Arrhenius & Enzell, 1975).

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DIMETHYL ANTHRANILATE (SUPPLEMENT)*

Biological data

Phototoxicity. Dimethyl anthranilate tested at a concentration of 5% in hydrophilic ointment produced phototoxic effects in eight out of ten human subjects (Kaidbey, 1978).

In tests on the skin of the hairless mouse (Forbes, Urbach & Davies, 1978), dimethyl anthranilate produced phototoxic effects when applied undiluted, while following application of various concentrations in methanol, the lowest level with a detectable phototoxic effect was found to be approximately 50%, or approximately 2 mg test agent/cm^2 of the skin (Forbes *et al.*).

Photoallergenicity. Dimethyl anthranilate did not produce any photoallergenic effects on human subjects when tested, at a concentration of 5% in hydrophilic ointment, by the photomaximization procedure (Kaidbey, 1978).

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*See original monograph: *Food and Cosmetics Toxicology* 1975, **13**, 791.

6-METHYLCOUMARIN (SUPPLEMENT)*

Biological data

Phototoxicity. 6-Methylcoumarin tested at a concentration of 5% in hydrophilic ointment did not have phototoxic effects on human subjects (Kaidbey, 1978), and no phototoxic effects were demonstrated with undiluted 6-methylcoumarin on the skin of the hairless mouse (Forbes, Urbach & Davies, 1978).

Photoallergenicity. 6-Methylcoumarin produced photoallergenic effects on 17 out of 18 subjects when tested, at a concentration of 5% in hydrophilic ointment, by the photomaximization test (Kaidbey, 1978).

A proprietary sunscreen induced photosensitivity reactions in a small number of users. Laboratory study revealed that the reactions were of the photoallergic type and were due to the presence of 6-methylcoumarin; by photomaximization testing, 6-methylcoumarin was found to be a potent photocontact allergen (Kaidbey & Kligman, 1978).

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Review Section

CRITICAL REVIEW OF THE TOXICOLOGY OF COUMARIN WITH SPECIAL REFERENCE TO INTERSPECIES DIFFERENCES IN METABOLISM AND HEPATOTOXIC RESPONSE AND THEIR SIGNIFICANCE TO MAN*

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Summary—Coumarin produces histological liver damage in the rat and dog, but not, following an intake of up to 67·5 mg/kg/day for 2 years, in baboons. Grave doubts have been expressed over the acceptability of the diagnosis of 'bile-duct carcinomas' in rats fed coumarin at 5000 or 6000 ppm in the diet for 2 years, especially in view of the absence of significant metastasis in these animals and of the negative carcinogenic findings obtained in an earlier study in rats. Coumarin does not act as a co-carcinogen in mouse skin and no evidence of carcinogenicity was obtained following repeated subcutaneous administration to rats in a limited experiment. Since the rat metabolizes coumarin quite differently from man, the suitability of the rat as a test species in predicting the hepatotoxic risk to man is questionable. Whether coumarin or a metabolite is the active hepatotoxin has not been established conclusively, but the evidence suggests that metabolism is an important factor in determining the hepatotoxic response. Therefore species differences in metabolism should be taken into account in evaluating the hepatotoxic hazard to man.

Introduction

Serious interest in the toxicology of coumarin dates back to the mid-1950s when hepatotoxic effects were demonstrated in rats and dogs (Hazleton, Tusing, Zeitlin, Thiessen & Murer, 1956). This finding prompted a series of investigations into the qualitative and quantitative aspects of coumarin metabolism, mainly in the rat and rabbit (e.g. Booth, Masri, Robbins, Emerson, Jones & DeEds, 1959; Feuer, Golberg & Gibson, 1966; Kaighen & Williams, 1961; Mead, Smith & Williams, 1958b; Van Sumere & Teuchy, 1971), and these studies have been supported by invaluable data obtained in man (Shilling, Crampton & Longland, 1969) and the baboon (Gangolli, Shilling, Grasso & Gaunt, 1974).

As part of a programme for examining the chronic toxicity of various natural and synthetic food flavourings, the US Food and Drug Administration (FDA) reported its findings on many compounds, including coumarin (Hagan, Hansen, Fitzhugh, Jenner, Jones, Taylor, Long, Nelson & Brouwer, 1967). Liver damage was confirmed in rats and dogs, but no effect was seen in rats given 1000 ppm in the diet for 2 years or in dogs given 10 mg/kg/day orally for up to 350 days. The FDA rat study revealed no evidence of coumarin-induced tumours (benign or malignant) in the liver or elsewhere, in striking contrast to the finding of 'bile-duct carcinomas' in coumarin-fed rats, reported in a preliminary paper by German workers in the same year (Bär & Griepentrog, 1967).

The diagnosis of the bile-duct lesion as carcinoma by Bär & Griepentrog (1967) and Griepentrog (1973) has been questioned (D. M. Conning, personal communication 1978; *Food and Cosmetics Toxicology*, 1969). Moreover the metabolic fate of coumarin differs considerably in man and the rat, and therefore doubts have been expressed as to the suitability of the rat as a test species in assessing the risk of coumarin to man (Shilling *et al.* 1969). In addition, relatively new evidence has come to light indicating that the hepatotoxicity of coumarin is less marked in animal species that resemble man in their metabolism of coumarin (Endell & Seidel, 1978; Evans, Gaunt & Lake, 1979).

It is against this conflicting background that a critical review of the toxicological status of coumarin was considered necessary, special attention being paid to the interspecies differences in metabolism and hepatotoxic response. This review is especially timely in view of the Occupational Safety and Health Administration's designation of coumarin as a 'category I carcinogen' on the apparent grounds of a carcinogenic response in two species—the controversial finding of 'bile-duct carcinomas' in rats and the erroneous citing by The National Institute for Occupational Safety and Health (1977) of a positive finding in the mouse-skin study by Roe & Salaman (1955), which yielded unequivocally a negative result (B. Buchner, private communication 1978; see this review, p. 288).

Occurrence, uses and legislative status

Coumarin occurs naturally in various plants and essential oils, including tonka beans, sweet clover, woodruff and oils of cassia and lavender. It is a white

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crystalline solid (m.p. 68–70°C). It is freely soluble in ethanol, chloroform, ether and oils, soluble in alkali hydroxide solutions and slightly soluble in water (0·25% in cold and 2% in hot). Coumarin is also known as 2*H*-1-benzopyran-2-one, 1,2-benzopyrone, *cis*-*o*-coumarinic acid lactone, coumarinic anhydride, *o*-hydroxycinnamic acid-δ-lactone, 2-oxo-2*H*-1-benzopyran and tonka bean camphor. It is used as a fixative and enhancing agent for the odour of essential oils in perfumes, is added to toilet soaps, toothpastes and hair preparations, is used in tobacco products to enhance and fix the natural taste, flavour and aroma, and is occasionally used in industrial products to mask disagreeable odours (International Agency for Research on Cancer, 1976).

Coumarin has been used since the 1900s, and its use in fragrances in the USA amounts to about 250,000 lb/year (Opdyke, 1974). The usual and maximum concentrations are 0·03 and 0·2% in soaps, 0·003 and 0·02% in detergents, 0·015 and 0·1% in creams and lotions and 0·3 and 0·8% in perfumes. The use of coumarin as a flavouring in food has been discontinued on account of the hepatotoxic effects induced in experimental animals (Hazleton *et al.* 1956). Coumarin usage in food ceased in the USA in 1954 (21 CFR 189.130) and the UK authorities recommended its withdrawal in 1965 (Food Standards Committee, 1965). However the Council of Europe in 1974 permitted its presence in food up to 5 ppm and in alcoholic beverages up to 10 ppm, and in the USA, the Bureau of Alcohol, Tobacco and Firearms has imposed since 1974 a 5 ppm limit on coumarin in alcoholic liquors (*Food Chemical News*, 1978). In West Germany, coumarin is permitted at levels up to 15 ppm in 'Maiwein' (an aromatic wine flavoured with woodruff).

Metabolism in animals and man

Absorption, distribution and excretion

The quantitative aspects of coumarin excretion in rats, rabbits and man are summarized in Table 1.

In one study in rats, orally-administered [3-^{14}C]-coumarin was rapidly absorbed from the intestinal tract, and ^{14}C appeared in the serum, liver and kidneys within 5 minutes of dosage, attaining a peak after 45–60 minutes (Feuer *et al.* 1966). Within 48 hours, 70% of this oral dose was eliminated in the urine and only 10% in the faeces. In other studies in rats, both the urinary and faecal routes of excretion assumed almost equal importance, regardless of the low or high dose administered orally (Kaighen & Williams, 1961). The difference in the extent of faecal excretion observed between the two laboratories cannot readily be explained (Table 1).

The biliary excretion of coumarin metabolites in the rat is of a high order. Within 24 hours of an oral or intraperitoneal dose of 50 mg coumarin/kg, 50% of the dose was excreted in the bile of rats as unidentified ring-opened compounds; unchanged coumarin was absent from the bile. When 7-hydroxycoumarin (50 mg/kg intraperitoneally) was given to biliary-cannulated rats, only 0·2% of the dose was excreted in the bile, unchanged and as the glucuronide conjugate (Williams, Millburn & Smith, 1965).

Utilization of [2-^{14}C]coumarin given intraperitoneally to rats facilitated the detection, in the expired air, of significant amounts of radiolabelled CO_2 (30% of dose), probably resulting from the decarboxylation of an intermediate preceding the formation of unlabelled *o*-hydroxyphenylacetic acid (Van Sumere & Teuchy, 1971).

In rabbits, the urinary route is the primary route of excretion, 90% of an oral dose of coumarin being eliminated within 48 hours (Kaighen & Williams, 1961).

In man, absorption of orally-administered coumarin is rapid, as evidenced by the urinary excretion of over 80% of a dose within 24 hours in a group of four men and four women. The urine was examined for only two specific metabolites of coumarin (7-hydroxycoumarin and *o*-hydroxyphenylacetic acid), so the urinary excretion may have exceeded 80% of the dose if other metabolites were also present in the urine. This rapid excretion, coupled with the absence of the

Table 1. *Percentage excretion of a dose of coumarin in various species including man*

Species	Route of administration	Coumarin dose	Percentage of dose excreted in			Reference*
			Urine	Faeces	Expired air	
Man†	Oral	200 mg/person	83 (1 day)	NE	NE	(1)
Rabbit	Oral	50 mg/kg‡	80 (1 day)	0·65	NE	(2)
			90 (2 days)	<0·2	ND	(2)
		1 mg/kg‡	51 (3 days)	11	NE	(2)
Rat	Oral	100 mg/kg‡	55 (3–6 days)	37	ND	(2)
		1 mg/kg‡	50 (2 days)	51	NE	(2)
		200 mg/kg‡	35 (1 day)	2	NE	(3)
			70 (2 days)	10	NE	(3)
	IP	7·5 mg/rat§	38 (16 hr)	13‖	30	(4)

NE = Not examined ND = None detected IP = Intraperitoneal

*References: (1) Shilling *et al.* (1969); (2) Kaighen & Williams (1961); (3) Feuer *et al.* (1966); (4) Van Sumere & Teuchy (1971).

†Four males and four females.

‡(3-^{14}C)-labelled.

§(2-^{14}C)-labelled.

‖Plus an additional 9% in the caecum.

two metabolites in the 48-hour urine, indicated the unlikelihood of enterohepatic circulation of coumarin or its metabolites (Shilling *et al.* 1969). In a pharmacokinetic study in man, Ritschel, Hoffmann, Tan & Sanders (1976) reported a half-life of about 1·5 hours in the blood of humans given intravenous doses of 0·125–0·25 mg coumarin/kg. Although the blood was analysed for 7-hydroxycoumarin (free and conjugated) no results were reported. In a subsequently-published pharmacokinetic study in man (Ritschel, Brady, Tan, Hoffmann, Yiu & Grummich, 1977), an oral (0·857 mg/kg) or intravenous (0·25 mg/kg) dose was administered to four male and two female volunteers and the blood was analysed at intervals from 5 minutes to 6 hours after dosage. After an oral dose, coumarin was rapidly absorbed from the intestinal tract but only 4% of the dose appeared as unchanged coumarin in the systemic circulation, the remainder being quantitatively transformed to 7-hydroxycoumarin (almost completely as the glucuronide conjugate). The biological half-lives of coumarin and 7-hydroxycoumarin glucuronide did not differ significantly between the oral and intravenous routes.

There is no evidence of any significant tissue accumulation of coumarin or any of its metabolites following coumarin administration either orally to rats (Feuer *et al.* 1966; Kaighen & Williams, 1961) or rabbits (Kaighen & Williams, 1961) or intraperitoneally to rats (Van Sumere & Teuchy, 1971). Following a single intraperitoneal injection of [3-^{14}C]coumarin (250 mg/animal) to rats, ^{14}C was taken up in various organs and tissues, especially the liver and kidneys, before declining steadily over 100 hours post-injection (Piller, 1977). Blood levels of ^{14}C were much lower than corresponding tissue levels at any given period. The half-life of coumarin and its metabolites was about 43 hours (Piller, 1977).

Percutaneous absorption

Rabbits dosed dermally or orally with coumarin showed a similar pattern of urinary excretion of coumarin metabolites (Table 2) but no quantitative data were presented (Pekker & Schäfer, 1969).

Metabolic transformations in vivo

The metabolism of coumarin has been studied in a variety of species including the baboon and man; most attention has, however, been focused on the rat and rabbit (Table 2). Although the earlier studies, which relied ostensibly on isolation and paper-chromatographic techniques, gave an insight into the metabolism of coumarin, a more complete understanding did not emerge until radiotracer techniques were applied. It then became possible to quantify the excretion of the various metabolites.

Coumarin is metabolized essentially by hydroxylation at all six possible ring positions to yield 3-, 4-, 5-, 6-, 7- and 8-hydroxycoumarin and by opening of the heterocyclic ring to yield eventually *o*-hydroxyphenylacetic acid and *o*-hydroxyphenyllactic acid. All these metabolites, together with unchanged coumarin, *o*-coumaric acid, *o*-hydroxyphenylpropionic acid (melilotic acid) and 6,7-dihydroxycoumarin have been identified in the urine of coumarin-treated animals (Table 2).

Considerable interest has arisen over the striking difference in the capacity of various species to 7-hydroxylate coumarin. This route of metabolism is far more prominent in man (79% of the dose excreted in the urine as 7-hydroxycoumarin) and the baboon (60%) than in the rat (<1%), rabbit and pig (12%), dog (3%) and other species (Table 2). The only other species approaching the 7-hydroxylating capacity of primates is one particular strain of mouse, the DBA/2J inbred strain, which excreted about 40% of an intraperitoneal coumarin dose in the urine as 7-hydroxycoumarin, albeit following phenobarbitone pretreatment. Two other strains of phenobarbitone-pretreated mice examined conformed to the more typical non-primate weak 7-hydroxylation (Wood & Conney, 1974; Table 2). The strain differences seen in respect of the urinary excretion of 7-hydroxycoumarin were reflected in the corresponding strain differences observed in the basal and phenobarbitone-induced levels of hepatic coumarin 7-hydroxylase determined *in vitro*. For example, urinary 7-hydroxycoumarin excretion was 25 times higher in DBA/2J mice than in AKR/J mice, whilst hepatic coumarin 7-hydroxylase was 14 times greater in the former strain than in the latter (Wood & Conney, 1974).

In species, such as the rat and rabbit, in which 7-hydroxycoumarin formation assumes a minor role, metabolism proceeds via a pathway leading ultimately to *o*-hydroxyphenylacetic acid (Table 2). This metabolite is usually excreted in the urine, but in one rat experiment in which ^{14}C excretion was prominent in the faeces, *o*-hydroxyphenylacetic acid was detected in the faeces together with unchanged coumarin (Kaighen & Williams, 1961). Although *o*-hydroxyphenylacetic acid was present in greater amounts than coumarin, the paper does not make clear whether the former accounted for a small or appreciable proportion of the faecal metabolites.

3-Hydroxycoumarin, the probable precursor of *o*-hydroxyphenylacetic acid, is excreted in the urine to a greater extent in the rabbit than in the rat (Table 2). Apart from 3- and 7-hydroxycoumarin and *o*-hydroxyphenylacetic acid, the remaining metabolites shown in Table 2 account for only a relatively small proportion of the coumarin dose.

It is of great interest that 7-hydroxycoumarin was detected by paper chromatography in the urine of normal human subjects (presumably not dosed with coumarin experimentally) and increased amounts were found in the urine of cancer patients treated with prednisone (Van Sumere, Teuchy & Massart, 1959).

Site of metabolism

Not unexpectedly, the liver is the major site of coumarin metabolism. Studies *in vitro* have demonstrated the ability of liver microsomes to transform coumarin into 3- and 7-hydroxycoumarin, *o*-hydroxyphenylacetic acid and *o*-hydroxyphenyllactic acid in experimental animals and into 7-hydroxycoumarin in man (Table 3). Feuer *et al.* (1966) showed that the coumarin metabolites, *o*-hydroxyphenylacetic acid and *o*-hydroxyphenyllactic acid, were formed rapidly in the liver of rats dosed orally with coumarin. In addition to hepatic involvement, the gut flora plays a role in the metabolism of coumarin. Rat and rabbit intestinal

Table 2. *Urinary excretion of coumarin and metabolites in various species, including man*

Species	Route of administration	Dose	Urinary metabolites and percentage of dose excreted*	Reference†
Man	Oral	200 mg/person	7-HC (79%); *o*-HPAA (4%)	(1)
Baboon	Oral	200 mg/kg	7-HC (60%)	(2)
Cat	Oral	200 mg/kg	7-HC (19%)	(2)
Dog	Oral	200 mg/kg	7-HC (3%)	(2)
Ferret	Oral	200 mg/kg	7-HC (1%)	(2)
		100 mg/kg	3-, 5-, 7- and 8-HC	(3)
Guinea-pig	Oral	200 mg/kg	7-HC (1%)	(2)
		140 mg/kg	3-, 5-, 7- and 8-HC	(3)
Hamster	Oral	200 mg/kg	7-HC (5%)	(2)
Mouse	Oral	200 mg/kg	7-HC (3%)	(2)
		400 mg/kg	3-, 5-, 7- and 8-HC	(3)
	IP	2 μmol/mouse	7-HC (40% in DBA/2J strain; 1·5% in AKR/J strain; 7% in hybrid AKD2F$_1$/J strain)‡	(8)
Pig	Oral	200 mg/kg	7-HC (12%)	(2)
Rabbit	Oral	170 mg/kg	3- (major), 7- and 8-HC	(3)
		0·5% in diet	3-HC, 7-HC and *o*-HPPA as major metabolites; *o*-HPPA, *o*-CA and *o*-HPLA as minor metabolites	(7)
		50 mg/kg	Unchanged coumarin (0·5%); 3-HC (22%); 4-HC (0·6%); 5-HC (0·4%); 6-HC (3%); 7-HC (12%); 8-HC (2%); *o*-HPAA (20%); *o*-HPLA (3%); acid-labile coumarin precursor (15%)	(4)
		100 mg/rabbit	3-, 4- and 7-HC; *o*-HPAA; *o*-CA; unchanged coumarin	(10)
	Dermal	100 mg/rabbit§	3-, 4- and 7-HC; *o*-HPAA; 6,7-dihydroxycoumarin; *o*-CA; unchanged coumarin	(10)
Rat	Oral	100 mg/kg	3-HC (2%); 4-HC (0·3%); 6-HC (0·3%); 7-HC (0·4%); 8-HC (0·4%); *o*-HPLA (0·8%) *o*-HPAA (19%)	(4)
	IP	7·5 mg/rat	5-, 7- (0·7%) and 8-HC; *o*-CA; *o*-HPPA; *o*-HPAA	(5)
	Oral or IP	100 mg/kg	*o*-HPAA (major); *o*-HPPA and *o*-CA (both minor metabolites)	(6)
	Oral	100 mg/rat	*o*-HPPA; *o*-HPAA; *o*-CA; *o*-HPLA	(7)
		200 mg/kg	3-, 7- and 8-HC	(3)
		200 mg/kg	3-, 4- and 7-HC; *o*-CA; *o*-HPAA (major); *o*-HPLA (major); *o*-HPPA (traces); unchanged coumarin	(9)
Squirrel Monkey	Oral	200 mg/kg	7-HC (1%)	(2)

IP = Intraperitoneal HC = Hydroxycoumarin *o*-HPAA = *o*-Hydroxyphenylacetic acid *o*-HPLA = *o*-Hydroxyphenyllactic acid *o*-HPPA = *o*-Hydroxyphenylpropionic acid *o*-CA = *o*-Coumaric acid

*Percentage of dose excreted in urine is given in brackets where available.

†References: (1) Shilling *et al.* (1969); (2) Gangolli *et al.* (1974); (3) Mead *et al.* (1958b); (4) Kaighen & Williams (1961); (5) Van Sumere & Teuchy (1971); (6) Scheline (1968b); (7) Booth *et al.* (1959); (8) Wood & Conney (1974); (9) Feuer *et al.* (1966); (10) Pekker & Schäfer (1969).

‡Each mouse strain also received phenobarbital for 8 days before coumarin dosage.

§2 g of 5% ointment.

microflora have been found to convert coumarin *in vitro* to *o*-hydroxyphenylpropionic acid, probably with the intermediate formation of dihydrocoumarin (Scheline, 1968b; Table 3).

A pharmacokinetic study of coumarin in man indicated that most of the metabolism of coumarin to 7-hydroxycoumarin occurs in the liver but glucuronidation is not confined to the liver and intestinal wall but also occurs in other tissues (Ritschel *et al.* 1977).

Several workers have studied the coumarin 7-hydroxylase activity in human liver (Conney, Kapitulnik, Levin, Dansette & Jerina, 1976; *Food and Cosmetics Toxicology*, 1966; Kapitulnik, Poppers & Conney, 1977; Kratz, 1976). Livers of healthy subjects exhibited a specific activity of coumarin 7-hydroxylase ranging from 0·16 to 0·65 nmol/mg/minute, but this activity was reduced in livers of patients with some, but not all, liver diseases (Kratz, 1976). Despite the possible loss of enzyme activity in liver samples between death and autopsy some 8–20 hours later, a study of coumarin 7-hydroxylase activity in homogenates of 32 adult human livers obtained at autopsy is of interest (Kapitulnik *et al.* 1977). Activity ranged from 0 to 496 nmol 7-hydroxycoumarin formed/g liver/hour. Correlation between coumarin 7-hydroxylase and benzo[*a*]pyrene hydroxylase in this experiment was lower ($r = 0·57$) than in another experiment (Conney *et al.* 1976) on ten human autopsy livers ($r = 0·84$).

Hepatic coumarin 7-hydroxylase activity in rabbits was over 60% lower at full-term pregnancy than in non-pregnant rabbits; enzyme activity was enhanced

Table 3. *Metabolism of coumarin* in vitro

Species	Incubation medium	Metabolic products identified	Reference*
Man	Liver homogenate and microsomes	7-HC	(6–9)
Cat	Liver microsomes	7-HC	(1)†
Coypu	Liver microsomes	7-HC	(1)
Guinea-pig	Liver microsomes	7-HC	(1)
Mouse	Liver microsomes	7-HC	(2)
Pigeon	Liver microsomes	7-HC	(1)
Rabbit	Liver microsomes	7-HC	(1, 3)
	Caecal extract	*o*-HPPA; DH	(4)
Rat	Liver microsomes	3-HC; 7-HC; *o*-HPLA; *o*-HPAA	(5)
	Caecal extract	*o*-HPPA; DH	(4)

HC = Hydroxycoumarin *o*-HPPA = *o*-Hydroxyphenylpropionic acid DH = Dihydrocoumarin
o-HPLA = *o*-Hydroxyphenyllactic acid *o*-HPAA = *o*-Hydroxyphenylacetic acid

*References: (1) Creaven, Parke & Williams (1965); (2) Wood & Conney (1974; (3) Mead *et al.* (1958b); (4) Scheline (1968b); (5) Gibbs *et al.* (1971); (6) Kapitulnik *et al.* (1977); (7) Kratz (1976); (8) *Food and Cosmetics Toxicology* (1966); (9) Conney *et al.* (1976).

†No coumarin 7-hydroxylase was found in rat or mouse liver; rat liver contained an inhibitor of rabbit-liver coumarin 7-hydroxylase (Creaven *et al.* 1965).

in both non-pregnant and pregnant rabbits by phenobarbital pretreatment. However, hydroxylation of biphenyl was not influenced by pregnancy, and in view of this difference and the known species difference in the reduction of hepatic metabolism of compounds in the rat and rabbit during pregnancy, it is difficult to speculate on the significance of these findings to man (Neale & Parke, 1973).

Pathways of metabolism

By following the biotransformation of various coumarin metabolites both *in vivo* and *in vitro*, it has been possible to elucidate the intermediary pathways leading to the end products of metabolism, although this approach may not reproduce exactly the pathways pursued by coumarin *in situ* (Table 4; Fig. 1).

In man, baboon and DBA/2J mice (phenobarbitone-pretreated), direct 7-hydroxylation of coumarin is the primary route of metabolism with 7-hydroxycoumarin appearing in the urine partly in free but mainly in conjugated form. In the rat and rabbit, 3-hydroxylation predominates, but whereas in the rat 3-hydroxycoumarin is extensively degraded to *o*-hydroxyphenylacetic acid, only partial degradation occurs in the rabbit, and this end product and 3-hydroxycoumarin are excreted in the urine in almost equal amounts. *o*-Hydroxyphenylacetic acid probably arises via the following pathway (Kaighen & Wil-

Table 4. *Biotransformation of coumarin metabolites* in vivo *and* in vitro

Compound	Species	Metabolites identified	Reference*
		In vivo	
3-HC	Rabbit	Mainly conjugated; *o*-HPPyA; *o*-HPLA; *o*-HPAA	(1)
	Rat	*o*-HPAA; *o*-HPLA	(1)
3-, 4-, 5-, 7- and 8-HC	Rabbit	Conjugated	(2)
6-HC	Rabbit	Conjugated; 6,7-dihydroxycoumarin	(2)
7-HC	Rat	Conjugated; β-resorcylic acid	(3)
o-CA	Rabbit	*o*-CA (free and conjugated); 4- and 7-HC	(4)
		Conjugated; *o*-HPPA; 4-HC; *o*-HPHA; *o*-HPAA	(5)
	Rat	Conjugated; *o*-HPPA; 4-HC; *o*-HPHA; *o*-HPAA	(5)
o-HPPA	Rabbit	4- and 7-HC; *o*-CA (free and conjugated)	(4)
	Rat	Conjugated; *o*-CA; 4-HC; *o*-HPAA; *o*-HPHA	(5)
		o-CA	(9)
o-HPLA	Rat	*o*-HPAA	(5)
		In vitro	
7-HC	Guinea-pig liver	Conjugated	(6)
o-HPPyA	Rat-liver microsomes	*o*-HPLA	(7)
o-CA	Rat-caecum extract	*o*-HPPA	(8)
Dihydrocoumarin	Admixture with laboratory diet	*o*-HPPA	(10)

HC = Hydroxycoumarin *o*-HPPyA = *o*-Hydroxyphenylpyruvic acid
o-HPPA = *o*-Hydroxyphenylpropionic acid *o*-HPLA = *o*-Hydroxyphenyllactic acid
o-HPAA = *o*-Hydroxyphenylacetic acid *o*-HPHA = *o*-Hydroxyphenylhydracrylic acid
o-CA = *o*-Coumaric acid

*References: (1) Kaighen & Williams (1961); (2) Mead *et al.* (1958a); (3) Van Sumere & Teuchy (1971); (4) Mead *et al.* (1958b); (5) Booth *et al.* (1959); (6) Creaven *et al.* (1965); (7) Gibbs *et al.* (1971); (8) Scheline (1968a); (9) Scheline (1968b); (10) Hagan *et al.* (1967).

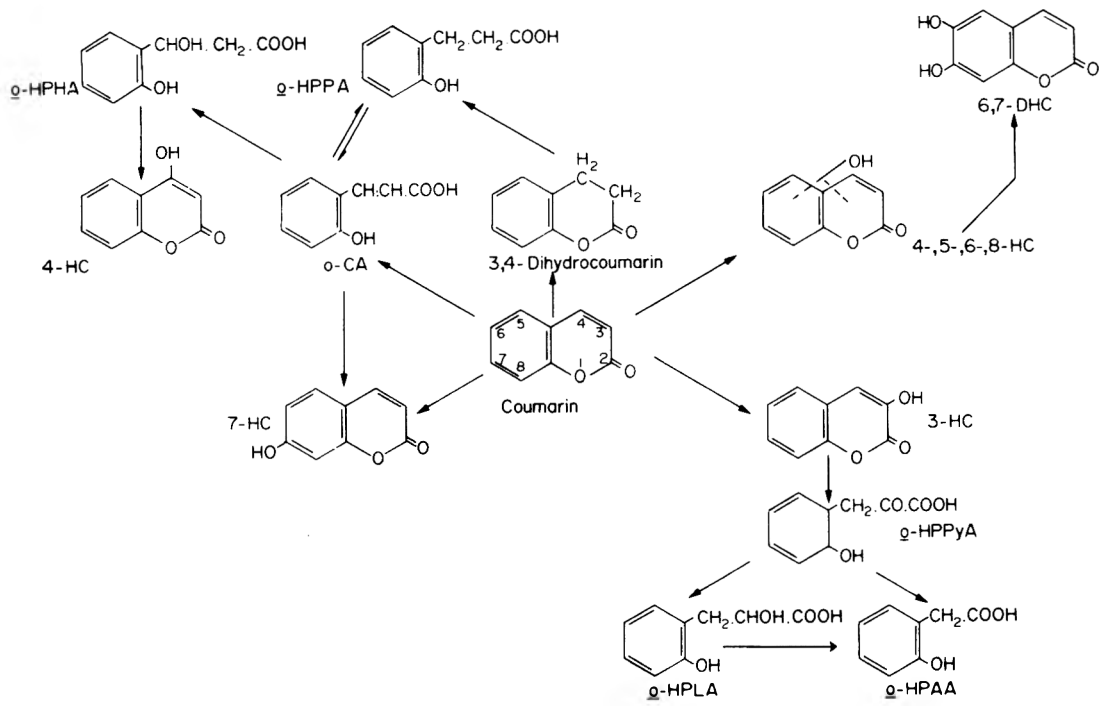

Fig. 1. Pathways of coumarin metabolism *in vivo* and *in vitro* (see also Tables 2–4) leading to the formation of: *o*-coumaric acid (*o*-CA); 6,7-dihydroxycoumarin (6,7-DHC); 3-hydroxycoumarin (3-HC; rabbit 22%, rat 2%); 4-hydroxycoumarin (4-HC); 4-, 5-, 6- and 8-hydroxycoumarin (4-, 5-, 6-, 8-HC; rabbit 6%, rat 1%); 7-hydroxycoumarin (7-HC; man 79%, baboon 60%, DBA/2J mouse 40%, rabbit 12%, dog 3%, AKR/J mouse 1·5%, rat <1%); *o*-hydroxyphenylacetic acid (*o*-HPAA; rabbit 20%, rat 19%, man 4%); *o*-hydroxyphenylhydracrylic acid (*o*-HPHA); *o*-hydroxyphenyllactic acid (*o*-HPLA; rabbit 3%, rat 0·8%); *o*-hydroxyphenyl propionic acid, (melilotic acid; *o*-HPPA); *o*-hydroxyphenylpyruvic acid (*o*-HPPyA). Figures in parenthesis indicate, for the given species, the percentage of a coumarin dose accounted for by urinary excretion of the particular metabolite or group of metabolites identified. Figures for mice are for phenobarbital-pretreated animals.

liams, 1961): coumarin → 3-hydroxycoumarin → *o*-hydroxyphenylpyruvic acid → *o*-hydroxyphenylacetic acid.

Hydroxylation of coumarin in the 4-, 5-, 6- and 8- positions are very minor routes of metabolism. In general, hydroxycoumarins are mainly excreted in the urine as glucuronic acid and ethereal sulphate conjugates (Kaighen & Williams, 1961; Mead, Smith & Williams, 1958a). *o*-Hydroxyphenylpropionic acid is also excreted as the glycine conjugate (Booth *et al.* 1959).

Several factors have complicated our understanding of coumarin metabolism. First, ring-opened metabolites may undergo cyclization (Booth *et al.* 1959), for example: coumarin → *o*-coumaric acid → *o*-hydroxyphenylhydracrylic acid → 4-hydroxycoumarin. Secondly, acid hydrolysis of the urine may destroy some urinary metabolites; Van Sumere & Teuchy (1971) have drawn attention to the sensitivity of *o*-coumaric acid and melilotic acid to boiling acid. Booth *et al.* (1959) also consider that *o*-coumaric acid found in the urine of coumarin-treated rats is probably derived from the acid-labile precursor, *o*-hydroxyphenylhydracrylic acid.

Toxicity studies in animals

Acute toxicity (single dose)

The acute oral LD_{50} was reported to be 196 mg/kg in mice of an unspecified strain (Kitagawa & Iwaki, 1963), 420 mg/kg in CH3/HeJ mice and 780 mg/kg in DBA/2J mice (Endell & Seidel, 1978), 290–680 mg/kg in rats (Hazleton *et al.* 1956; Jenner, Hagan, Taylor, Cook & Fitzhugh, 1964) and 202 mg/kg in guinea-pigs (Jenner *et al.* 1964). The subcutaneous LD_{50} was reported as 310–342 mg/kg in mice (Kitagawa & Iwaki, 1963). Rats showed depression and mottled livers, whilst guinea-pigs showed depression, ataxia and severe gastro-intestinal-tract irritation (Jenner *et al.* 1964). Lethal doses of coumarin had a narcotic effect in rats, whilst sublethal doses produced spotted and mottled livers, enlarged adrenals, spotted kidneys and hyperaemia of the intestines, stomach, testes and pancreas (Hazleton *et al.* 1956).

Repeated oral doses in dogs (8 days)

Eight daily oral doses of coumarin (100 mg/kg) given to nine dogs by Hazleton *et al.* (1956) produced toxic signs (vomiting, anorexia, weight loss, salivation, depression, incoordination and jaundice), impaired liver function, gross and histological changes in the liver (cytoplasmic vacuolation, fatty change and necrosis) and changes in the kidneys (swelling and granularity of the epithelial cells of the convoluted tubules and hyaline casts stained with bile). Two dogs observed for 40–61 days following this coumarin dosage showed recovery from toxic signs, regeneration and repair of liver cells and disappearance of the renal lesions.

Repeated injections in rabbits (8 days)

Four injections of 100 mg coumarin/kg given on alternate days to rabbits produced no histological changes in the liver, kidney, spleen, lungs or heart; decreased blood cholesterol and increased liver glycogen were observed (Patyra, Ziolo & Nagorna-Stasiak, 1965).

Feeding studies in rats (1–29 weeks)

In a 90-day rat study (Hazleton *et al.* 1956), coumarin at 50 or 250 ppm in the diet exerted no effect on weight gain, efficiency of food utilization or organ weights and pathology, but a dietary level of 2500 ppm impaired food efficiency and produced liver enlargement and damage (mottled and spotted liver, fatty change, swelling and granularity of the cells and cell rupture). Feeding of coumarin (10,000 ppm in diet) for 1–8 weeks caused growth retardation, death, slight to moderate degenerative changes in the liver, cell necrosis, bile-duct proliferation and testicular atrophy (Hagan *et al.* 1967). No effect was seen at 1000 ppm for 14 or 28 weeks but a level of 2500 ppm given for 29 weeks caused growth retardation and liver changes (mottling of liver, fatty change).

Oral studies in dogs (9–350 days)

Daily oral doses of 25, 50 and 100 mg/kg body weight for 133–330, 35–277 and 9–16 days, respectively, produced emaciation, jaundice, liver damage (focal necrosis, fibrosis and bile-duct proliferation) and pathological changes to the spleen (haemosiderosis), bone marrow (pale and fatty) and gall bladder (distension), but no effect was seen at 10 mg/kg/day for 297–350 days (Hagan *et al.* 1967).

Oral study in baboons (3 weeks)

Two baboons (one male and one female) were given 50 mg coumarin/kg/day* for 3 weeks followed in succession by a treatment-free period of 3 months, further treatment with 100 mg/kg/day* for 3 weeks and another treatment-free period for 3 months (Gangolli *et al.* 1974). Bromsulphthalein (BSP) assays and liver biopsies were carried out at the end of each coumarin treatment and at the end of the experiment. No histological changes were seen in the liver but some biochemical, histochemical and ultrastructural evidence of hepatotoxicity was presented. Thus BSP excretion was prolonged in the female (but not the male) following each coumarin regime, returning to normal at the end of the corresponding treatment-free period. Lysosomal changes (translocation and autophagic-vacuole formation) and dilatation of the smooth and rough endoplasmic reticulum were seen in both animals at the end of each coumarin period but were not discernible at the end of each 3-month treatment-free period.

Feeding study in baboons (2 years)

Groups of between four and eight male baboons of different ages (6–13·4 kg) and of different species [approximately equal numbers of Olive (*Papio anubis*) and Hamadryas (*Papio hamadras*) and a single Yellow (*Papio cynocephalus*)] were maintained on diets providing 0, 2·5, 7·5, 22·5 and 67·5 mg coumarin/kg body weight/day for 16–24 months (Evans *et al.* 1979), the latter intake being the highest compatible with normal food consumption. No histological changes were seen in the liver or in a wide range of other organs. Biochemical (ethylmorphine *N*-demethylase, aniline 4-hydroxylase, coumarin 7-hydroxylase, glucose 6-phosphatase and cytochrome *P*-450 determinations), histochemical (glucose 6-phosphatase, aniline hydroxylase and lysosomal acid phosphatase) and ultrastructural studies of the liver revealed no significant changes, with the exception of dilatation of the endoplasmic reticulum in three of the four baboons on 67·5 mg/kg/day, a change that first became apparent after 10 months of feeding. This effect was regarded as evidence of early cell damage and a no-effect level of 22·5 mg/kg/day was established; on the basis of conventional histological evidence, the no-effect level would have been 67·5 mg/kg/day.

Carcinogenicity studies

Feeding studies in rats (2 years)

In a 2-year rat study using groups of 6 males and 6 females (Hagan *et al.* 1967), no effect was seen with 1000 ppm coumarin in the diet, but growth retardation and liver damage (slight fatty change and bile-duct proliferation and minimal focal necrosis) occurred at 2500 and 5000 ppm. In addition the 5000 ppm group alone showed focal proliferation of bile ducts of atypical appearance with associated fibrosis (cholangiofibrosis). No details are given, however, of survival rates in the test groups.

The long-term feeding study in rats described in preliminary form by Bär & Griepentrog (1967) and more fully by Griepentrog (1973) merits close scrutiny in view of the serious allegation of coumarin-induced 'bile-duct carcinomas'. Both reports refer to the same study, the findings of which are summarized in Table 5. These malignant tumours were reportedly found at 5000 and 6000 ppm but not at 1000 or 2500 ppm. None of the control rats developed 'bile-duct carcinomas'. Liver tumours did not metastasize to any distant organ and only in a few cases could metastasis into neighbouring liver tissue be observed (Griepentrog, 1973). It is not clear what happened to the liver at the two lowest levels. According to the 1967 paper, no pathological changes were seen at 1000 or 2500 ppm, but the 1973 paper claims that bile-duct proliferation and some benign adenomas were observed at these levels.

Another source of ambiguity lies in the actual intake of coumarin in the 6000 ppm groups. Because of the unpalatability of the diet, food intake was impaired and the coumarin intake averaged only an amount equivalent to a normal intake of diet containing 3500 ppm (the authors also refer to a 6000/7500 ppm group). It is also not clear whether the 40 controls were equally divided by sex—this is of importance in view of the much higher incidence of 'carcinomas' in males than in females. The full significance of the German findings is evaluated later (p. 288).

* Incorrectly expressed as ppm in original paper (S. D. Gangolli, private communication 1978).

Table 5. *Incidence of 'bile-duct carcinomas' reportedly diagnosed in coumarin-treated rats**

Dietary level (ppm)	Initial group size	Group no.	Incidence of carcinomas in rats surviving at least 18 months	No. of survivors at 2 yr
0	40	C‡	0	
1000†	20 M + 20 F	1	0/32	17
5000	20 M + 20 F	2	11/12 M + 1/12 F	14
0	50	C‡	0	
2500†	25 M + 25 F	3	0/28	12
6000§	25 M + 25 F	4	2/20 M + 1/8 F	20
6000§	32 M	5	2/5 M	5

M = Males F = Females

*Data from Bär & Griepentrog (1967) and Griepentrog (1973).

†No liver damage appeared to have occurred at 1000 or 2500 ppm (Bär & Griepentrog, 1967) but some benign adenomas or proliferation of the bile-duct were reported at these levels by Griepentrog (1973).

‡Control; no data on survival or sex distribution.

§Rats on 6000 ppm apparently showed a reduced food intake; coumarin intake was on average equivalent to normal consumption of diet containing about 3500 ppm.

Absence of tumour-initiating activity in mouse skin (21 weeks)

A single application of 15% coumarin in acetone to mouse skin failed to produce epidermal hyperplasia within 3 days of dosage, and no tumour-initiating activity was seen after either a single dose of 45 mg coumarin (15% in acetone) or a total dose of 150 mg coumarin (one dose of 10% in acetone followed by 12 weekly doses of 3·3% in acetone) to mouse skin followed 10 or 21 days later by weekly treatment with the tumour promoter, croton oil, for up to 18 weeks (Roe & Salaman, 1955). Benign skin tumours were confined to one of 20 survivors on croton oil alone and one of 17 survivors of the group on 45 mg coumarin plus croton oil. No skin tumours appeared in 13 survivors in the group given repeated coumarin (total 150 mg) plus croton oil.

Lack of subcutaneous sarcoma development in rats (2 years)

Twice-weekly subcutaneous injections of 2 mg coumarin in 0·5 ml arachis oil for 65 weeks to a group of six rats did not induce local sarcomas or tumours remote from the injection site, although only one rat survived 2 years (Dickens & Jones, 1965).

Teratogenicity, mutagenicity and other studies

Teratogenicity studies

The offspring of mice fed dietary levels of 0·05–0·25% coumarin on days 6–17 of pregnancy exhibited no malformations, but an increase in stillbirths and delayed ossification were seen at the 0·25% level and mortality up to 3 weeks of life was increased at all levels (Roll & Bär, 1967). No effect on the resorption rate, foetal mortality or incidence of foetal malformations was seen following oral administration to rats of 10–400 times the therapeutic dose of a coumarin–rutin combination (30 mg coumarin plus 150 mg rutin) on days 4–14 and 18–21 of pregnancy (Grote & Günther, 1971) or intravenous administration to rabbits of 10 or 100 times the therapeutic dose of coumarin or a coumarin–rutin combination on days 6–18 of pregnancy (Grote & Weinmann, 1973).

Mutagenicity testing (Ames test)

In three tests (two incorporating tests and one spot test), coumarin at 1, 10, 100 and 1000 μg/plate, with and without liver homogenate, failed to induce *in vitro* mutagenic activity in the histidine-requiring (auxotrophic) *Salmonella typhimurium* strains TA1535, TA1537, TA98 and TA100 (F. Benazet and J. R. Cartier, personal communication to B. Buchner, Rhodia Inc., 1978).

Other studies

Coumarin has been found to exert an anti-carcinogenic action in rats, suppressing the development of mammary carcinomas when administered at 10 mg/kg/day in the drinking-water or at 146 mg/kg/day by gavage for 18 days prior to the injection of the carcinogen 7,12-dimethylbenz[*a*]anthracene (Feuer, Kellen & Kovacs, 1976). Coumarin exerted no protective effect when administered after the 7,12-dimethylbenz[*a*]anthracene treatment. The coumarin administration resulted in a dose-related decrease in coumarin 3-hydroxylase and an increase in serum prolactin and it was suggested that coumarin partly inhibited the metabolism of 7,12-dimethylbenz[*a*]-anthracene to carcinogenic metabolites.

Coumarin interferes with excision repair processes in ultraviolet-damaged DNA and with host-cell reactivation of ultraviolet-irradiated phage T1 in *Escherichia coli* WP2 (Grigg, 1972). Exposure of *Drosophila melanogaster* to coumarin resulted in an almost 50% decrease in the population (Beldowska & Guzewska, 1977).

Sensitization study in human volunteers

A maximization test (Kligman, 1966) carried out on 25 human volunteers using an 8% concentration in petrolatum produced no sensitization reactions (Greif, 1967).

Subcellular changes in the liver of coumarin-treated rats

An investigation into the establishment of biochemical criteria for the detection of early manifestations of liver damage revealed that administration of various hepatotoxic compounds, including cou-

Table 6. *Comparative hepatotoxic response in various species*

Species	Urinary 7-HC (% of dose)	Route	Highest dose causing no histological liver damage	Lowest dose causing histological liver damage	Lowest dose causing hepatic changes*	Reference†
Baboon	60	Oral	100 mg/kg/day for 3 wk	Not known	50 mg/kg/day for 3 wk	(1)
		Diet	67·5 mg/kg‡/day for 2 yr	Not known	67·5 mg/kg‡/day for 2 yr	(2)
Dog	3	Oral	10 mg/kg/day for 350 days	25 mg/kg/day for 133 days	Not known	(3)
				100 mg/kg/day for 8 days	Not known	(4)
Mouse	1·5–40	Oral	Not known	Not known	250 mg/kg (1 dose)	(5)
Rabbit	12	Injection	100 mg/kg or 8 days‖	Not known	100 mg/kg for 8 days‖	(10)
Rat	<1	Diet	1000 ppm for 2 yr	2500 ppm for 29 wk		(3)
			1000 ppm for 90 days	2500 ppm for 90 days		(4)
			1000 ppm for 2 yr	5000 ppm for 2 yr		(6)
			Not established	1000 ppm for 2 yr		(9)
		Oral	135 mg/kg/day for 7 days	405 mg/kg/day for 7 days	135 mg/kg/day for 7 days	(7)
					10–20 mg/kg/day for 7 days	(8)

7-HC = 7-Hydroxycoumarin

*Biochemical histochemical or ultrastructural.

†References: (1) Gangolli *et al* (1974); (2) Evans *et al.* (1979); (3) Hagan *et al.* (1967); (4) Hazleton *et al.* (1956); (5) Endell & Seidel (1978); (6) Bär & Griepentrog (1967); (7) Grasso *et al.* (1974); (8) Feuer *et al.* (1965b); (9) Griepentrog (1973); (10) Patyra *et al.* (1965).

‡Bodyweight.

‖Four doses.

marin, to rats brought about a reduction in liver-microsomal glucose-6-phosphatase activity and an increase in cytoplasmic glucose-6-phosphate dehydrogenase activity. In general, glucose-6-phosphatase proved to be the more sensitive to the action of the hepatotoxic agents (Feuer, Golberg & Le Pelley, 1965a). These hepatic enzyme changes became apparent within 2–3 days of administration of daily oral doses of 100–200 mg/kg or within 7 days of daily oral doses of 10–20 mg/kg to rats (Feuer, Golberg & Le Pelley, 1965b). The latter dose is about five times lower than the dose required to produce histological damage to the liver in rats (Hazleton *et al.* 1956; see also Table 6). When rat-liver microsomes were treated *in vitro* with coumarin or various hydroxycoumarins, glucose-6-phosphatase was unaffected, but enzyme inhibition did occur following treatment with *o*-hydroxyphenylacetic acid and *o*-hydroxyphenylactic acid, especially the former (Feuer *et al.* 1966). Glucose-6-phosphatase inhibition by *o*-hydroxyphenylacetic acid was also demonstrated in liver microsomes from rabbits, monkeys and man. Oral and parenteral administration of *o*-hydroxyphenylacetic acid (50–100 mg/kg/day for 7 days) also decreased glucose-6-phosphatase activity and it seems probable that the effect of coumarin in reducing glucose-6-phosphatase is mainly due to its metabolite, *o*-hydroxyphenylacetic acid (Feuer *et al.* 1966).

In order to throw more light on the significance of glucose-6-phosphatase depression in terms of hepatocellular damage, Grasso, Wright, Gangolli & Hendy (1974) followed the biochemical, histological and ultrastructural changes taking place in the liver of coumarin-treated rats. It was found that seven daily oral doses of 15 or 45 mg/kg had no effect on liver weight, liver histology or any of the biochemical, histochemical or ultrastructural criteria examined. By increasing the dose to 135 mg/kg, liver enlargement occurred, accompanied by biochemical changes (reductions in cytochrome *P*-450 and amidopyrine-demethylase activity), histochemical changes (centrilobular loss of glucose-6-phosphatase and aniline hydroxylase, and changes in lysosomal acid phosphatase) and ultrastructural changes (gross hypertrophy and dilatation of the rough endoplasmic reticulum, an increase in the number and size of lysosomes and an increased incidence of autophagic vacuoles). When the dose was increased still further, to 405 mg/kg, these biochemical, histochemical and ultrastructural changes were accompanied for the first time by histological liver damage. The hypertrophy of rough endoplasmic reticulum indicated an enhancement of protein synthesis in agreement with the biochemical evidence of increased protein synthesis in the liver of rats given 50–450 mg coumarin/kg/day for 3–7 days (Nievel, 1969). From their study of the cytopathological changes in the enlarged but histologically normal liver of coumarin-treated rats, Grasso *et al.* (1974) concluded that doses producing liver enlargement also produce histochemical and electron-microscopic indications of liver damage, while doses insufficient to cause liver enlargement are unable to elicit such changes.

Discussion

The current review has highlighted three crucial issues worthy of discussion:

(1) Classification of coumarin by OSHA as a Category I carcinogen on the basis of the controversial finding of 'bile-duct carcinomas' in rats (Bär & Griepentrog, 1967; Griepentrog, 1973) and of the erroneous citing in the NIOSH Registry (National Institute for Occupational Safety and Health, 1977) of a positive result for a study on mouse-skin tumour-

initiating activity although the data obtained were unequivocally negative (Roe & Salaman, 1955);

(2) The questionable diagnosis of 'bile-duct carcinomas' in rats given dietary levels of 5000 or 6000 ppm coumarin for 2 years (Bär & Griepentrog, 1967; Griepentrog, 1973);

(3) The validity of the rat as a test species in predicting the hepatotoxic response of coumarin to man, in view of the striking difference in metabolism now known to exist between these two species and of the role of metabolism as a determining factor in coumarin's hepatotoxicity.

OSHA classification—category I carcinogen

The NIOSH Suspected Carcinogens 1976 list and the NIOSH Registry of Toxic Effects of Chemical Substances 1977 (National Institute for Occupational Safety and Health, 1976 & 1977) cite in the entry for coumarin:

"GN 42000 Coumarin
orl-rat TDLo: 2190 mg/kg/2YC TFX: CAR TXCYAC 1, 93, 73
skn-mus TDLo: 1800 mg/kg TFX: NEO BJCAAI 9, 177, 55"

The first citation relates to the work of Griepentrog (1973). TFX denotes "Toxic effects—used to introduce the pathology or the principal organ system affected". CAR denotes "Carcinogenic effects—producing cancer, a cellular tumor the nature of which is fatal, or is associated with the formation of secondary tumors (metastasis)". The controversy over the diagnosis of the 'bile-duct carcinomas' is discussed below; this will reveal uncertainty as to whether the bile-duct lesion represented a true carcinoma associated with metastasis.

The second citation relates to the work of Roe & Salaman (1955). NEO denotes "Neoplastic effects—the production of tumors not clearly defined as carcinogenic by the author(s) of the cited reference". As described earlier, Roe & Salaman (1955) observed that coumarin failed to produce epidermal hyperplasia and to exhibit tumour-initiating activity when applied to mouse skin subsequently treated with the tumour promoter, croton oil. The results obtained were clearly negative and provided no grounds whatsoever for, or even suspicion of, any action by coumarin as an incomplete or complete carcinogen to mouse skin.

If coumarin is deemed a Category I carcinogen on the basis of a carcinogenic response by this additional route of administration (mouse skin), then the justification for so doing is invalid and the Category I classification should be revoked.

Diagnosis of 'bile-duct carcinomas' in rats

Hagan *et al.* (1967) found no evidence of malignancy in rats fed up to 5000 ppm coumarin in the diet for 2 years but observed focal proliferation of bile ducts of atypical appearance with associated fibrosis at the 5000 ppm level. However, Bär & Griepentrog (1967) and Griepentrog (1973) claim to have observed 'bile-duct carcinomas' in similarly-treated rats. The rationale underlying this diagnosis is not clear, especially since convincing evidence of metastasis to extra-hepatic tissues was not forthcoming. Indeed a note in *Food and Cosmetics Toxicology* (1969) argued that the photomicrographs published by Bär & Griepentrog (1967) were more consistent with the interpretation of cholangiofibrosis observed by Hagan *et al.* (1967) than with a diagnosis of malignancy. In contrast, the International Agency for Research on Cancer (1976), in its evaluation of coumarin, appears to have accepted the German findings without reservation but makes no reference to the basis on which a diagnosis of malignancy was made or to the lack of consistent metastasis.

Examination, by BIBRA pathologists, of the liver slides, made available by Dr. Griepentrog, revealed severe chronic hepatic damage, including fatty degeneration, necrosis and an extensive bile-duct proliferation accompanied by fibrosis in the 5000 ppm group. Hyperplastic nodules were occasionally seen. However the cytological changes in the bile ducts were not regarded as unequivocal evidence of a carcinomatous process and the possible occurrence of metastasis were excluded (D. M. Conning and J. G. Evans, personal communication 1978).

Various true carcinogens are known to produce, *inter alia*, biliary hyperplasia and fibrosis and, with the exception of azo dyes, the lesions do not progress to bile-duct carcinoma and are regarded as toxic manifestations of the compound (Farber, 1976). Similar cytological changes in bile-duct epithelium can be produced by bile-duct ligations or the feeding of lithocholic acid (Palmer & Hruban, 1966). Cholangiofibrosis is often mistaken for cholangiocarcinoma, and areas of cholangiofibrosis frequently persist for months or years without any evidence of neoplastic development (Farber, 1976). Bannasch (1976) describes the processes leading to the development of multiple foci of cholangiofibrosis induced by certain agents. The cells of the proliferated bile ducts may show an atypical structure but despite these pronounced cellular alterations invasive growth or metastases never occur. Bannasch (1976) concludes that most workers do not view cholangiofibrosis as a neoplastic lesion.

Returning to the findings of Bär & Griepentrog (1967) and Griepentrog (1973), it is difficult to explain why a 90% incidence of 'bile-duct carcinomas' was seen in males on 5000 ppm and a 0% incidence in males on 2500 ppm. The lower incidence of carcinomas in males of the 6000 ppm groups could be explained by the reduction in food intake which was said to have resulted in an estimated coumarin intake equivalent to the normal consumption of food containing 3500 ppm.

It is noteworthy that Schmähl (1977), in a review of environmental carcinogens, states that, whilst certain compounds capable of producing tumours in animals are possibly human carcinogens, no judgement can be passed on the possibility of coumarin being carcinogenic to man; no specific reasons are given for this, however.

Relevance of metabolism to coumarin's hepatotoxicity

The striking difference in metabolism between man and non-primate species (Shilling *et al.* 1969) is of obvious toxicological importance. First, formal toxicity tests so far published have been carried out mainly in the rat and to a lesser extent in the dog. Both species are poor 7-hydroxylators of coumarin.

in contrast to man who relies on the formation and urinary excretion of 7-hydroxycoumarin as the major route of metabolism. Secondly, there is evidence to suggest that the hepatotoxic action of coumarin may be mediated by one of its metabolites, possibly *o*-hydroxyphenylacetic acid, a major metabolite in the rat.

The first indication that coumarin may owe its hepatotoxicity to a metabolite stemmed from the work of Feuer *et al.* (1965a,b). Initially, these authors found that liver glucose-6-phosphatase activity was depressed by administration of a variety of hepatotoxic agents, including coumarin, to rats, the depression thus being regarded as a useful biochemical index of early toxic liver damage. It then became apparent that the enzyme could be inhibited *in vitro* markedly by *o*-hydroxyphenylacetic acid and to a lesser extent by *o*-hydroxyphenyllactic acid (both metabolites of coumarin in the rat), but not by coumarin or known hydroxycoumarin metabolites. Moreover, enzyme inhibition by *o*-hydroxyphenylacetic acid was demonstrated following its oral or intravenous administration to rats, albeit in high doses of 50–100 mg/kg which were probably required to attain inhibitory concentrations *in situ* (Feuer *et al.* 1966).

A further indication that metabolism plays an important role in coumarin's hepatotoxicity is provided by the finding of curious strain differences in coumarin 7-hydroxylation in the mouse (Wood & Conney, 1974) and of the ability of the DBJ/2A strain, a good 7-hydroxylator, to resist the hepatotoxic action of coumarin (assessed biochemically) relatively better than the CH3/HeJ strain, a poor 7-hydroxylator (Endell & Seidel, 1978). In this study, serum levels of glutamic-oxalacetic transaminase and succinic dehydrogenase were elevated in mice treated with a single oral dose of 350 mg coumarin/kg, but more markedly in the CH3/HeJ strain than the DBA/2J strain. It remains to be seen, however, whether the same strain difference in hepatotoxic response is observed on histological assessment.

On the other hand, other workers have subscribed to the view that hepatotoxicity is due to coumarin *per se* (Gibbs, Janakidevi & Feuer, 1971) and that species differences in coumarin metabolism do not appear to be relevant to its hepatotoxicity (Gangolli *et al.* 1974).

Gibbs *et al.* (1971) based their conclusion on the observations by Mead *et al.* (1958a,b) and Feuer *et al.* (1966) that administration of 3-hydroxycoumarin, *o*-hydroxyphenyllactic acid and *o*-hydroxyphenylacetic acid to rats produced no toxic effects (presumably no hepatotoxic effects). However, the reviewer could find nothing in these three reports to suggest that these compounds had been tested for hepatotoxicity either *in vivo* or *in vitro*, let alone that they had produced negative results; indeed in the case of *o*-hydroxyphenylacetic acid, liver enlargement was produced in rats given doses of 100 mg/kg/day for 7 days orally or intravenously (Feuer *et al.* 1966).

Gangolli *et al.* (1974) based their conclusion on their somewhat limited baboon study. They argued that although the baboon was an efficient 7-hydroxylator of coumarin it succumbed to the hepatotoxic action of coumarin. But did it? No histological changes were seen in the liver after oral administration of 50 mg/kg/day for 3 weeks followed later by 100 mg/kg/day for a further 3 weeks. Liver malfunction (prolonged BSP excretion) was seen in the single female but not in the single male baboon tested. The histochemical changes (lysosomal changes) and ultrastructural changes (autophagic vacuoles and dilatation of the smooth and rough endoplasmic reticulum), although reversible, were regarded as being indicative of hepatotoxicity. Thus this study on baboons presented somewhat tenuous evidence of hepatotoxicity in only two animals. Whilst it may justify the conclusion that coumarin is possibly hepatotoxic to the baboon, this finding alone cannot justify the authors' claim that species differences in the metabolism of coumarin are probably of no relevance to its hepatotoxicity. Indeed, in a more formal baboon study carried out in the same laboratory (Evans *et al.* 1979), coumarin given in the diet at levels providing an intake of up to 67·5 mg/kg body weight/day for 2 years failed to produce any histological damage to the liver and the only effect seen was dilatation of the endoplasmic reticulum at the highest dose level. Whether higher doses are capable of producing frank histopathological damage in the baboon is not known.

The direct testing of coumarin metabolites for hepatotoxic potential may not necessarily unmask the potential hepatotoxic metabolite, in view of the difficulty of ensuring attainment of hepatotoxic concentrations of the metabolite at the target site *in situ*. Dihydrocoumarin, a very minor metabolite, produced no liver damage in rats given dietary levels of 100 or 1000 ppm for 14 weeks or in dogs dosed orally with 50 or 150 mg/kg/day for 2 years (Hagan *et al.* 1967). No major metabolites of coumarin appear to have been tested for hepatotoxicity.

As can be seen from Table 6, coumarin is more hepatotoxic to the dog than to the rat following short-term administration. Both these species are poor 7-hydroxylators of coumarin (Table 2). The baboon, a good hydroxylator, did not exhibit histological liver damage at dietary intakes of up to 67·5 mg/kg/day for 2 years (Evans *et al.* 1979). DBJ/2A mice, the only non-primate species so far shown to possess a 7-hydroxylating capability, appears to be more resistant to the hepatotoxic action of coumarin than does another mouse strain with limited 7-hydroxylating potential (Endell & Seidel, 1978).

The rat and man also appear to differ in the extent of biliary excretion of coumarin metabolites. Shilling *et al.* (1969) excluded the possibility of enterohepatic circulation of coumarin or its metabolites in man whilst Williams *et al.* (1965) observed a considerable biliary excretion of coumarin metabolites in the rat. This latter finding, taken together with the slower urinary excretion of coumarin metabolites in the rat compared with man and with the significant faecal excretion of coumarin in the rat (Table 1), suggests the importance of enterohepatic circulation in coumarin-treated rats. Moreover, although Williams *et al.* (1965) did not identify the ring-opened metabolites in the bile, *o*-hydroxyphenylacetic acid was possibly present. If a ring-opened metabolite proves to be the causative hepatotoxin, then the presence of enterohepatic circulation in the rat might confer a greater susceptibility to the hepatotoxic agent, in contrast to man in whom enterohepatic circulation is unlikely.

Other tests for carcinogenicity

The oral tests in dogs (Table 6) were of insufficient duration to reveal any potential carcinogenic response. The subcutaneous study in rats by Dickens & Jones (1965) was inadequate for evaluating carcinogenicity (only one small initial group; poor survival) although no tumours were found either locally or at sites remote from the injection site.

Recommendations

In view of the erroneous National Institute of Occupational Safety and Health (1977) report of tumours in the study by Roe & Salaman (1955), who demonstrated quite clearly the failure of coumarin to possess tumour-initiating activity in mouse skin, OSHA should consider deleting coumarin from the list of Category I carcinogens, if this classification was based in part on an adverse mouse-skin finding.

Because of the uncertainties over the diagnosis of the bile-duct proliferations as carcinomas in coumarin-fed rats, a third rat study may be considered desirable to resolve whether the lesions are consistent with a diagnosis of non-malignancy, as reported by Hagan *et al.* (1967), or of malignancy, as reported by Griepentrog (1973). Before this, however, it is recommended that the relevant sections of tissue from both studies be submitted for re-examination, preferably by an international panel of pathologists.

Further study is needed of the histogenesis of the bile-duct proliferations so that a distinction can be drawn between proliferated bile-ducts of cholangiofibrotic areas and true cholangiocarcinomas.

The urinary excretion of 7-hydroxycoumarin in DBA/2 mice given coumarin should be examined in the absence of phenobarbitone pretreatment and compared with other mouse strains found to be poor 7-hydroxylators. The livers of these mouse strains should also be examined histologically to see if an inverse relationship exists between the degree of liver damage and the extent of 7-hydroxylation.

Experiments should be undertaken to see whether coumarin metabolism in the rat via the 3-hydroxycoumarin pathway can be diverted to 7-hydroxylation by the intervention of a chemical agent, with attendant suppression of hepatotoxicity.

The study of coumarin metabolism in man should be extended to a wider sample of volunteers to strengthen the currently available evidence that man is an efficient 7-hydroxylator of coumarin.

Summary and conclusions

Coumarin produces histological liver damage in the rat and dog, but not, following an intake of up to 67·5 mg/kg/day for 2 years, in baboons.

Grave doubts have been expressed over the acceptability of the diagnosis of 'bile-duct carcinomas' in rats fed coumarin at 5000 or 6000 ppm in the diet for 2 years, especially in view of the absence of significant metastasis in these animals and of the negative carcinogenic findings obtained in an earlier study in rats.

Coumarin does not act as a co-carcinogen in mouse skin and no evidence of carcinogenicity was obtained following subcutaneous administration to rats in a limited experiment.

Since the rat metabolizes coumarin quite differently from man, the suitability of the rat as a test species in predicting the hepatotoxic risk to man has been questioned. Whether coumarin or a metabolite is the active hepatotoxin has not been established with certainty. However, the evidence so far obtained suggests that metabolism is an important factor in determining the hepatotoxic response and therefore species differences in metabolism should be taken into account in evaluating the hepatotoxic hazard to man.

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REVIEWS OF RECENT PUBLICATIONS

Patty's Industrial Hygiene and Toxicology. Third revised edition. Volume I: General Principles. Edited by G. D. Clayton and F. E. Clayton. John Wiley & Sons, New York, 1978. pp. xviii + 1466. £53.00.

"Patty" has been the bible of industrial hygiene and toxicology since it first appeared in 1948, although the recent great advances in this field have inevitably diminished its value. The last edition of Volume I was published 20 years ago, making the task of updating immense. It is thus no surprise to find that the new edition is nearly twice the length of its predecessor (and incidentally so heavy that it might have been better in two separate parts). New fields such as epidemiology, ergonomics and quality control are included for the first time, and agricultural hazards have also been brought within its scope. Other newly-introduced chapters deal with the important subject of industrial hygiene engineering controls, with odour measurement and control, and with calibration.

Of the revised chapters, only the first is still by F. A. Patty himself, now in his eighties but obviously still interested in recent US developments. Unfortunately, he seems less interested in the progress of industrial hygiene in other countries, the summary of this aspect having been transferred unchanged from the second edition. However, this is not characteristic of the book as a whole, most of which has been extensively updated by authorities who are still working in their chosen fields.

As the title implies, the first volume is largely concerned with general principles rather than with specific toxicants. A notable exception is the chapter on air pollutants by G. D. Clayton, which now contains a valuable review of the health effects of nitrogen dioxide, photochemical oxidants, carbon monoxide, hydrocarbons, sulphur dioxide and particulates. G. W. Wright also surveys the effects of inhaled inorganic dust, with particular reference to crystalline silica, carbonaceous dust, asbestos and fibrous glass. However, most such discussions have quite properly been deferred to Volume II (Toxicology), the publication of which, scheduled for 1979, is eagerly awaited.

Environmental Health Criteria 4: Oxides of Nitrogen. Published under the joint sponsorship of the United Nations Environment Programme and the World Health Organization. WHO, Geneva, 1977. pp. 79. Sw.fr. 9.00 (available in the UK through HMSO).

A WHO Task Group met in August 1976 to consider the health hazards presented by the oxides of nitrogen. The monograph resulting from the group's deliberations, limited in effect to a consideration of nitrogen dioxide and nitric oxide, has now been published as the fourth volume in the Environmental Health Criteria series.

Following a summary and recommendations for further work, the monograph contains chapters on the chemistry and analysis of these nitrogen oxides, their sources, both natural and man-made, the atmospheric concentrations likely to be encountered under various environmental conditions and the effects of the gases in experimental animals and man. The final chapter presents the Working Group's evaluation of the reviewed data, involving some 180 references in all. Although these data indicate that nitrogen dioxide is the only nitrogen oxide with any significant biological activity, they were said to provide an insufficient basis for the prediction of a safe level for long-term exposure. It was tentatively agreed, however, that a maximum 1-hour exposure to a nitrogen dioxide concentration of 0·1–0·17 ppm should not prove hazardous to the public health. A further recommendation was that this exposure should not be exceeded more than once a month.

As with earlier volumes in this series, this brief document provides a useful and succinct review of the data on an important group of environmental contaminants.

Environmental Health Criteria 5: Nitrates, Nitrites and *N*-Nitroso Compounds. Published under the joint sponsorship of the United Nations Environment Programme and the World Health Organization. WHO, Geneva, 1977. pp. 107. Sw.fr. 10.00 (available in the UK through HMSO).

This contribution to the Environmental Health Criteria series is a concise account of the data available for evaluating the health risks associated with exposure to nitrates, nitrites and *N*-nitroso compounds. The material is well organized into sections that consider, in turn, analytical methods, environmental sources and levels, metabolism, effects on animals and man and an evaluation of risks to human health. In each section, data on nitrates and nitrites precede those on *N*-nitroso compounds, so that an area of interest can rapidly be identified. It was recognized that it would be an impossible task to summarize all available data on this vast subject, but there is frequent reference to important review articles in addition to the more specific papers.

Some cause for concern must be expressed over the reluctance of a distinguished WHO Task Group to offer any conclusions concerning human exposure to nitrosamines. While it is reasonable to identify a lack of human data and to issue a cautionary note regarding the over-zealous application of animal data to the human condition, some qualified estimate of the likely order of hazard might have been expected from such a panel of experts. Instead, we are confronted with familiar maxims, typified by the conclusion that "as the dose is reduced, the tumour incidence decreases and the time for tumour induction

increases and may exceed the life-span of the animals".

It is hard to identify a topic in toxicological research that has provided more fertile ground for the proliferation of review articles, monographs and expert deliberations than that of nitroso compounds. Viewed against such a background, this monograph may be regarded as an excellent summary of the vast literature on this subject up to 1976, when the Task Group met, but inevitably it has rapidly been overtaken by subsequent events and authoritative documents from the USA and Europe.

Clinical Chemistry and Chemical Toxicology of Metals. Developments in Toxicology and Environmental Science Vol. 1. Edited by S. S. Brown. Elsevier/North-Holland Biomedical Press, Amsterdam, 1977. pp. xi + 398. Dfl. 96.00.

This initial volume in a new series reproduces the texts of a selection of invited lectures and communications given at an international symposium sponsored by the International Union of Pure and Applied Chemistry, the International Federation of Clinical Chemistry and the Association of Clinical Scientists.

The form of the book is rather unusual. It is divided into eleven sections each introduced by the text of a plenary lecture, which is followed by a selection of shorter communications on more specific topics. Some of the latter are obviously relevant to the introductory paper, although in other cases the connection is far from clear. One of the most interesting papers is the first—on the "essentiality versus toxicity" of metals. In this, Dr. Klaus Schwarz outlines and explains the somewhat paradoxical situation that many metals generally considered as highly toxic, such as cadmium and arsenic, are at low concentrations essential for normal body functions, and conversely that each essential trace element is toxic when given in excess, as has been clearly demonstrated in the cases of iron, copper and selenium.

Other sections of the book are concerned with metabolic targets of metal toxicity, analytical methods, tissue and cellular toxicology and poisoning with cadmium, mercury, lead, nickel and arsenic.

Although this compilation appears at first sight to cover the field comprehensively, the communications are, for the most part, on very specific topics and hence the book is likely to appeal mainly to workers involved directly in research relevant to the toxicology of heavy metals.

Trace-element Contamination of the Environment. By D. Purves. Fundamental Aspects of Pollution Control and Environmental Science 1. Edited by R. J. Wakeman. Elsevier Scientific Publishing Company, Amsterdam, 1977. pp. xi + 260. Dfl. 85.00.

Dispersion of trace elements from their localized and limited sources is a form of environmental pollution which, while contributing to the general ecological crisis, is receiving little attention; this is the view expressed by the author of this monograph. The book reports on the contamination of rural and urban soil, air and water by trace elements and identifies sources of contamination and their consequences. Seven of the eight chapters provide a critical review of the published literature. In the final chapter, the author expounds his own hypothesis and in doing so suggests, on the one hand, that international standards be established to reduce pollution of the environment by trace elements, and on the other, that methods be developed for the recovery of these metals from wastes, thus conserving exhaustible reserves. These are among the many points on which action is advocated.

A comprehensive bibliography of 446 references means that the book will be welcomed as a worthwhile addition to the reference shelves.

Environmental Health Criteria 3. Lead. Published under the joint sponsorship of the United Nations Environment Programme and the World Health Organization. WHO, Geneva, 1977. pp. 160. Sw.fr. 16.00 (available in UK from HMSO).

This is the third volume in the series of books on environmental contaminants published under the auspices of WHO and the United Nations Environment Programme. In common with the previous volumes (on mercury and polychlorinated biphenyls), this contribution reviews and assesses the biological effects of lead in a comprehensive but extremely concise manner. The aspects of the subject covered include sources, levels of distribution and transport of lead in the environment, analysis and metabolism and a review of epidemiological and clinical studies of the effects of lead in man.

The emphasis of the book is placed strongly on elucidating the effects of lead in man; experimental studies in animals are usually mentioned only when these throw light upon data from humans. In fact researchers would do well to take note of the opening paragraph of chapter 7 (on experimental studies on the effects of lead) which includes the statement: "The major part of published experimental work on animals...does not contribute much to the understanding of the relationship between the dose administered, its distribution in a period of time, and the biological effect."

This is an ideal book for anyone who wishes to be brought rapidly up to date in the field of lead toxicology and environmental effects. It can be highly recommended.

IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man. Some Aromatic Amines and Related Nitro Compounds—Hair Dyes, Colouring Agents and Miscellaneous Industrial Chemicals. Vol. 16. IARC Working Group. International Agency for Research on Cancer, Lyon, 1978. pp. 400. Sw.fr. 50.00.

Included in this collection of monographs are some aromatic amines and related nitro compounds that have not come within the bounds of previous monographs. Many of the compounds considered are colouring agents, used as hair dyes, as industrial dyes or as additives in the manufacture of plastics and polymers; others are dye intermediates or inter-

mediates in the production of a variety of industrial chemicals.

In a general chapter, the Working Group stresses that data from studies in which a chemical was administered as part of a mixture could not be used as collateral evidence of the carcinogenicity of the pure compound. A similar problem arose with epidemiological studies on several occupational groups, such as dye-workers, printers and textile workers; although such studies possibly involved exposure to some of the substances under evaluation by the IARC, they were not reviewed, because of the non-specific nature of the exposure. Although the problem of mixed exposure also affects professions such as hairdressing—where hazards may include the inhalation of aerosol propellants—studies on these occupations were reviewed, since the specificity of exposure was thought to be much greater. The Working Party concluded that although further epidemiological studies would be necessary before firm conclusions could be drawn, the epidemiological evidence suggested an elevated risk of cancer both for users of hair dyes and for those with occupational exposure to hair preparations. The evidence of an increased risk of cancer at certain sites was stronger for persons with occupational exposure to hair-care products, including dyes.

Of the hair-dye constituents examined, only 2,4-diaminotoluene was definitively branded a carcinogen in experimental animals; for another five (4-amino-2-nitrophenol, 2,4-diaminoanisole sulphate, 1,2-diamino-4-nitrobenzene, 1,4-diamino-2-nitrobenzene and 2,5-diaminotoluene) no evaluation of carcinogenicity could be made in the absence of data on the individual compounds, and in the case of *m*- and *p*-phenylenediamine, studies were considered inadequate for evaluation. However, the Working Group was aware that studies were being carried out on some of these chemicals.

There was a more positive outcome from the data on the colourings. Eight out of nine colourings were deemed carcinogenic in experimental animals. These were Benzyl Violet 4B, Blue VRS, Brilliant Blue FCF disodium salt, Fast Green FCF, Guinea Green B, Light Green SF, Rhodamine B and Rhodamine 6G. For acridine orange the data available were not sufficient to permit an evaluation.

In the section covering 15 miscellaneous industrial chemicals, four were considered to be carcinogenic in experimental animals, namely *N*,*N*′-diacetylbenzidine, 3,3′-dichloro-4,4′-diaminodiphenyl ether, 5-nitroacenaphthene and 4,4′-thiodianiline. For six compounds, *p*-chloro-*o*-toluidine, 4,4′-diaminodiphenyl ether, *N*-phenyl-2-naphthylamine, *o*-toluidine, 2,4-xylidine and 2,5-xylidine, there was evidence suggestive of carcinogenicity. There were insufficient data for evaluation of the remaining compounds, 5-aminoacenaphthene, *p*-aminobenzoic acid, anthranilic acid, cinnamyl anthranilate and 2,4-diphenyldiamine. Epidemiological data were available to the Working Group only for *p*-chloro-*o*-toluidine, *N*-phenyl-2-naphthylamine and *o*-toluidine, but in no case did these data permit an assessment of carcinogenic hazard.

This volume, similar in style and format to previous publications in this series, is a valuable collection of data on the cancer hazard of aromatic amines.

Environmental and Health Aspects of Selected Organohalide Compounds: an Information Overview. By R. H. Ross, L. B. Yeatts, Jr., E. B. Lewis, G. A. Dailey, D. S. Harnden, D. C. Michelson and L. M. Frogge. ORNL/EIS-105, Oak Ridge National Laboratory, Oak Ridge, Tennessee, 1978. pp. xxxi + 475. Available from National Technical Information Service, Springfield, Virginia, at $15.25 (printed copy) or $3.00 (microfiche).

The environmental persistence of certain halogenated organic compounds has long been a source of concern, which has been heightened in some cases by recent findings of carcinogenicity and mutagenicity. The document named above reviews data from the open literature on difluorodichloromethane and trichlorofluoromethane (fluorocarbons 11 and 12), chloroform, methyl chloroform, carbon tetrachloride, perchloroethylene, hexachlorobutadiene, vinyl chloride, allyl chloride and ethylene dibromide. These were chosen from an initial list of about 80 compounds because of their significant potential environmental and health impact. The survey was prepared by the Information Division of the Oak Ridge National Laboratory under contract from the US National Science Foundation.

Separate sections review analytical methods, environmental aspects (including production and emission volumes, levels in air and water, persistence and degradation), effects on plants and levels in foods, microbiological effects, and metabolism and toxicity in animals and man. This last section is by far the longest (263 pages), and presents a fairly comprehensive survey of the literature, including papers from Eastern Europe which are not always readily accessible in the West.

It is a pity that the survey was evidently completed in 1976, although one can appreciate the difficulties of getting a document like this into print. Some sections give signs of having been prepared even earlier. Why, for instance, is mention made only of the 1974 proposed OSHA standard for vinyl chloride, when a final standard was issued in the same year? Other surprising omissions include the marked inter-species differences in chloroform metabolism and the reports (some published before 1960) of the development of hepatomas following repeated oral administration of carbon tetrachloride. Nevertheless, provided that it is treated as a useful guide to the literature rather than as an exhaustive review, the publication should be of considerable value to those concerned with the environmental and toxicological effects of these ten compounds.

Long-term Toxic Effects: A Study Group Report. The Royal Society. 1978. pp. 16. Gratis (single copies) or £0.35 (multiple copies).

The Royal Society Study Group on Long-term Toxic Effects was set up in 1975, under the Chairmanship of Sir Richard Doll, to review knowledge of the toxic effects of long-term exposure to substances present in the environment at levels not adverse in the short term, and to identify areas where research was needed. The main questions before the group were whether man-made chemicals or processes were

a major environmental factor in disease, and how their effects might be predicted and prevented. During its ten meetings the Group discussed the monitoring of health, the effects of environmental lead, the environmental causes of cancer and testing procedures for the identification of carcinogens, the significance of mutagenic and teratogenic chemicals in the environment, sources of exposure to toxic materials in everyday life, the value and limitations of evidence derived from wild life, linkage of records on health, employment and exposure to chemicals, and the major gaps in existing knowledge and research.

One of the Group's most important conclusions was that toxicity testing of new chemicals and drugs tended to be a "sterile routine" of exposure of many animals, without attention to mechanisms. Yet an understanding of toxic mechanisms was essential for any extrapolation of test findings across species and from large doses to levels of human exposure. Nutritional and other variables that could alter susceptibility to toxic effects by an order of magnitude or more were also considered to be poorly understood and little studied. It was thought unlikely that any group of currently available tests for carcinogenicity could provide a quantitative measure of the human risk, and although mutagenicity tests promised to be of value, they needed to be validated by animal studies and correlated with human experience.

There was disagreement about the extent to which environmental carcinogens were likely to be industrial in origin, and about the possible importance of other co-carcinogenic factors, and some members thought that research and prevention should be focused on food intake and other aspects of life style. All agreed, however, that the social cost of removing many chemicals from industrial use could be far greater than any potential benefit.

The Group regarded existing systems for monitoring trends in human disease as inadequate, and suggested that the Office of Population Censuses and Surveys should monitor not only mortality, as at present, but also other aspects of health such as haemoglobin and cholesterol levels. Improvements were also considered necessary in the current systems of cancer registration and notification of congenital anomalies, and in the health monitoring of industrial workers exposed to new chemicals. To facilitate the detection and measurement of human risks, records of exposure to industrial and other hazards should be linked with hospital discharge and mortality data. Manufacturers should be required to publish toxicity data on compounds about to be marketed, with appropriate provisos to protect patents, and data should also be published on related chemicals that have not been commercially developed, especially when development has been stopped because of toxic effects.

It is to be hoped that the conclusions and recommendations of this succinct report will be given serious and immediate consideration by the appropriate authorities.

Microbial Ecology of the Gut. Edited by R. T. J. Clarke and T. Bauchop. Academic Press Inc. (London) Ltd., 1977. pp. xvii + 410. £13.50.

The intestinal tract is a complex ecological system, since microorganisms are present not only in a bewildering variety of types but often also in enormous numbers, sometimes 10^{10}–10^{11} organisms per gram of gut contents. Allied to this complexity are the difficulties involved in culturing, isolating and identifying the members of the gut flora. Suitable techniques have only recently been developed fully and the opening chapters of this book are therefore devoted to reviews of the methods available for studying gut microbes and to brief descriptions of the various types encountered.

The longest chapter in the book is, not surprisingly, on the biochemical activities of the gut flora of several animal species and includes an interesting section on the unusual methods of energy transformation in anaerobic organisms. Although there is a good deal of emphasis on the gut flora of ruminants, the floras of other animals are considered in some detail, and afficionados of the esoteric will no doubt be thrilled by the (albeit short) accounts of foregut fermentations in the hippopotamus and in macropod marsupials such as the quokka.

Any attempt to describe the gut flora from an ecological standpoint must obviously consider host–microbe interactions. Hence the chapter on the interaction of the host and its microbes, by D. C. Savage, and that on the gnotobiotic animals, by M. E. Coates and R. Fuller, are perhaps the most important and certainly the most fascinating in the book. They also serve to highlight some large gaps in our knowledge of the gut-flora ecosystem.

Carcinogenicity Testing: Principles and Problems. Edited by A. D. Dayan and R. W. Brimblecombe. MTP Press Ltd., Lancaster, 1978. pp. xi + 128. £8.95.

A short symposium which covered most of the important aspects of carcinogenicity testing was organized in London, at the Royal College of Physicians, in December 1977. An edited account of this meeting has now been published. The topics discussed feature most of the pertinent problems, ranging from the biochemical mechanisms involved in carcinogenicity to long-term and short-term tests to evaluate carcinogenic and mutagenic potential, the cost to industry of screening for carcinogens and last, but not least, the attitude of the UK regulatory authorities. The contributors to the symposium were all experts in their respective fields and their experience has been harnessed into compact, concise chapters which form complete entities in themselves. Each chapter is followed by a short commentary which forms an effective summing up.

The central theme is the assessment of carcinogenic hazard, and there is only one chapter concerning carcinogenic mechanisms, a topic that would, in its own right, sustain a complete book. Nevertheless it keys the underlying problem of the number of variables involved in the production, detoxification and removal of carcinogenic molecules to the question of designing tests for carcinogenicity.

The conventional long-term tests for carcinogenicity are considered from the point of view of experimental design, pathological interpretation, mathematical analysis and extrapolation of data to man. Con-

tributors discuss succinctly the problems presented by the standard type of test currently in use, involving prolonged exposure of one or more species of animal, and its value as a technique for detecting carcinogenesis and for the assessment and minimization of risk.

Increasing interest is now being focused on the use of short-term screening tests for the detection of carcinogenic potential, as a faster, less expensive and, to a certain sector of the public, more ethical means of testing large numbers of chemicals. The underlying principles involved in both mammalian short-term tests and in microbiological mutation tests are well presented and are amply supported by discussion of the difficulties encountered in their evaluation and interpretation. The present status of such test systems is considered, together with the problems that must be solved in the future if these tests are to be recommended for adoption in routine screening procedures.

The contributions to this volume are in every case informative and well presented and should be of interest to all those concerned with the difficult and expensive exercise of carcinogenicity testing.

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Information Section

ARTICLES OF GENERAL INTEREST

LUNG DAMAGE AND BUTYLATED HYDROXYTOLUENE

There have been a number of reports in recent years concerning the pulmonary reactions in mice given large doses of the antioxidant butylated hydroxytoluene (3,4-di-*tert*-butyl-4-hydroxytoluene; BHT), which is widely used as a food additive. An early observation was made by Marino & Mitchell (*Proc. Soc. exp. Biol. Med.* 1972, **140**, 122), who noted substantial proliferation of pulmonary cells within 3–5 days of an ip injection of BHT. Later these cellular changes were shown to be accompanied by increases in lung weight and in total DNA (Witschi & Saheb, *ibid* 1974, **147**, 690) and the DNA changes, which were dose-dependent, were found to be associated with an increase in total pulmonary RNA and lipids (Saheb & Witschi, *Toxic. appl. Pharmac.* 1975, **33**, 309; Witschi & Cote, *Fedn Proc. Fedn Am. Socs exp. Biol.* 1976, **35**, 89). Hypertrophy, hyperplasia and a general thickening of the alveolar walls were also reported in these studies. Meanwhile it was reported in 1974 that the dietary administration of BHT to 18-month-old mice at a level of 0·75% for 16 months resulted in a 63·6% incidence of lung tumours compared with a 24·0% level in the control animals (Clapp *et al. Fd Cosmet. Toxicol.* 1974, **12**, 367).

Now several Canadian papers have been published, which investigate further the nature of the pulmonary changes resulting from a single ip injection of BHT in mice. An attempt has been made to correlate the morphological changes observed in both light- and electron-microscopic studies and accompanying cytodynamic changes with certain biochemical effects, such as changes in the activity of thymidine kinase and uridine kinase, which reflect the cellular proliferative response to cell injury.

In all of the experiments, male mice were given 400 mg BHT/kg in corn oil as a single ip injection and killed at intervals within the following 9 days. The sequential morphological and cytodynamic events that occurred following an ip injection of BHT have been described by Adamson *et al.* (*Lab. Invest.* 1977, **36**, 26). An initial perivascular oedema and cellular infiltration in type I (squamous) epithelial cells was followed by multifocal necrosis and destruction of the air–blood barrier and by fibrin exudation into the alveoli by day 2 after the injection. The alveolar wall was repopulated by proliferation type II epithelial cells, but these differed in form from the type II cuboidal cells typically found in repair of the lung, and were instead giant forms with large nuclei and abundant cytoplasm.

The ultrastructural changes that accompanied the sequence of type I cell damage and subsequent type II proliferation and differentiation were noted in all alveolar septal cells and were analysed systematically at intervals from 6 hours to 9 days by Hirai *et al.* (*Expl mol. Path.* 1977, **27**, 295). Damage was evident in type I cells as early as day 1, with some swelling, a decrease in cytoplasmic density and breaks in the plasmalemma. In some focal areas, cell destruction was complete within 2–3 days, although in others it was comparatively slight at this time. Changes in the cytoplasm of type II cells were evident from days 2 to 7, the cuboidal cells tending to become squamous, forming pseudopodia and elongating to cover the damaged area. Within the cells, cytoplasmic movement was indicated by the presence of microfilaments and microtubules, and there was a reduction in lamellar bodies as a result of their expulsion into the alveolar lumen. Cell proliferation was reflected in numerous mitotic figures and, following cell division, other cellular changes such as a decrease in the number of microvilli, an increase in free ribosomes and changes in the mitochondria were observed. By day 5, the majority of cells were typical of the type I epithelium, although some were representative of a newly formed type II cell. From days 7 to 9, types I and II cells were normal in appearance.

The capillary epithelium did not show any sign of damage until day 3, and it was not until day 7 that the appearance of duplicated centrioles and mitotic figures indicated regeneration of damaged cells. Of the other tissue components, interstitial cells did not appear to be damaged by BHT, but signs of proliferation were apparent as early as day 4, invading empty alveolar spaces and indicating the beginning of a fibrosis. Macrophages were involved from the onset of cellular damage, phagocytizing activity being maximal on day 5.

Tissue repair was followed by incorporation of thymidine into DNA, as was shown by administration of an ip injection of 2 μCi tritiated thymidine/g 2 hours before the mice were killed (Adamson *et al. loc. cit.*). The activities of two enzymes involved in nucleic acid synthesis, thymidine kinase and uridine kinase, showed a similar pattern over the 9-day period. Activity was increased at day 2, reached a peak by days 3 and 4 and then returned to control levels by day 9. Differential counts of labelled cells showed that the initial rise was due almost entirely to the proliferation of epithelial cells, followed by that of interstitial and endothelial cells, the latter showing a late peak of activity at day 6 and then a steady decline to day 9.

A similar technique was used to determine whether BHT was directly toxic to lung cells or whether this activity was a consequence of its metabolic transformation. The latter is at least a theoretical possibility, since rat-liver microsomes, for example, are known to convert BHT into several ring-oxygenated metabolites. Williamson *et al.* (*Toxic. appl. Pharmac.* 1978,

43, 577) measured the *in vivo* incorporation of thymidine into DNA and the activity of pulmonary thymidine kinase after a BHT injection in mice pretreated with inducers or inhibitors of microsomal enzymes, or exposed after the BHT injection to 100% oxygen, which would allow for non-enzymic formation of metabolites. Incorporation measurements, using BHT labelled with ^{14}C at the toluene methyl group, demonstrated that maximum concentrations of BHT were present in the lung between 4 and 8 hours after the injection. Pretreatment with phenobarbital diminished the toxic effect of BHT, but 3-methylcholanthrene and α-naphthylisothiocyanate had no effect on its toxicity. Similarly the oxygen inhalation was without effect.

Although they point out that the evidence is circumstantial, the authors conclude that the results indicate that metabolic activation is not a prerequisite for BHT toxicity in the lung. The phenobarbital reduction of BHT toxicity was due in all probability to its known capacity for increasing biliary excretion. Moreover, it was not possible to prevent cell death by suppressing protein synthesis in the target organ by pretreatment with cycloheximide, a procedure that has been used to abolish the toxic effects of some other compounds requiring metabolic activation.

The mechanism of BHT toxicity, however, still remains unclear. The susceptibility of the squamous epithelium to injury is similar to that seen after oxygen exposure, radiation and treatment with blood-borne bleomycin, but the recovery pattern is quite different. Thus, for example, the repair cell profile is not typical and even at high BHT dosage, capillary destruction was always found to be secondary to that of the type I epithelium. Cellular regeneration as reflected by DNA synthesis is also different. In both cases there is early endothelial injury followed by type I cell necrosis but the peak of DNA synthesis in the epithelium precedes that in the endothelium following BHT toxicity whereas the reverse is true after oxygen poisoning. Adamson *et al.* (*loc. cit.*) suggest that this may be related to the severity of damage; after oxygen poisoning there is early endothelial necrosis, while after BHT the changes appear to be related rather to increased permeability.

BHT is thought to cause cell lysis and death as a result of interaction with the cell membrane and Williamson *et al.* (*loc. cit.*) comment that a similar mechanism may account for the early disruption of type I alveolar cell membranes. However, the reason has yet to be found why these are the first target cells; the capillary endothelial cells have earlier contact with BHT but only become damaged 4–6 days later when BHT has virtually disappeared from the circulation.

[R. Hawkins—BIBRA]

STYRENE BEFORE THE FRAY

Paranoia may become an occupational hazard in the plastics industry. Findings confirming vinyl chloride's carcinogenicity were quickly followed by those implicating acrylonitrile. The preliminary report from a long-term inhalation study (conducted at Dow Chemical's Toxicology Laboratory, Midland, MI) indicating that styrene may be carcinogenic in the rat must have given members of the industry a feeling of *déja vu*. The 2-year inhalation study, conducted on behalf of the Manufacturing Chemists' Association, will provide the first opportunity for assessing the carcinogenicity of styrene, previous animal studies having used much shorter treatment periods. Chronic oral toxicity data will also be available soon. Long-term studies are being conducted in the rat and mouse by the IARC at Lyon, and a similar programme is being sponsored by the National Cancer Institute at Litton Bionetics.

Mutagenicity

Styrene oxide, a possible alkylating agent, may provide the clue to styrene's carcinogenic activity. The oxide induced gene conversion in *Schizosaccharomyces cerevisiae* and forward mutations in *S. pombe* and hamster cells, whereas styrene was inactive in all three systems even in the presence of a weak metabolizing system (*Cited in F.C.T.* 1978, **16**, 300). In host-mediated assays, both styrene and its oxide induced gene conversion in *S. cerevisiae*, but had no effect on *S. pombe* (*ibid* 1978, **16**, 300).

In the more established *Salmonella typhimurium* system, styrene has given conflicting results. In one study (*ibid* 1978, **16**, 397) it was not mutagenic at levels up to 1 mg/plate against strains TA1535, 1537, 1538, 98 and 100 in the presence of a liver metabolizing system obtained from rats and hamsters pretreated with polychlorinated biphenyls. However, according to the results of the Ames test conducted by De Meester *et al.* (*Mutation Res.* 1977, **56**, 147), who used the same metabolizing system, styrene was mutagenic in strain TA1535 although there was no evidence of mutagenicity in the absence of the metabolizing system. At concentrations from 1 to 11 μmol (1 mg)/plate the number of revertants increased with dose; an appreciable dose-related toxic effect was also observed. Both in the absence and the presence of a metabolic activating system, styrene oxide was shown to be mutagenic towards the TA1535 and TA100 strains.

A number of recent studies have indicated that styrene is mutagenic in mammalian systems. In an *in vitro* study (de Raat, *Chemico-Biol. Interactions* 1978, **20**, 163) the monomer induced sister chromatid exchanges in Chinese hamster ovary cells. Styrene's mutagenic activity was only observed, however, in the presence of both a metabolic activation system (an S_9 fraction from phenobarbitone-treated rats) and the epoxide-hydrase inhibitor cyclohexene oxide. Styrene oxide was a potent inducer of sister chromatid exchanges even in the absence of the activation system. Nevertheless when the S_9 fraction was present, the mutagenicity by styrene oxide was increased by the addition of cyclohexene oxide. The lack of mutagenic activity of styrene even in the presence of the metabolic activation system can be ascribed to a very

rapid decomposition of styrene oxide, the decomposition being inhibited by the cyclohexene oxide.

Meretoja *et al.* have shown styrene to be mutagenic *in vivo* (*Toxicology Lett.* 1978, **1**, 315). Male rats inhaling 300 ppm styrene for 6 hours/day, 5 days/week, for up to 11 weeks first exhibited an increased rate of chromosomal aberrations, almost totally chromosome-type breaks, in their bone-marrow cells at week 9. This increased rate, the aberrant cell incidence ranging from 8 to 12% in the exposed group compared with 1 to 6% in the control group, was maintained until the end of the experiment. Styrene exposure was also associated with an increase in polyploid cells.

The same group has studied styrene's mutagenic activity in man. The chromosomes in cultured lymphocytes from ten men occupationally exposed to the monomer were compared with those from five control individuals (Meretoja *et al. Mutation Res.* 1977, **56**, 193). Styrene exposure was associated with a significant increase in the rate of chromosomal aberration, a control incidence of 3% or less being increased into the 11–26% range. The men, aged from 20 to 41 years, had been employed in the lamination industry for up to 8·5 years and all felt they were in good health.

Epidemiology

Extensive investigations into the effects of styrene on man have not been attempted although limited epidemiological data are available. Western contributions in this sphere include a study by Zielhuis *et al.* (*Fourteenth International Congress on Occupational Health*, Madrid 1963, **3**, 1092), who found no significant difference between the health of workers exposed to styrene (at an average concentration below 100 ppm) and that of a control population, apart from an increased occurrence of subjective symptoms, such as irritation of the mucosae and headache. In the study of Wink (*Ann. occup. Hyg.* 1972, **15**, 211), styrene exposure was associated with a decrease in urinary 17-oxosteroid levels but no other objective adverse effects. In a small group of Swiss workers, high exposures to styrene were shown to result in conjunctivitis (*Cited in F.C.T.* 1976, **14**, 201). Four out of the nine workers who underwent haematological examination had a lymphocytosis.

Scientists at the Institute of Occupational Health, Helsinki, have investigated the psychological and neurophysiological consequences of working with styrene. A group of about a hundred workers employed for an average of 5 years in the manufacture of reinforced plastics were compared from the psychological angle with a group of 43 workers in the concrete industry (Lindström *et al. Scand J. Work Envir. Hlth* 1976, **3**, 129). Exposure to styrene was assessed by means of the urinary mandelic acid levels; these varied from 7 to 4715 mg/dm^3 with an average value of 808 mg/dm^3. There were statistically significant differences between the two groups in visuomotor accuracy and psychomotor performance. Disturbances in visuomotor accuracy and psychomotor activity were related to urinary mandelic acid level, whilst performance in tests measuring visuomotor speed and visual memory was related to the length of service in the styrene industry. Since at least 20 hours had elapsed between the last workshift and the examination, the authors considered that the findings were indicative of more than an acute change. In the neurophysiological investigation (Seppäläinen & Hörkönen, *ibid* 1976, **3**, 140), abnormalities in the electro-encephalograms of workers illustrated the toxic effect of styrene on the central nervous system. Abnormalities were found in 24% of the workers exposed to styrene, a significant increase over the control incidence. Low-level exposure to styrene did not increase the prevalence of abnormal electro-encephalograms, but almost one third of those subjected to higher exposures (again judged from the urinary mandelic acid level) were adversely affected.

Neurotoxic effects of styrene have been investigated by Lilis *et al.* (*Envir. Res.* 1978, **15**, 133) in workers involved in its production and polymerization. As expected, prenarcotic symptoms such as light-headedness, were found in 13% of the 488 workers, and these symptoms were seen more frequently in those classified as having a high exposure to the monomer. Distal hypo-aesthesia of the lower extremities occurred in 8·5% of the 180 workers with over 20 years experience in the industry, but in only 4·1% of the 121 subjects with less than 7 years exposure. As no control group was included in the study it is uncertain whether the difference between the two groups was merely a consequence of increased age. In a further examination of 80 of the workers, reductions in the conduction velocity of the radial nerves (< 55 m/second) and peroneal nerve (< 40 m/second) were seen in 18·8 and 16·4%, respectively, of those tested. Although the authors noted that there was a steady decrease in the peroneal nerve velocity with duration of styrene exposure, the small number of subjects in each exposure subgroup largely undermine the significance of this result. By contrast, there was no evidence from the study of Seppäläinen & Hörkönen (*loc. cit.*) that peripheral nerves are susceptible to styrene exposure: nerve conduction velocities were comparable in an exposed and control group.

A single case of retrobulbar neuritis tentatively attributed to styrene exposure was reported in 1964 (*Cited in F.C.T.* 1965, **3**, 534). Ocular examination of 345 workers exposed to styrene produced no confirmatory evidence of styrene's activity in this respect (Kohn, *Am. J. Ophthal.* 1978, **85**, 569). Conjunctival irritation affected 22% of this workforce and this irritation was correlated with intensity of exposure.

Consumer exposure

Some attempt has been made to assess possible consumer exposure to styrene. A report from the Canadian Health Protection Branch (Withey & Collins, *Bull. env. contam. & Toxicol.* (*U.S.*) 1978, **19**, 86) has shown that styrene levels from as low as 698 ppm up to 3285 ppm occur in samples of polystyrene packaging material. No clear relationship between the monomer content of the packaging and the amount leached into the foodstuffs was identified, although for food of approximately similar composition, the extent to which migration occurred was proportional to the original monomer content. Relatively large amounts of styrene migrated during the short period immediately after the food was placed in the container but after this initial effect there was a regular increase in monomer contamination of the food with

time. The maximum level of styrene in any of the foods tested was 246 ppb (in sour cream), with a more usual level of the order of 20 ppb.

The epidemiological data published so far provide no direct evidence that styrene is carcinogenic to man. However carcinogens typically have latent periods of 20 years or more and few of the workers studied had an occupational history of this order. The data currently available do suggest, however, that workers in a number of industries are exposed to styrene concentrations that produce subjective complaints such as eye irritation and headache and evidence of neurotoxicity. This does not necessarily indicate that the present TLV of 100 ppm is too high; compliance with the limit may be the problem.

Styrene's carcinogenic status in animals will presumably be resolved in the near future. Any evidence of a positive response in animals will need to be answered by adequate epidemiological data. If styrene does prove carcinogenic in animals, there is likely to be an immediate call for polystyrene food-packaging materials to contain "zero" monomer, while even the suspicion of carcinogenicity may provide enough impetus to lower the present TLV.

[J. Hopkins—BIBRA]

GENETIC EFFECTS OF FORMALDEHYDE

Formaldehyde is widely used in chemical syntheses and in the manufacture of textiles, papers and synthetic resins. It is also used in agricultural chemicals, particularly in insecticides, fungicides and disinfectants and under some circumstances is present at very low levels in certain foods. Formaldehyde has been shown to be a mutagen in Drosophila (*Cited in F.C.T.* 1966, **4**, 99), but this effect has been detected only in male larvae whose early spermatocytes appear to be sensitive (Auerbach *et al. Mutation Res.* 1977, **39**, 317). Moreover, it has been argued that, because formaldehyde is able to damage DNA in DNA polymerase-deficient strains of *Escherichia coli*, it may have significant carcinogenic potential (*Cited in F.C.T.* 1973, **11**, 923). There is, however, no experimental evidence for this, carcinogenicity studies in mammals having produced, at most, only equivocal results, while indications of mutagenicity in bacteria and other simple organisms remain of very questionable relevance to man. Nevertheless, studies of the type of genetic damage induced in such organisms by formaldehyde are of considerable interest.

Auerbach *et al.* (*loc. cit.*) considered in 1976 that the widespread use of formaldehyde and related compounds in industry called for a keen assessment of their potential hazard and produced an extensive review (citing 189 papers) of available data. In biological materials, the reaction of formaldehyde with amino groups is likely to be of prime importance. With amino acids and polypeptide chains, the first step in this reaction is the formation of unstable methylol derivatives, while the second produces stable condensation products with methylene cross-links. With nucleosides, nucleotides and nucleic acids a similar progression may occur, and it appears that with aminopurines the methylene bridges can actually link the purine groups. The primary lesions in the genetic effects of formaldehyde are likely to be the formation of cross-links between two amino acids in a protein, between two nucleic acid bases or between an amino acid and a nucleic acid base (Auerbach *et al. loc. cit.*).

In micro-organisms, formaldehyde-induced genetic effects have been found to be subject to excision repair, which is dependent on the activity of DNA polymerase I. When wild strains of *Escherichia coli* and a strain deficient in this DNA polymerase were incubated with formaldehyde or with the product of its reaction with glycine (Poverenny *et al. Mutation Res.* 1975, **27**, 123), cell survival was depressed to a similar degree by the two additions. However, both treatments resulted in a much lower survival of the mutant strain, deficient in excision-repair capacity, than in the wild strain, the mutant strain showing a large number of single-stranded breaks in DNA, which could be repaired in the wild-type cells. Spectrophotometric studies of the reaction between nucleotides and formaldehyde in the presence of varying concentrations of glycine showed that a threefold molar excess of the amino acid, such as was used in these incubations of the two compounds with *E. coli*, almost completely bound the formaldehyde present. This study indicated, therefore, that the action of formaldehyde on bacterial DNA was exerted not directly by formaldehyde itself but by the products of its reaction with amino-containing compounds, either the glycine added with it or free amino acids present in the bacterial cells.

The possibility of excision repair of formaldehyde-induced lesions has also been demonstrated in strains of yeast (*Saccharomyces cerevisiae*), a eukaryotic organism (Chanet *et al. ibid* 1976, **35**, 29). This group (*idem, ibid* 1975, **33**, 179) also showed that, in contrast to yeast cells exposed to radiations, cells in the stationary phase were more resistant to the action of formaldehyde than were exponentially growing cells. In synchronized populations, the lag and G_1 phases of the cell cycle were the most resistant to both the lethal effect and the recombinant-inducing action of formaldehyde, while the end of the G_2 phase and mitosis were the most sensitive stages. Discussing the possible reasons for this fluctuation in sensitivity to formaldehyde, the authors put forward the hypothesis that this may be related to the variations in the cellular pool of free amino acids. The nitrogen pool is known to reach a maximum roughly in mid-cycle, and this could yield a maximum level of formaldehyde–amino acid reaction products, resulting in more DNA damage and a higher level of cell inactivation and recombination.

More recent work on haploid strains of *S. cerevisiae*, including two ultraviolet-sensitive mutants, has provided further information on the lesions induced in DNA by formaldehyde (Magaña-Schwencke *et al. ibid* 1978, **50**, 181; Magaña-Schwencke & Ekert, *ibid* 1978, **51**, 11). The technique used for much of this

work involved isotopic labelling of DNA with [6-^{3}H]uracil or [2-^{14}C]adenine over five or six generations followed by conversion of the cells to spheroblasts (swollen cells), either immediately (for controls) or after treatment with formaldehyde, or after further incubation of control or formaldehyde-treated cells. In some cases the formation of spheroblasts (carried out to facilitate subsequent lysis) preceded the formaldehyde treatment. These procedures were followed by lysis of the spheroblasts on the top of alkaline sucrose gradients through which the DNA was subsequently sedimented.

Analysis of the sedimentation profiles obtained under the various experimental conditions showed that formaldehyde caused a dose-dependent incidence of single-strand breaks in the DNA of exponential-phase cells of the wild-type yeast through a metabolic process, a finding in line with that in *E. coli* reported by Poverenny *et al.* (*loc. cit.*). Further evidence was also obtained in support of the indications of this process presented by Chanet *et al.* (1976, *loc. cit.*). Mutant strains of *S. cerevisiae* defective in excision repair of pyrimidine dimers induced by ultraviolet irradiation showed a reduced capacity to undergo single-strand breaks after treatment with formaldehyde, but as is the case with ultraviolet irradiation, they were also more sensitive than the wild-type cells to the lethal effect of the treatment. These findings suggest a common step in the mechanisms for the repair of damage induced by irradiation and by formaldehyde. Incubation of wild-type cells in growth medium after exposure to formaldehyde showed a lag in cell division during which rejoining of single-strand breaks occurred, leading to a low recovery of DNA of normal molecular weight.

Single-strand breaks are not the only lesion produced in yeast DNA by formaldehyde. Other work by this group (Magaña-Schwencke & Ekert, *loc. cit.*), involving conversion of labelled cells of a wild-type haploid yeast to protoplasts (cells denuded of the cell wall), before or after treatment with formaldehyde, and subsequent lysis and sedimentation on a neutral sucrose gradient, demonstrated a dose-related induction of cross-links between DNA and proteins by formaldehyde. Ultraviolet irradiation also produced cross-links between DNA and proteins in this strain. However, the degree of cell survival for a comparable degree of cross-linking was much higher after formaldehyde treatment than after radiation, suggesting either that formaldehyde-induced cross-links were repaired more efficiently than those due to radiation exposure or that another type of damage, such as the pyrimidine dimers induced by radiation, were responsible for the observed differences.

These studies shed useful additional light on the biochemical mechanisms underlying the genetic effects of formaldehyde in simple organisms, knowledge that may, in the long run, help to establish the extent to which effects of this type may be relevant to the mammalian situation.

[P. Cooper—BIBRA]

HAIR DYES AND THE CHROMOSOMES

The controversial debate over the safety-in-use of commercially available hair dyes continues apace. Ever since Ames and his colleagues (*Proc. natn. Acad. Sci.* 1975, **72**, 2423) produced evidence that certain hair-dye constituents were mutagenic when tested in short-term laboratory tests designed to detect mutagens and potential carcinogens, these materials have been subjected to an intensive programme of toxicological examination. Already a number of these compounds, including 2,4-toluenediamine (2,4-TD), 2,4-diaminoanisole (4-methoxy-*m*-phenylenediamine; 2,4-DAA), Direct Blue 6, Direct Black 38, Direct Brown 95 and 4,4'-thiodianiline have been shown to have a carcinogenic effect in studies that involved the feeding of each material to rats (and mice in some cases) at high dose levels (*Fd chem. News* 1978, **19** (46), 60; *Federal Register* 1978, **43**, 19923 & 20562). Whilst the use of some ingredients has been voluntarily abandoned by some manufacturers, the United States authorities have now proposed a label warning for hair dyes containing ingredients that have been shown to cause cancer in laboratory animals (*Fd chem. News* 1978, **19** (43), 38).

Although the findings of Ames *et al.* (*loc. cit.*) have since been substantiated by the experimental animal studies, efforts to refine and demonstrate the validity of short-term predictive tests have led to an examination of the effects of various hair-dye components in eukaryotic systems. Using L5178Y mouse lymphoma cells, Palmer *et al.* (*J. envir. Path. Toxicol.* 1977, **1**, 87) were able to assess chemically-induced mutations at the thymidine kinase (TK) heterozygous locus. The test system was exposed to 2,5-diaminoanisole sulphate (2,5-DAA) at 3·125, 4·69 and 6·25 μg/ml, to 2,4-DAA at 12·5, 18·75 and 25 μg/ml, to 2-nitro-*p*-phenylenediamine (2-NPPD) at 25, 50 and 75 μg/ml, to *m*-phenylenediamine (MPD) at 25, 50 and 100 μg/ml, and to 4-nitro-*o*-phenylenediamine (4-NOPD) at 50, 100 and 200 μg/ml, in each case for a period of 24 hours. All three phenylenediamines produced dose-related increases in the total number of mutants, in mutation frequency and in the ratios of induced to spontaneous mutation frequency, MPD being the most potent and 4-NOPD the least. Although the diaminoanisoles produced similar effects, the results proved rather more difficult to interpret because of the narrow range of dose levels used. As a result of this, the investigators suggested that 2,4-DAA had produced a questionable mutagenic response while 2,5-DAA had evoked a negative response. They also concluded that, in the absence of an activation component for the system, enzymes actually present in the cells were probably involved, during the long exposure period, in the production of mutagenic intermediates responsible for the positive response. Alternatively they suggested that the compounds underwent a structural alteration to produce the active compound during the long incubation period at 37°C. The responses to 2,4-DAA and 2,5-DAA seemed to indicate that the enzymes that

activated these latter compounds were different from those that activated the phenylenediamines. A similarly long exposure period also gave rise to a positive response to 2-NPPD and 4-NOPD in a morphological transformation system used by Benedict (*Nature, Lond.* 1976, **260**, 368).

The same investigator (*loc. cit.*) also examined chromosomal aberrations in a hamster cell line, $A(T_1)C1$-3, being developed as a rapid screen for chemical carcinogens. Both 2-NPPD and 4-NOPD produced chromatid breaks. In addition, chromosomal aberrations of a type considered by the author to be indicative of malignant transformation and oncogenesis resulted from treatment with 2-NPPD but not 4-NOPD.

Unfortunately the scoring of chromosomal aberrations has a number of disadvantages. It is a highly skilled and subjective procedure and very tedious at low concentrations of the chemical. Recently, however, new cytological techniques have been developed which permit an assessment of chromosomal damage by scoring the frequency of sister chromatid exchanges (SCEs). When Chinese hamster cells are allowed to pass through two cell cycles in the presence of 5-bromodeoxyuridine and are then stained, SCEs, showing a dose-dependent response following exposure to a range of chemical mutagens, may be observed. Using this method, Perry & Searle (*Mutation Res.* 1977, **56**, 207) examined the induction of SCEs by the hair-dye components 2-NPPD and 4-NOPD. Exponentially-growing cultures of a near diploid Chinese hamster cell line growing in McCoy's 5A medium with HEPES buffer were exposed to 10 μM 5-bromodeoxyuridine together with varying concentrations of the dye components. Ethyl methanesulphonate (EMS) was used as a positive control. Both dye components at each concentration produced high frequencies of SCEs and were somewhat more potent than EMS. The 2-NPPD proved to be slightly more potent than 4-NOPD, but the latter exhibits a poor solubility in tissue-culture media.

Negative results were obtained by Hossack & Richardson (*Experientia* 1977, **33**, 377) in micronucleus tests on 12 hair-dye constituents. The compounds examined were 2,4-DAA, *p*-phenylenediamine, 4-NOPD, 2-NPPD, *p*-aminophenol, *m*-aminophenol, 2-amino-4-nitrophenol, 2,5-toluenediamine sulphate, resorcinol, 4-chlororesorcinol, 4-amino-2-hydroxytoluene and 1-naphthol. Groups comprising five male and five female rats were dosed by gastric intubation with the test compounds in 0·5% (w/v) gum tragacanth containing 0·05% sodium sulphite. The total dosages were close to the lethal doses and were administered in two equal parts separated by an interval of 24 hours. Six hours after the second dose the animals were killed and bone-marrow smears were prepared. The incidence of micronucleated cells per 2000 polychromatic erythrocytes was compared with the values for the control group and with laboratory standard values. The mean values and ranges of micronucleated cells found were essentially similar to those of the control group and were within the laboratory standard range. None of the compounds showed evicence of mutagenicity in this test.

Understandably the examination of effects of hair-dye components on chromosomal material has been extended to involve human cell types. Searle *et al.* (*Nature, Lond.* 1975, **255**, 506) have looked into the events following exposure of cultures of human peripheral blood lymphocytes to 2-NPPD. At concentrations between 50 and 100 μg/ml a considerable number of chromosome and chromatid gaps and breaks were evident. Mitotic delay and toxicity were evident at 100 μg/ml but it was still possible to demonstrate that up to 45% of the cells contained damaged chromosomes. Cultures treated with 25 μg 2-NPPD/ml did not differ significantly from controls. Similar experiments with 4-NOPD at concentrations up to 100 μg/ml did not produce any chromosomal damage.

More recently such investigations of chromosomal damage have been utilized in an epidemiological study of the potentially genotoxic effects of hair dyes (Kirkland *et al. Lancet* 1978, **II**, 124). Sixty hair tinters and 36 control individuals were investigated. The tinters were volunteers from salons, whilst controls were drawn both from within the hairdressing profession and from other occupations. All volunteers had to provide details of smoking habits, alcohol consumption, medical history, use of drugs, infections, vaccinations, X-ray exposure and whether or not their own hair was dyed. Information on occupational exposure was also gathered. Results from the study indicated that (i) there were no significant differences in chromosomal damage in the cultured lymphocytes from the peripheral blood of tinters and controls closely matched for age and sex, (ii) when age-matched women were re-grouped according to whether their own hair was dyed or not, a significant excess of chromatid and chromosome gaps and breaks was observed in women with dyed hair, (iii) men whose hair was not dyed (mean age 31·5 years) had significantly more chromosome breaks than men with dyed hair (mean age 22·9 years). Discussing these findings, the investigators pointed out that tinters normally wear gloves during the dyeing operation and, even in the absence of gloves, percutaneous absorption of dye components may be impeded by the horny surface of the hands and the lack of sebaceous glands in the palms. They added that dye constituents are more readily absorbed through the scalp and would therefore be more likely to induce chromosomal aberrations by this route. The authors felt that an age effect might be responsible for the lower frequency of chromosome aberrations in the young men with dyed hair compared that recorded in slightly older men without dyed hair. Furthermore, it was suggested that the frequency of hair-dye use by the younger men was too low to reverse this age effect. In conclusion it was emphasized that the excess of cytogenetic damage in women with dyed hair could not be considered definitive at this stage.

The study by Kirkland *et al.* (*loc. cit.*) has given rise to some controversy. In a brief comment on the paper (*ibid* 1978, **II**, 271), Van Abbé extracted the data for subjects who had recently been exposed to X-rays or viral infections, whether or not their hair had been dyed. On analysis, it appeared that exposure to X-rays or a recent viral infection significantly increased the proportion of damaged cells. In contrast, the effect of the dyeing of the subject's own hair was not significant nor was there any significant interac-

tion between dyeing and recent exposure to X-rays or virus infection. On the basis of these findings, Van Abbé suggested that a lady who had her hair tinted 90 times with a permanent hair dye and ten times with a semi-permanent one, was unlikely to suffer greater chromosomal damage than if she underwent routine dental X-rays or occasionally caught a cold.

In their reply, Kirkland and his colleagues (*ibid* 1978, **II**, 272) pointed out that their definition of X-ray exposure did not cover procedures like dental X-rays; they were referring to diagnostic X-ray of considerable dosage, and the viral exposure considered were recent influenza, chicken pox, heavy vaccination or glandular fever but not the common cold. Van Abbé acknowledged these points in a subsequent letter (*ibid* 1978, **II**, 368), commenting that if the chromosome damage associated with hair dyeing was similar in degree to that caused by routine dental or chest X-rays or common-cold infection, the risk would not seem to be unreasonable. In the same issue of the Lancet, the statistical methods used by both sides in this discussion were the subject of further questioning, with a suggestion that the data would be better analysed using a *G*-test (Price, *ibid* 1978, **II**, 368). Use of this method confirmed that there was a statistically significant excess of damaged cells in the women with dyed hair as well as in those exposed to X-rays or recent viral infection, but that there was no interaction between the two factors.

A detailed criticism of the study was added to the debate by Feinstein (*ibid* 1978, **II**, 627) but all his points were answered by Kirkland *et al.* (*ibid* 1978, **II**, 628), who reiterated their original statement that their findings were "not definitive" and that further studies were needed. This important point seemed in danger of becoming lost in the lengthy arguments.

An important comment comes from Burnett (*ibid* 1978, **II**, 685). Accepting these data at face value, there remain serious doubts about their significance in terms of human hazard from cancer or mutation. Observations of chromosome gaps and breaks alone cannot be taken as an indication of heritable damage, nor is there any strong evidence that they are closely associated with malignancy.

Both statistical and risk analyses are an important aspect of any interpretation of the safety-in-use of a chemical, whether it be a food additive, a cosmetic or an industrial chemical. Already the relevance for man of the results from the animal carcinogenicity studies on 2,4-DAA has been questioned (*Fd chem. News* 1978, **20** (9), 32). Dr. H. J. Eiermann of the FDA Division of Cosmetics Toxicology has indicated that normal use of hair dyes would be equivalent to a dietary dose of 2,4-DAA of only 0·00033 mg/kg/day. The CTFA, which claims that even the proposed label warning is unjustified and should be withdrawn, has estimated the risks of the various dyes at between 1 in 16 million and 1 in 2400 million. FDA estimates are considerably higher, ranging from 1 in 31,000 to 1 in a million. All of the projected risk assessments are worded in a rather conservative fashion and admit the need for better data to make more reasoned analyses. In an attempt to resolve the difficulties of making risk assessments on such materials, an interagency workshop on percutaneous toxicology has now been set up in the United States.

The results from epidemiological studies have an important contribution to make to the debate. In this respect, the IARC has already stated that the epidemiological evidence currently available suggests an elevated risk for both users of hair dyes and those (barbers and hairdressers) occupationally exposed to hair preparations (this issue, p. 294). The report goes on to suggest that epidemiological studies, which should include workers employed in the production of hair dyes, are required before any firm conclusions can be drawn. These could in turn be supplemented by a further examination of exposed individuals for genotoxic effects, along the lines of the studies undertaken by Kirkland *et al.* (*loc. cit.*).

[S. P. Johnson—BIBRA]

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TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS

FLAVOURINGS, SOLVENTS AND SWEETENERS

Dealing with coumarin

Piller, N. B. (1977). Tissue levels of (3-^{14}C) coumarin in the rat: distribution and excretion. *Br. J. exp. Path.* **58**, 28.

Ritschel, W. A., Brady, M. E., Tan, H. S. I., Hoffmann, K. A., Yiu, I. M. & Grummich, K. W. (1977). Pharmacokinetics of coumarin and its 7-hydroxy-metabolites upon intravenous and peroral administration of coumarin in man. *Eur. J. clin. Pharmac.* **12**, 457.

One of the major problems in any assessment of the possible hazard that coumarin may present to man is the wide divergence between the metabolism of this compound in man and the rat, the species in which most testing has been carried out. In rats, 7-hydroxylation of coumarin has been shown to occur to only a very limited degree (*Cited in F.C.T.* 1972, **10**, 714), while in man it is the main metabolic pathway (*ibid* 1969, **7**, 681). Coumarin is rapidly eliminated from the latter species, half-lives in the region of 1·5 hr having been demonstrated with iv doses of about 0·2 mg/kg (*ibid* 1977, **15**, 365). More information about coumarin elimination in man is now provided, and more is revealed of its tissue distribution in the rat.

The first paper cited above describes the distribution of [3-^{14}C]coumarin in rat tissues after a single ip injection of 25 mg/kg. Samples of blood, cerebrum, heart, kidney, liver, lung, thigh muscle, skin, small intestine and spleen were examined at intervals between 2 and 100 hr after administration of the dose. At 6 hr, body tissues retained 35% of the dose, with a descending order of concentration in muscle, gut, liver and skin. At 10 hr, 23% remained in the tissues, distributed principally in muscle, liver, skin and gut, in that order. At 24 hr, an anomalous 38% was estimated for the ^{14}C retention, the high figure obtained for the gut probably reflecting a sampling problem. At 48 hr, 21% remained, mostly in skin, with less in muscle, gut and liver. At 72 hr the residue was 9% and at 100 hr it was 7%, distributed mainly in muscle, with less in the liver, skin and gut. Faecal excretion accounted for 1·1% of the dose during the first 2 hr, rising to 30·9% in 100 hr and urinary excretion accounted for 1·7% in 2 hr and 46·9% in 100 hr. The highest tissue concentrations detected were in kidney and liver.

The second paper reports on the pharmacokinetics of coumarin after administration of single doses of 0·857 mg/kg by mouth and 0·25 mg/kg iv to six healthy volunteers, four of whom participated in a crossover study. The oral doses were dissolved in propylene glycol and the iv doses in water–propylene glycol (10:1, v/v). Despite rapid absorption of coumarin after oral administration, only about 3·4% of the dose was found as such in the systemic circulation. The rest appeared in the systemic circulation as 7-hydroxycoumarin (7-HC) and its glucuronide (7-HCG), indicating an extensive first-pass effect.

The biological half-life of coumarin was similar after oral and iv administration (0·80 and 1·02 hr, respectively) as was that of 7-HCG (1·47 and 1·15 hr, respectively). The amount of 7-HC present was at all times only a small fraction of the amount of 7-HCG (1–2%, irrespective of the route of administration), although more was converted to the glucuronide after oral dosing. The fact that in two cases the rate of appearance of the 7-HCG was faster than that of 7-HC itself suggests that the formation of these two metabolites may sometimes be simultaneous rather than sequential. Elimination of 7-HCG was almost exclusively into the urine, active tubular secretion—a process commonly associated with glucuronides—being indicated by a clearance rate about ten times greater than the glomerular filtration rate.

EMULSIFIERS AND STABILIZERS

Suspension of the suspending media

Bachmann, E., Weber, E., Post, M. & Zbinden, G. (1978). Biochemical effects of gum arabic, gum tragacanth, methylcellulose and carboxymethylcellulose-Na in rat heart and liver. *Pharmacology* **17**, 39.

The thickening agents gum arabic, gum tragacanth, methylcellulose and sodium carboxymethylcellulose are used very widely both as food additives and as suspending media for the oral administration of water-insoluble compounds. Little toxicological information is available on the two vegetable gums. In 1974, the Joint FAO/WHO Expert Committee on Food Additives was unable to set an acceptable daily intake (ADI) for tragacanth because of a lack of adequate data, but put gum arabic usage in the 'no limit' category on the basis of its widespread traditional usage, at the same time making recommendations for further studies (*Cited in F.C.T.* 1975, **13**, 565). The ADI for total modified celluloses was set at 25 mg/kg body weight.

Previous observations that gum tragacanth impaired oxidative phosphorylation and electron-transfer activities in rat-liver mitochondria have now led to a further detailed study of the effects of gum

tragacanth, gum arabic, methylcellulose and sodium carboxymethylcellulose on the function of heart and liver mitochondria and liver mixed-function oxidases of rats.

The agents were given to female rats by gavage twice daily for 5 days/wk over a period of 4 wk– gum arabic at dose levels of 2 × 20, 2 × 40 and 2 × 200 mg/kg/day, gum tragacanth at 2 × 10, 2 × 20 and 2 × 40 mg/kg/day, methylcellulose at 2 × 10 and 2 × 20 mg/kg/day and sodium carboxymethylcellulose only at 2 × 10 mg/kg/day. Oxygen consumption, as measured by electron-transfer reactions, and oxidative phosphorylation were assayed in mitochondrial preparations from heart and liver, and the mixed function oxidase system of the hepatic endoplasmic reticulum was measured by assay of biphenyl hydroxylation.

It was found that all four substances caused uncoupling of oxidative phosphorylation and partial inhibition of the mixed-function oxidases, but there were considerable differences between them with regard to the potency and reversibility of these effects.

In liver mitochondria the uncoupling developed slowly, but whereas that caused by gum tragacanth became progressively worse with time, indicating a cumulative effect, with the other three substances it became stabilized or tended to return towards normal with continued dosing. The damage caused by gum arabic was dose-dependent, and a secondary effect on electron-transfer activities was seen when significant uncoupling was present, but uncoupling caused by gum tragacanth was not clearly dose-dependent. The two gums also differed with respect to their effect on heart mitochondrial function. Gum tragacanth caused severe initial damage at all doses, but on continued treatment normal function was re-established, while with gum arabic the uncoupling effect developed slowly and progressed on continued treatment. A moderate degree of uncoupling in heart mitochondria was caused by sodium carboxymethylcellulose and also by the higher dose of methylcellulose tested. In the former case, the uncoupling developed slowly; in the latter, its development was, in contrast, relatively rapid.

Inhibition of mixed-function oxidases occurred with gum arabic, with the highest dose of gum tragacanth and with sodium carboxymethylcellulose, but no adverse effect on this system was observed following administration of methylcellulose.

The authors point out that the effects observed could have been due to the formation of small amounts of breakdown products or to undetected impurities, or indeed to the creation of an intracellular metabolic imbalance. They also stress that the tests were carried out *ex vivo*, that is, in cell organelles isolated from the organs of the treated or control animals, and that further *in vivo* assays should be carried out for a full evaluation of these substances. However, these results do indicate significant changes in the function of heart and liver mitochondria and liver microsomes, and the fact remains that in biochemical pharmacology and toxicology, enzyme assays are frequently performed using these suspending media, which may themselves affect the results of the assay. Of the four substances tested, only the 0·5% level suspension of methylcellulose was without effect, and the authors recommend that this should be the suspending medium of choice.

ANTIOXIDANTS

Hepatic effects of ethoxyquin

Skaare, J. U., Nafstad, I. & Dahle, H. K. (1977). Enhanced hepatotoxicity of dimethylnitrosamine by pretreatment of rats with the antioxidant ethoxyquin. *Toxic. appl. Pharmac.* **42**, 19.

Nafstad, I. & Skaare, J. U. (1978). Ultrastructural hepatic changes in rats after oral administration of ethoxyquin (EMQ). *Toxicology Lett.* **1**, 295.

In common with certain other antioxidants, ethoxyquin (EQ) protected mice and rats against the carcinogenicity of benzo[*a*]pyrene and dimethylbenz-[*a*]anthracene (*Cited in F.C.T.* 1973, **11**, 328). Protection has also been reported against the hepatotoxicity of selenium (*ibid* 1972, **10**, 869) and carbon tetrachloride (Cawthorne *et al. Biochem. Pharmac.* 1973, **22**, 783) in rats and against the lethal effects of ethyl methanesulphonate in mice (Cumming & Walton, *Fd Cosmet. Toxicol.* 1973, **11**, 547).

These protective powers have generally been attributed to EQ's ability to induce certain liver enzymes and suppress others, as demonstrated after the feeding of a 0·5% dietary level to rats for 14 days (*Cited in F.C.T.* 1976, **14**, 67; Kahl & Netter, *Toxic. appl. Pharmac.* 1977, **40**, 473). The position is complicated, however, by the facts that dietary levels below 0·05% are not enzyme-inducing (Kahl & Netter, *loc. cit.*) and that a single intragastric dose of 500 mg/kg may inhibit enzymes that are induced by repeated feeding of EQ (Parke *et al. Biochem. Pharmac.* 1974, **23**, 3385). The two papers cited above report different stages of a study concerned with the effect of EQ pretreatment on the acute hepatotoxicity of *N*-nitrosodimethylamine (NDMA) to rats and with the ultrastructural effects of EQ on the rat liver.

In rats given either a single dose of 500 mg EQ kg by stomach tube or 0·015% in the drinking-water (equivalent to an intake of about 37·5 mg/kg/day) for 60 days, liver weight increased but the hepatic content of protein and of cytochrome *P*-450 decreased. Moreover, both EQ pretreatments significantly enhanced the liver necrosis and rise in plasma glutamic-oxalacetic-transaminase (GOT) activity produced by ip injection of NDMA, single-dose gavage being more potent than dietary administration in these respects. No correlation was demonstrated between the grade of necrosis and the EQ concentrations in liver and plasma but there seemed to be some correlation between the enhancement of NDMA-induced toxicity and the increase in liver weight. EQ alone caused

no significant increase in GOT or in hepatic lesions detectable by light microscopy, and a detailed investigation by electron microscopy was therefore undertaken.

EQ given by gavage or in the drinking-water increased liver weight by 38 and 23%, repectively, while levels of the antioxidant in the liver and plasma were about 6·5 and 8·5 times greater after intubation than after drinking-water treatment. Ultrastructural changes were obvious only in the livers of intubated rats, and consisted of a proliferation of the smooth endoplasmic reticulum, dilatation of the perinuclear space, disorganization of mitochondrial membranes and desquamation and fragmentation of living cells. In rats treated orally, a slight proliferation of the endoplasmic reticulum was the only finding.

Endoplasmic reticular proliferation is generally regarded as a sign of microsomal-enzyme induction, which in this case was associated with a decrease rather than an increase in cytochrome *P*-450 and protein synthesis. EQ is apparently not unique in this respect (Meldolesi, *Biochem. Pharmac.* 1967, **16**, 125), and, as NDMA can have similar effects, it is suggested that the additive effects of the two compounds may have been responsible for the observed enhancement of NDMA hepatotoxicity.

[However, the reductions in liver proteins and cytochrome *P*-450 concentrations induced by ethoxyquin plus nitrosodimethylamine were not significantly greater than those induced by either compound alone, and a more plausible explanation for the antioxidant's effects may lie in the BIBRA finding that nitrosodimethylamine metabolism is not mediated entirely by a microsomal mixed-function oxidase system dependent on cytochrome *P*-450 (Phillips *et al. Fd Cosmet. Toxicol.* 1975, **13**, 611; Lake *et al. Toxicology* 1976, **5**, 297). It seems likely that, in the above study, ethoxyquin induced a different enzyme system, which led to a greater evolution of hepatotoxic metabolites from the nitrosamine.]

BLEACHING AND MATURING AGENTS

More effects of chlorinated cake flour

Cunningham, H. M. & Lawrence, G. A. (1978). Effect of chlorinated lipid and protein fractions of cake flour on growth rate and organ weight of rats. *Bull. envr. contam. & Toxicol. (U.S.)* **19**, 73.

Chlorinated cake flour lipids, produced during the bleaching of flour with chlorine gas, have been shown to remain in the adipose tissue of rats for about the same period as normal lipids (Cunningham & Lawrence, *Fd Cosmet. Toxicol.* 1977, **15**, 105). Chlorination of cake flour, flour lipids and wheat gluten yields products that have been found to produce toxic effects when fed to rats for 2 wk (*Cited in F.C.T.* 1978, **16**, 191). The work cited above extended the studies in rats and investigated the effects of baking on the toxicity of the chlorinated cake flour lipids.

Groups of ten male rats were fed for 10 wk on 87·4% cake flour containing 7·93% protein and 0·2 or 1·0% chlorine. The animals given the 1%-chlorinated flour had reduced weight gains, and the relative weights of liver, kidney, heart and brain were increased. The only effects of 0·2%-chlorinated flour were to reduce weight gain and increase relative kidney weight. Lipid and lipid-free fractions of the flour were separately chlorinated with 2·0 g chlorine/kg of original flour and fed to rats for 2 wk. The chlorinated lipid fraction significantly reduced growth rate and increased the relative weights of liver, kidney and heart, while chlorine in the lipid-free fraction produced only an increase in relative kidney weight.

Wheat gluten, freed from lipids and chlorinated at 2 or 5%, was fed at a level of 10% in the rat diet for 10 wk. At 2% chlorinated it significantly increased relative kidney weight and at 5% it also decreased growth rate. There was again an increase in relative brain weight, but this is normally expected when growth is markedly restricted. Lipids extracted from sponge cakes baked using 0·2%-chlorinated cake flour were incorporated into ground rat chow at a level of about 6%. The chow was fed *ad lib.* to ten rats for 2 wk, controls being pair-fed. The treated rats showed reduced growth rate and increased absolute liver weight compared with controls; brain weight unadjusted for body weight was significantly lower in the exposed rats, providing additional evidence for a reduction in growth. Liver lipids were not altered in any of these experiments.

It appears, therefore, that both the lipid and non-lipid fractions of chlorinated wheat flour have a harmful effect when fed to rats for extended periods, the lipid fractions having the more deleterious effect for the same amount of chlorine. Baking did not counteract these effects.

MISCELLANEOUS DIRECT ADDITIVES

The teratogenicity of crude papain

Singh, S. & Devi, S. (1978). Teratogenic and embryotoxic effect of papain in rat. *Indian J. med. Res.* **67**, 499.

Papain, a proteolytic enzyme obtained from unripe papaya fruit, was found to deplete the cartilage matrix when given intravenously to young rabbits, and its direct injection into the tibial medulla produced epiphyseal arrest (*Cited in F.C.T.* 1964, **2**, 95). However, the only known effect of its industrial handling is the occasional induction of allergy, particularly asthma

(*ibid* 1977, **15**, 261). The FDA has recently proposed to affirm the GRAS status of papain as a processing aid and texturizer, on the basis of a FASEB Select Committee report which concluded that there was no evidence of hazard from current or reasonably anticipated future use levels (*Fd chem. News* 1978, **20** (19), 39). In one of the studies on which this conclusion was based, the oral administration of up to 820 mg/kg to pregnant rats and up to 1080 mg/kg to pregnant mice on days 6–15 of gestation produced no evidence of maternal or foetal toxicity or teratogenicity (*Federal Register* 1978, **43**, 31349). A further study which appears to conflict with this last finding is now reported.

Crude papain was given to pregnant rats at various times between days 8 and 17 of gestation as a single oral dose of 375, 750 or 1000 mg/kg, as two or three oral doses each of 750 mg/kg, or as one or two ip doses each of 375 mg/kg, the multiple doses being given on consecutive days. The foetuses were examined on day 20. All the rats given 1000 mg/kg died within 24 hr, and some deaths also occurred after two ip doses. There were no foetal resorptions after single oral doses, or after two oral doses given on days 10 and 11, but in other orally-treated groups the incidence of resorptions was in the range of 8–9% after two doses and of 17–30% after three. Injected papain was more severe in its effects, producing a 12–20% level of resorptions after one dose and 56–65% after two.

In all groups there were some foetal abnormalities, with an incidence ranging from 12% after a single oral dose of 375 mg/kg to 100% after two ip or three oral doses. Except after a single oral dose the foetuses were frequently stunted, and oedema occurred in some cases. Subcutaneous and visceral haemorrhages were found at all levels of treatment, the latter being about twice as frequent as the former. Organs affected were the liver, kidney and brain, which were often also oedematous. The rim of the placenta was frequently necrotic, and marked stunting of the long bones and ribs and delayed ossification of the skull bones were evident on staining with alizarin red.

It is suggested that the skeletal effects of papain may have been due to its ability to deplete the cartilage matrix, while its proteolytic action may have increased capillary permeability and so led to haemorrhage. Increased vascular permeability may also have been responsible for the oedema. Another possibility is that its antigenic properties may have led to maternal immunization against the offspring.

[Whatever the mechanisms behind these findings, a difference in specification would seem the most likely explanation for the conflicting results of the two teratogenicity studies. The purification process specified in the proposed FDA regulation (Sec. 184.1585) makes the embryotoxicity and teratogenicity of crude papain of doubtful relevance to the use of this enzyme as a food additive.]

PROCESSING AND PACKAGING CONTAMINANTS

A diet of phthalates

Tomita, I., Nakamura, Y. & Yagi, Y. (1977). Phthalic acid esters in various foodstuffs and biological materials. *Ecotoxic. envir. Safety* **1**, 275.

The leaching problems, distribution, retention, excretion and toxicology of phthalic acid esters commonly used as plasticizers in plastics film have been studied in some detail (Cooper, *Fd Cosmet. Toxicol.* 1976, **14**, 501) and particular attention has been paid to the migration of phthalates into blood from storage packs and transfusion equipment (Cooper, *Fd Cosmet. Toxicol.* 1979, **17**, 88). The transfer of phthalates into packaged foodstuffs has been studied in Japan by the authors cited above.

Samples of non-fatty foods were extracted with ether, while fatty foods were subjected to *n*-hexane–acetonitrile partition. Column chromatography of the acetonitrile and ether solutions was used to separate polychlorinated biphenyls, chlorinated pesticides and other organic contaminants from the phthalates prior to analysis by gas–liquid chromatography. Di-*n*-butyl phthalate (DNBP) and di-(2-ethylhexyl) phthalate (DEHP) were the most prevalent of these esters in the foods investigated.

Among 55 samples of Japanese foodstuffs, four fatty and 12 non-fatty foods each contained DNBP and DEHP totalling more than 1·0 ppm. The highest level of DNBP (6·35 ppm) in fatty foods occurred in an instant vegetable cream soup contained in a paper–aluminium–plastics pack. One tempura (frying) powder among the non-fatty foods contained 9·33 ppm DNBP and 16·26 ppm DEHP. Powdered or granulated foods wrapped in plastics film showed a high phthalate content, whereas lower levels occurred in paper-packed foods.

A close correlation was found to exist between the levels of phthalate esters in the food wrap or container and the amounts present in the food. Foods wrapped in plastics films or in plastics laminates containing cellophane, aluminium and/or paper, particularly those carrying printing, were more contaminated than those contained in plastics bottles. Samples in contact with printed areas of laminated plastics film had much higher phthalate content than those contacting non-printed areas of the same film. In one tempura powder consisting of 99·3% wheat flour plus starch, egg yolk and salt and packed in various plastics films, both DNBP and DEHP increased rapidly during storage and were found in higher concentrations as the combined phthalates in the film increased. Comparison of the levels of phthalate in food samples with the levels in the corresponding packs suggested that the food contamination could be kept below 1 ppm if the packaging materials did not contain more than 10 ppm.

As an extension of this study, phthalate analyses

were carried out on a small number of blood samples taken from Japanese subjects. Both DNBP and DEHP were detectable in the blood of all but two of the 25 subjects sampled. The levels were some 30 times higher than those of polychlorinated biphenyls and were found to be considerably higher in samples taken 2 hr after meals than in those taken before meals. In only a few cases, however, were the before- and after-meal samples directly comparable in other respects.

THE CHEMICAL ENVIRONMENT

Arsenic and cancer

Reymann, F., Møller, R. & Nielsen, A. (1978). Relationship between arsenic intake and internal malignant neoplasms. *Archs Derm.* **114**, 378.

Skin cancer has developed after the chronic ingestion of potassium arsenite (Fowler's solution) for the treatment of psoriasis or asthma (*Cited in F.C.T.* 1972, **10**, 101) and several cases of angiosarcoma of the liver have been reported after arsenic treatment of psoriasis or after exposure to arsenical pesticides (*ibid* 1976, **14**, 507). The paper cited above analyses the treatment records of patients with various skin diseases in an attempt to assess the contribution of arsenic to subsequent malignant neoplasms.

Records of 161 patients with lichen planus, 72 with verruca plana and 93 with psoriasis, all of whom had been treated internally with arsenic during the period 1930–1939, were examined. Included in the survey were small groups of arsenic-treated patients with multiple basal-cell carcinoma or Bowen's disease, giving a total group of 389. Between 1943 and 1974, internal malignant neoplasms occurred in 41 of these patients, as against an expected 44·6 calculated from figures produced by the Danish Cancer Registry. The tumour incidence was apparently not affected by the total dose of arsenic or by the form in which it was administered (potassium arsenite, allylarsonic acid or unspecified arsenicals).

A further 53 arsenic-treated patients were considered separately, as they had arsenical keratoses. On average, this group had received higher doses of arsenic than the group of 389 patients, although in about 43%, of both groups, the total dose ingested could not be estimated. Other studies have also indicated that the development of arsenical keratoses is generally dose-dependent. The group with arsenical keratoses showed a markedly high incidence of internal malignancies, but the preselection of these patients prevented any assessment of the statistical significance of this finding. There was no indication that the internal tumours developed more rapidly in the keratosis patients. In the main group, the only significant increase in the incidence of internal malignancies appeared in women who suffered from multiple basal-cell carcinoma.

While these data fail to establish any clear pattern connecting past arsenical therapy with later internal malignancies, the authors conclude that evidence for the carcinogenicity of arsenic, even in the relatively low doses that have been used in dermatology in the past, is sufficiently strong to justify cessation of the use of arsenicals in human therapy.

The fate of inhaled chromate

Langård, S., Gundersen, N., Tsalev, D. L. & Gylseth, B. (1978). Whole blood chromium level and chromium excretion in the rat after zinc chromate inhalation. *Acta pharmac. tox.* **42**, 142.

Workers handling chrome pigments, particularly zinc chromate, have been reported to have an increased incidence of respiratory cancer (*Cited in F.C.T.* 1979, **17**, 97). The possible use of urinary or faecal excretion of chromium (Cr) as an index of exposure to chromate dusts has been considered as a step in the control of this hazard.

In a pilot study, three pairs of rats were exposed in an inhalation chamber to 7·35 mg zinc chromate/m^3 for 100, 250 or 350 min, and their blood, urine and faeces were assayed for Cr for the next 2 days. In the main exposure experiment, two groups of eight rats were exposed to zinc chromate for 6 hr on each of 4 consecutive days or nights, and their urinary and faecal excretion of Cr was determined for 48 hr in the case of rats exposed in the daytime, and for 24 hr for those exposed at night. The zinc yellow pigment used, with 99% of the particles smaller than 5 μm, had a respirable fraction of 76%. The zinc chromate concentration in the chamber varied from 6·33–8·09 mg/m^3 during the day and 9·98–10·5 during the night.

The Cr concentration in whole blood rose rapidly with increased exposure time. After exposure to zinc chromate dust for 100 min there was a five-fold increase in the whole-blood level of Cr, and a further similar increase was demonstrated over the next 150 min of exposure. Blood Cr appeared to be reaching a plateau level by day 4 of exposure.

There were marked variations in individual urinary and faecal Cr concentrations, but the differences were smaller in the urine. Faecal Cr excretion appeared to reflect zinc chromate that was ingested rather than inhaled during exposure, and was probably useless as an index of inhalation exposure. Urinary Cr excretion increased rapidly from the start of each exposure and fell rapidly afterwards, but there was still a surplus of chromium in the urine 3 days after exposures had ended. The chromium level in the urine may thus give an indication of recent inhalation exposure levels. There was no significant difference in the absorption and excretion of Cr between day and night exposures to zinc chromate.

Chromate carcinogenicity in Japan

Ohsaki, Y., Abe, S., Kimura, K., Tsuneta, Y., Mikami,

H. & Murao, M. (1978). Lung cancer in Japanese chromate workers. *Thorax* **33**, 372.

Lung cancer has been recognized as a hazard among workers in the chromate industry (*Cited in F.C.T.* 1979, **17**, 97) but the study cited above is apparently the first of its kind to be reported from Japan.

The major products from the factory in question were chromic acid and sodium and potassium dichromate, and the processes produced several noxious dusts or fumes, including the ore, sodium carbonate, lime, sulphuric acid, monochromates and dichromates. The equipment was old-fashioned and little attempt was made to protect the workers from the dusts.

The authors examined the records of 554 men who had worked in the factory between 1936 and 1973. Four cases of lung cancer were identified from these records and a further ten cases were diagnosed during the period 1972–1976. Eight of the latter individuals were found to have nasal perforations, which provided direct evidence of exposure to soluble chromate. In these fourteen lung-cancer patients, the period of exposure to chromate dust had ranged from 10 to 36 yr. Twelve of the affected individuals were heavy smokers. From the 133 employees who had worked at the plant for more than 10 yr (the shortest exposure period among those with lung cancer), the authors calculated that the annual cancer incidence was 657·9/100,000 population, compared with an overall death rate from lung cancer in Japan of 13·3/100,000 in 1975.

It is believed that the main carcinogenic activity of chromium is due to the hexavalent ion, although exposure to insoluble chromium may also play a part. In these Japanese cases, in many of which smoking may have been a contributory factor, the primary site was limited to the large bronchi, obstructive pneumonia being the first symptom of disease.

Tin under the skin

Bischoff, F. & Bryson, G. (1976). Toxicologic studies of tin needles at the intrathoracic site of mice. *Res. Commun. chem. Path. Pharmac.* **15**, 331.

Bischoff, F. & Bryson, G. (1977). Intraperitoneal foreign body reaction in rodents. *Res. Commun. chem. Path. Pharmac.* **18**, 201.

Many studies in the field of tin toxicology have concentrated on alkyl-tin compounds. The use of tin-lined containers for food is not generally considered any threat to health, but the inertness of the metal when introduced parenterally has not been closely investigated. These two studies indicate that, as has been demonstrated with a number of other materials (*Cited in F.C.T.* 1972, **10**, 570), the physical form and particle size of implanted tin govern the reaction of tissues to it.

In the first experiment, 3-month-old male mice were given a single intrathoracic injection of 4 mg tin (about 180 mg/kg) in the form of needles 1 μm in diameter. About 46% of the needles were 3·3 μm or less in length and about 34% measured 9·9 μm or more. The size and form of the needles were similar to those of asbestos particles that are known to have induced mesotheliomas and lung tumours in rodents. The animals were observed for up to 19 months.

There was a marked reduction in water and food intake over the first 24 hr. Of the 43 mice treated, 41 survived for 10–19 months. Tin particles were detected in the thorax of 16, in the peritoneum of nine and in both the thoracic and peritoneal cavities of one. There were six lung adhesions, seven pericardial adhesions, two diaphragmic adhesions and five thoracic-wall adhesions, while six diffuse adhesions occurred at the injection site. In contrast, three female mice given an ip injection of 10 mg tin needles showed after 40 days six liver adhesions, three diaphragmic adhesions and three diffuse local adhesions.

The ip foreign-body reaction to tin was primarily lymphohistiocytic. At the intrathoracic site, the tin needles were largely engulfed by giant cells and remained in the cytoplasm; some adjacent nodular fibroplasia appeared, and in every instance a network of capillaries developed locally. The terminal incidence of bladder distension and inflammatory changes in the liver and kidney showed no significant difference between the tin-treated mice and saline-treated controls, and neoplasms were detected no more frequently in the treated than in the control animals. The survival rate of injected mice was higher than that of controls, a finding tentatively associated with an enhancement of the immunological defence against respiratory infection.

In the second experiment, open-ended cylinders of tin (measuring 12 × 4 and 25 × 8 mm) were implanted into mice and rats, respectively, and the animals were observed for 18–24 months. The local foreign-body reaction differed from that reported earlier after the sc implantation of tin, in that some cylinders failed to become anchored and there was commonly no fibrous capsule outside the implant. Some lymphohistiocytic involvement and adenomatous hyperplasia in the fibrosis within the tin cylinder also contrasted with the avascular, cell-poor fibrosis with fibroblasts associated with the sc treatment.

The treated rats developed significantly more local sarcomas than did controls, and 30–50% of these sarcomas metastasized. In the mice, the relative significance of local sarcoma induction was less clear because of the tendency of the controls to develop pneumonia, but the eight local tumours in 31 treated animals (compared with one in 23 controls) included four fibro-sarcomas and one spindle-cell sarcoma. The lymphoid tumours that occurred spontaneously in the controls showed no higher incidence in the tin-implanted group. The results demonstrate that solid-state carcinogenesis associated with ip implantation of tin in rodents is not accompanied by chemical carcinogenesis.

An eyeful of lead

Ali, A. R., Smales, O. R. C. & Aslam, M. (1978). Surma and lead poisoning. *Br. med. J.* **2**, 915.

Surma is a cosmetic applied to the conjunctival surface of the eye and is chiefly used on the young. In 1968, the Home Office (*Lead Poison Warning, Home*

Office Press Notice) warned that use of surma might lead to lead poisoning, and this warning has recently had to be repeated. The above-cited study provides some scientific basis for this concern.

When 29 samples of surma were analysed, little or no lead was detected in the six white samples, whereas of eighteen grey samples all but four had a lead content greater than 80%. The five black samples contained 12–32% lead.

Blood samples were obtained from 62 Asian children, of whom 37 (60%) had had surma applied to their eyes. The mean level of lead (±SD) in the blood of the 25 control children was 0·98 ± 0·42 μmol/litre, whereas in the 37 surma users it was 1·65 ± 0·68 μmol/litre, the difference between the two groups being statistically significant. Concern has been expressed about children in whom blood lead concentrations rise above 1·8 μmol/litre, although none of the children in this study showed any clinical evidence of lead toxicity.

United Kingdom regulations to be made in the near future will prohibit the sale of cosmetics products containing lead (*Trade & Industry*, 1978, 9 June, p. 520). In this case, however, the regulations may have little effect as most of the surma is not manufactured in this country but obtained directly from abroad by relatives or friends of the users. The authors suggest that a more appropriate approach in this situation may be to present all the information simply and clearly to the leaders of the Asian communities, who can then advise their people of the possible hazard of using surma as a cosmetic.

Mercury in the blood

Cherian, M. G., Hursh, J. B., Clarkson, T. W. & Allen, J. (1978). Radioactive mercury distribution in biological fluids and excretion in human subjects after inhalation of mercury vapor. *Archs envir. Hlth* **33**, 109.

There is little information on the uptake of inhaled mercury by man, its distribution to different organs and the kinetics of its loss. Stable mercury has been used to determine fractional retention in the lung as a function of the mercury concentration in inhaled air (Hursh *et al. Archs envir. Hlth* 1976, **31**, 302) and the above-cited study looks at metabolism in man, using [^{197}Hg]mercuric nitrate and [^{203}Hg]mercuric chloride reduced with stannous chloride as sources of radioactive mercury.

Five male volunteers, aged 22–27 yr, inhaled radioactive mercury vapour from a reservoir, the content of which was regulated to 0·1 μg mercury/litre. Their 14–24 min exposures resulted in doses of 4–10·7 μCi ^{197}Hg or 1 μCi ^{203}Hg. The subjects were monitored on a whole-body counter and the total amounts of stable mercury in red blood cells (RBCs), plasma, urine and faeces were recorded for up to 7 days.

The whole-body monitoring showed a loss in radioactivity over the 7 days at levels that were found to be in reasonable agreement with the urinary and faecal levels. The amount of radioactive mercury found in RBCs was maximal (at 98% of the total blood content) immediately after exposure, and showed a linear decline over the 5-day period, whereas the levels in the plasma were initially very low, reached a maximum 20 hr after exposure and then declined at a rate similar to the decline recorded for the RBCs. The 24-hr excretion of radioactive mercury in the urine and faeces showed no definite trend with time, and faecal excretion after the first 24 hr appeared to be four times the urinary excretion. There was little correlation between the level of mercury in the urine and that in the plasma.

In view of these results the authors suggest that blood sampling should be used in the evaluation of recent exposure to mercury, the present monitoring of mercury levels in the urine being a less sensitive test.

Air versus urine for nickel concentrations

Bernacki, E. J., Parsons, G. E., Roy, B. R., Mikac-Devic, M., Kennedy, C. D. & Sunderman, F. W., Jr. (1978). Urine nickel concentrations in nickel-exposed workers. *Annls clin. Lab. Sci.* **8**, 184.

Occupational exposure to nickel involves both metallic nickel and its carbonyl, and the latter has been incriminated as an acute pulmonary irritant and carcinogen (*Cited in F.C.T.* 1966, **4**, 211). NIOSH recommends that industrial exposure for up to 10 hr be limited to a time-weighted average concentration of 15 μg nickel/m^3 (*Federal Register* 1977, **42**, 49472). Nickel excretion in the urine has been investigated in man after the inhalation of nickel carbonyl vapour (Hagedorn-Götz *et al. Arch. Tox.* 1977, **38**, 275), and studies with radioactive nickel administered iv have indicated that this element is rapidly eliminated in the urine (Smith & Hackley, *J. Nutr.* 1968, **95**, 541).

The study cited above used electrothermal atomic absorption spectrometry to develop a sensitive and improved method for measuring nickel levels in urine (Mikac-Devic *et al. Clin. Chem.* 1977, **23**, 948).

Ten groups of 5–21 workers, divided according to the type of nickel exposure to which they were subjected and totalling 101 individuals in all, were compared with two control groups of 19 hospital and 23 office workers. The mean levels of nickel in the urine were greatest in nickel-refinery workers who were exposed to inhalation of aerosols of soluble nickel salts. They were also high in those involved in nickel-plating operations. Less marked increases were found in the groups of metal sprayers, nickel-battery workers, bench mechanics and arc welders, while no significant increases were observed in the groups of external grinders and buffers and polishers.

In seven of the ten exposed groups, atmospheric concentrations of nickel were estimated by the use of individual air samplers. Air sampling was carried out during an 8-hr period but no correlation was found between atmospheric exposure to nickel and the nickel concentration in the urine.

The authors recommend that industrial monitoring for nickel exposure should include analyses of both air and urine.

Beryllium in reverse

Sprince, N. L., Kanarek, D. J., Weber, A. L., Chamberlin, R. I. & Kazemi, H. (1978). Reversible respira-

tory disease in beryllium workers. *Am. Rev. resp. Dis.* **117**, 1011.

Exposure to beryllium and its salts continues to be an occupational hazard in a variety of industries. Beryllium most commonly enters the body via the respiratory system and may cause acute or chronic pulmonary disease. Local involvement with production of dermatitis or chronic skin ulcers may also occur. The likelihood that the chronic disease may be a manifestation of delayed hypersensitivity has already resulted in the development of a hypersensitivity test that may be used for the diagnosis and monitoring of workers exposed to beryllium (*Cited in F.C.T.* 1978, **16**, 80).

The above-cited study reports a combined medical and environmental survey undertaken in a beryllium extraction and processing plant in 1971 and a follow-up study carried out in 1974. Peak air concentrations of beryllium as much as 50 times the accepted peak limit value (25 $\mu g/m^3$) were found in the plant in 1971, whereas by 1974, following workplace improvements, almost all peak air concentrations of beryllium were less than the acceptable figure. In 1971, radiographic changes indicative of interstitial disease were found in 31 workers, hypoxaemia was found in 20, and in 11 workers a combination of hypoxaemia and radiographic indications of interstitial disease were reported. Smoking habits of the workers were taken into account. The follow-up study in 1974 revealed an improvement in hypoxaemia and a decrease in alveolar–arterial oxygen tension difference at rest in the 13 men still available out of the 20 who had hypoxaemia in 1971. In addition, the radiographic abnormalities related to interstitial disease reported in 1971 were reversed in some workers.

Although the underlying factors determining the type of physiological impairment in chronic beryllium disease remain unknown, reversible inflammatory reactions with little or no fibrosis may have caused the interstitial abnormalities, a possible early sign of the chronic disease. Further exposure might have led to the emergence of workers with obstructive and restrictive defects as well. The authors conclude that the approach of combining an examination of all exposed workers with industrial hygiene evaluations of the workplace was effective both in detecting early disease and in documenting its subsequent improvement following a reduction in the atmospheric concentrations of beryllium in the plant.

Asbestos in the gut

Storeygard, A. R. & Brown, A. L., Jr. (1977). Penetration of the small intestinal mucosa by asbestos fibers. *Mayo Clin. Proc.* **52**, 809.

Rosen, P. P. (1978). Asbestos fibers in animal tissue. *Mayo Clin. Proc.* **53**, 131.

Jacobs, R., Dodgson, K. S. & Richards, R. J. (1977). A preliminary study of biochemical changes in the rat small intestine following long-term ingestion of chrysotile asbestos. *Br. J. exp. Path.* **58**, 541.

An increased frequency of gastro-intestinal tumours among asbestos workers has previously been indicated (*Cited in F.C.T.* 1972, **10**, 575). In addition, asbestos, as a constituent of talc, has been a suspect in the aetiology of stomach cancer in Japan, where it may be eaten in rice (*ibid* 1972, **10**, 123). Doubts have been expressed about this association, however, and some previously reported investigations (*Cited in F.C.T.* 1976, **14**, 361; Harley *et al. Lab. Invest.* 1974, **30**, 375) produced no evidence of penetration or transmigration of the gut wall by asbestos fibres.

The first paper cited above describes experiments to investigate the penetration by finely ground amosite asbestos into an isolated section of rat jejunum *in vivo*. The concentration of the fibres, which ranged in length from 0·5 to 25 μm and in diameter from 0·12 to 1·8 μm, was in the region of $9{\cdot}4 \times 10^9$ fibres/ml of saline. This saline suspension was left in the jejunum for 1 hr, after which the rats were killed. Scanning electron microscopy of the mucosa showed that in each 4 × 9 mm segment of jejunum from three of the five rats, between one and six fibres had penetrated the epithelium, mostly at an angle between 45 and 90°. Fibres did not penetrate the intercellular junctions but they were occasionally seen within the interstices of the lamina propria. The fibres seen in the epithelial cells or lamina propria were 0·4–1·4 μm in diameter, and the length of the visible portion ranged from 5 to 30 μm.

In a letter criticizing the lack of information concerning the fate of asbestos fibres after their penetration of the epithelial layer of the gut, Rosen (cited above) suggested that the possible formation of ferruginous bodies or asbestos bodies resembling those isolated from lung tissue should be investigated. The importance of the long-term fate of the asbestos fibres and the potential carcinogenic effects in the intestine were emphasized, although existing evidence suggests that appreciable numbers of ferruginous bodies do not form as a result of intestinal penetration by asbestos.

The cited paper by Jacobs *et al.* describes the effects in rats of ingesting chrysotile asbestos, or asbestos pretreated with cigarette smoke, in diets based on commercially-available rat pellets and exposed in some cases to cigarette smoke during preparation. After administration of these diets for 10 months, mucosal cells and lumen washings from the small intestine were examined. Exposure to asbestos, with or without tobacco smoke, significantly lowered the RNA level and raised the DNA level in the lumen but not in the mucosal cell layer. Total mucosal-cell protein was consistently but not significantly higher in animals that had ingested low levels (0·5 mg daily) of asbestos, while those fed high levels (50 mg daily) of smoke-pretreated asbestos showed a significantly lower protein concentration in the lumen than did controls. Cigarette smoke alone in the diet reduced the amount of RNA in mucosal cells and reduced the RNA/DNA ratio, even compared with the combined effect of asbestos and smoke. Cigarette smoke alone increased adenosine triphosphatase (ATPase) activity in the lumen and in mucosal cells without affecting other brush-border and hydrolytic enzymes. Low concentrations of asbestos alone increased β-glucuronidase, sucrase and alkaline-phosphatase activi-

ties in the lumen and β-glucuronidase activity in mucosal cells. High concentrations of asbestos increased β-*N*-acetylglucosamidase, sucrase, ATPase and *p*-nitrophenyl acetate hydrolase in the lumen. The alterations in enzyme activity when tobacco smoke and asbestos were fed together did not differ notably from those produced by asbestos alone.

The authors comment that the higher level of DNA in the lumen of rats that had ingested asbestos suggests that the sloughing-off of mucosal lining cells into the lumen may be accelerated by mineral-induced cell damage. This possibility is consistent with the finding of Storeygard & Brown that fibres do penetrate epithelial cells. Alternatively asbestos in the gut lumen or in the layer of mucus on the brush-border may preferentially bind DNA and thus delay its progress through or degradation in the lumen. But neither of these possibilities explains why the protein level in the lumen is unaffected by ingestion of asbestos alone or why RNA in the lumen is lower in rats fed any diets containing asbestos.

n-Hexane gets the boot?

Buiatti, E., Cecchini, S., Ronchi, O., Dolara, P. & Bulgarelli, G. (1978). Relationship between clinical and electromyographic findings and exposure to solvents, in shoe and leather workers. *Br. J. ind. Med.* **35**, 168.

The neurotoxicity of low-boiling solvents has been evident over a number of years in workers in various industries. Episodes of polyneuropathy, for example, have been reported in Italian shoemakers exposed to a mixture of solvents and in workers exposed to *n*-hexane in a printing room (*Cited in F.C.T.* 1977, **15**, 258; *ibid* 1977, **15**, 492).

This latest paper again reports the prevalence of polyneuropathy in shoe and leather workers in Italy. Workers from 31 factories in the area around Florence were assessed for objective signs of peripheral nervous involvement (muscle tone, tendon reflexes, muscle wasting and sensory disorders) and subjective symptoms were recorded. Electromyographical recordings were used to measure maximum motor conduction velocity (MCV) and other parameters of polyneuropathy. Workers with a history of exposure to other neurotoxic compounds were excluded, and laboratory investigations were carried out to exclude those with other diseases associated with neurotoxic signs.

The results showed that there was no statistically significant difference in the occurrence of polyneuropathy between the sexes. However, the incidence was significantly higher in the older workers, aged 40–70 yr, and was found to be progressively greater with more prolonged exposure. The degree of exposure was an important factor in the occurrence of polyneuropathy. Thus a significantly higher incidence was found in workers who used more than 1·3 kg glue/day, and in factories in which the air volume was less than 40 m^3/worker.

The MCV is known to be linearly related to age. In this study exposure to the glue solvents was found to accentuate this physiological age-related fall; there was a significant decrease in MCV not only in polyneuropathic workers compared to the 'normal' workers but also between this latter group and the general population. This indicated that it was possible to detect the neurotoxic action of the solvents in the 'normal' worker population, although the authors point out that the MCV alone is not a reliable criterion for the diagnosis of polyneuropathy.

Comparison of the MCV in a group of workers of the same age-group demonstrated a clear relationship between duration of solvent exposure and the MCV; moreover, polyneuropathic workers showed a greater susceptibility to this action of solvents with increased duration of exposure. The correlation between the occurrence of subjective symptoms and polyneuropathy was also studied. Only four symptoms were significantly associated with polyneuropathy; these were muscle spasms, leg weakness, leg pain and arm paraesthesia.

Owing to the very variable composition of the glues, it was not possible to correlate the concentration of any individual solvent with the occurrence of polyneuropathy. The contributions of individual solvents to the mixtures varied within the following ranges: *n*-hexane 40–99·5%, *n*-heptane 7–10%, other aliphatic, branched or linear, low-boiling hydrocarbons 7–54%, ethyl acetate 15–44% and trichloroethylene 0·42–45·3%. However, it was possible to exclude the highly neurotoxic agent tri-*o*-cresyl phosphate (TOCP), as this was not found in any of the glues.

In presenting this additional evidence of the neurotoxicity of solvents used in the Florentine leather and shoe industry, the authors stress the need either for improvements in factory ventilation and hygiene or for changes in the composition of the glues used.

UV Ink spots

Nethercott, J. R. (1978). Skin problems associated with multifunctional acrylic monomers in ultraviolet curing inks. *Br. J. Derm.* **98**, 541.

Acrylic monomers have long been recognized as potential skin sensitizers, and with the increasing use of acrylic compounds in industry a greater incidence of skin problems is being reported. The printing industry is no exception, and contact with ultraviolet-curable printing inks (UV inks) containing polyfunctional acrylates has resulted in dermatitic allergies (*Cited in F.C.T.* 1978, **16**, 297).

The above-cited paper provides both clinical and experimental evidence for induction of contact dermatitis by polyfunctional acrylic monomers in ultraviolet curing inks.

Clinical observations on 19 individuals exposed to UV inks and their components are detailed. All except one individual worked in one ink-manufacturing plant. Eleven patients had eye or skin problems but did not demonstrate positive patch-test reactions. They were believed to have had an irritant contact dermatitis (eight cases) or irritant contact conjunctivitis (three cases). Two with dermatitis and one with conjunctivitis had these problems when they had no direct contact with UV ink but were working near mills in which it was being ground, suggesting contact with airborne material. A twelfth patient, with chronic urticaria, was eliminated from the study as his condi-

tion was apparently unrelated to the occupational exposure. Of the seven who gave a positive patch test with acrylic monomers, all reacted to pentaerythritol triacrylate (PETA) and two were also sensitive to trimethylolpropane triacrylate (TMPTA), whilst none reacted to hexanediol diacrylate or methyl methacrylate (MMA).

In a sensitization study, guinea-pigs were exposed to PETA by intradermal injection. After 1 wk, 25% PETA in petrolatum was applied to the skin for 48 hr, and after 2 wk the animals were challenged with 10% PETA in petrolatum for 24 hr. Thirteen of fifteen animals (87%) reacted positively to the monomer. Intradermal irritancy tests performed on twenty control animals revealed that PETA at concentrations greater than 10% produced inflammation in a majority of the animals.

Human sensitization studies on PETA, again in petrolatum, involved an initial application of 10% PETA to the arm. In the subsequent challenge patch tests, seven of the nine subjects reacted. None of these seven sensitized patients reacted to MMA.

The original patients returned to work in the ink plant after use of the polyfunctional acrylates had been discontinued, and in more than a year they have had no further skin problems. These observations appear to demonstrate the sensitization potential of PETA and TMPTA, although previous animal studies have given negative results for the former (Buehler, *Archs Derm.* 1965, **91**, 171; Magnusson & Kligman, *Allergic Contact Dermatitis in the Guinea-pig*; Charles C. Thomas, Springfield, IL, 1970).

Another mutagenic flame retardant

Gold, M. D., Blum, A. & Ames, B. N. (1978). Another flame retardant, tris-(1,3-dichloro-2-propyl)phosphate, and its expected metabolites are mutagens. *Science, N.Y.* **200**, 785.

The flame retardant tris-(2,3-dibromopropyl) phosphate (TDBP), commonly known as 'Tris', has been banned from use in children's clothing in the United States, on the grounds that it is carcinogenic, mutagenic and readily absorbed through the skin (*Federal Register* 1977, **42**, 18850). In an Ames test TDBP was mutagenic to *Salmonella typhimurium* strains TA100 and TA1535, particularly when liver microsomes from rats pretreated with a polychlorinated biphenyl (PCB) were used for metabolic activation (Prival *et al. Science, N.Y.* 1977, **195**, 76). The closely-related flame retardant tris-(1,3-dichloro-2-propyl) phosphate (Fyrol FR2), which is being used as a substitute for TDBP, has now been subjected to similar testing.

In the presence of PCB-induced rat-liver homogenate, Fyrol FR2 gave little evidence of mutagenicity to *S. typhimurium* strain TA100, although a weak dose-related response was sometimes observed. However, with phenobarbital-induced mouse-liver homogenate there was a much more consistent mutagenic response, and some positive results were also obtained in tests with liver homogenates from PCB-pretreated mice and hamsters and from phenobarbital-pretreated rats. The expected enzymatic hydrolysis product, 1,3-dichloro-2-propanol, was a somewhat stronger mutagen in the presence of phenobarbital-induced mouse-liver homogenate, while the predicted product of oxidative dealkylation, 1,3-dichloro-2-propanone, was a potent mutagen even in the absence of metabolic activation.

Studies elsewhere also showed Fyrol FR2 to be mutagenic in a sister chromatid exchange test, although its activity was weak in comparison with that of TDBP. As National Cancer Institute feeding tests on Fyrol FR2 are still a long way from completion, the authors recommend that this flame retardant should be replaced now with safer alternatives, such as inherently fire-resistant fabrics.

NATURAL PRODUCTS

Terata and tumours from potato sprouts

Keeler, R. F., Young, S., Brown, D., Stallknecht, G. F. & Douglas, D. (1978). Congenital deformities produced in hamsters by potato sprouts. *Teratology* **17**, 327.

Ivankovic, S. (1978). Gastric cancer in rats after chronic intraperitoneal application of sap of green parts of potatoes (*Solanum tuberosum* L.). *Experientia* **34**, 645.

The hypothesis that blighted potatoes may be responsible for anencephaly and spina bifida in man has now been refuted (*Cited in F.C.T.* 1974, **12**, 772). This hypothesis, based originally on epidemiological indications, was strengthened by the finding of defects in the cranial bones of four of 11 foetal marmosets, the mothers of which had been fed blighted potato concentrate also containing the potato sprouts (*Cited in F.C.T.* 1973, **11**, 313). Induction of anencephaly, spina bifida and related defects by sprouts from 'Kennebec' potatoes was subsequently demonstrated in hamsters, and the solanum alkaloid solasodine produced similar malformations (*ibid* 1977, **15**, 260). A further study of the teratogenicity of potato sprouts, peel and tubers has now been undertaken by Keeler *et al.* (cited above).

Simonsen hamsters were gavaged, during the primitive streak/neural plate stage of gestation, with dried sprout material from potatoes of the 'Kennebec', 'Russet', 'Pioneer', 'Targhee', 'Sebago', 'Nampa' and 'Norchip' varieties, at dose levels in the range 2·7–6·3 g/kg. These levels were highly toxic, producing an 11·5–48·5% incidence of maternal deaths, and there was a significant increase in litters that included malformed offspring. The incidence of such litters was only 1·34% in controls (given water by gavage), but ranged from 8·3% with 'Pioneer' to over 25% with 'Russet' and 'Kennebec' sprouts. The last two varieties greatly increased the average number of resorptions per litter and the percentage of totally resorbed litters.

and 'Sebago' and 'Nampa' sprouts were also effective in the latter respect. The malformations consisted largely of cranial blebs and exencephaly after dosing on day 8 of gestation, with some microphthalmia and spina bifida after dosing on day 7. The alkaloid dose in the sprouts was in the range 0·09–0·35 g/kg, but teratogenicity did not vary directly with alkaloid content.

In contrast to the sprouts, dried 'Kennebec' peel and tubers (unsprouted or sprouted) were not teratogenic or embryo-lethal when given in doses as high as 10–20 g/kg, although they caused some maternal mortality. Preliminary trials with the peel and tubers of 'Russet', 'Nampa' and 'Sebago' potatoes also gave negative results. Solasodine (1·2–1·6 g/kg) was as potent as 'Kennebec' and 'Russet' sprouts in its teratogenic and embryo-lethal effects, and produced similar deformities. The veratrum alkaloid jervine (80–170 mg/kg) was an even stronger teratogen but produced mainly cebocephaly and harelip, suggesting that the teratogen in potato sprouts may be more closely related in structure to solasodine. Both alkaloids were also teratogenic in Engle hamsters, but 'Kennebec' and 'Russet' sprouts were not.

The second paper cited above briefly reports the finding of four carcinomas of the stomach in 12 rats given weekly ip injections of 0·25 ml of the sap of green potato tops. After a median induction time of 850 days, two had developed adenocarcinomas of the glandular stomach that had metastasised to the liver, and two others died of papillomas and squamous-cell carcinomas of the forestomach. The remaining rats died of pneumonia after about 850 days. Analysis of the sap revealed several volatile *N*-nitroso compounds, but it is not yet clear whether these were the responsible agents. Injection studies on the sap of potato tubers will be reported separately, as will recently initiated feeding studies on the sap of potato tops.

[This raises the interesting possibility that nitroso compounds may play a role in the teratogenicity of potato sprouts. *N*-Methyl and *N*-ethyl-*N*-nitrosoureas, for instance, have been shown to be potent teratogens in the rat and hamster respectively (*Cited in F.C.T.* 1971, **9**, 926), and the former compound produces stomach tumours in the rat and guinea-pig (*ibid* 1971, **9**, 720).]

A haemolytic factor in tea

Benjamin, L. J., Goldstein, B. D., Distenfeld, A. & Troll, W. (1977). Production of paroxysmal nocturnal hemoglobinuria-like red blood cells by tea. *Am. J. Hemat.* **2**, 245.

Tea-drinking has been held responsible for a variety of effects in recent decades, but 'teas' made not from leaves of the tea plant but from similar infusions of the dried leaves of other plants have been more commonly implicated. The capacity of tea to alter red-cell membranes in such a way as to produce haemolysis resembling that of paroxysmal nocturnal haemoglobinuria (PNH) is another phenomenon to add to the list.

A young man developed acute intravascular haemolysis after drinking, over a 2-wk period, large quantities of a herbal tea made from hops. The ways in which red blood cells isolated from this patient and from normal healthy individuals reacted towards the herbal tea and to standard tea were therefore investigated *in vitro*. Red cells were incubated for 2 and 16 hr with saline-based brews of the preparations, and were then subjected to acid- and sucrose-haemolysis tests in the presence of complement.

Both hop tea and standard commercial tea infusions prepared either from the leaf or from 'instant' granules produced a complement-dependent haemolysis of red cells derived from the patient and of cells from the controls. This occurred whether the pH was kept at 4–5 or whether the incubating mixture was buffered at physiological pH. Antisera to C3 and to C3 activator, but not antisera to C4, inhibited the haemolysis, suggesting that incubation with tea may alter the red-cell membrane in a way that specifically potentiates the lytic effect of the alternate complement pathway rather than the classic pathway. Leupeptin, a protease inhibitor which prevents complement-dependent haemolysis of red cells from PNH patients, prevented the haemolysis of normal red cells incubated with tea infusions.

The mechanism underlying these effects remains obscure and the clinical implications of the observations are unknown, but it seems that infusions of both herbal tea-substitutes and tea itself are capable of producing an *in vitro* effect resembling that of PNH.

The tea constituent responsible for the observed effect has not been identified; no haemolysis followed incubation of cells with caffeine, theophylline or tannic acid, but there could remain some 40 other possibilities. Meanwhile the young man who prompted this investigation has given up drinking tea and has had no further episode of intravascular haemolysis.

[More support for John Selden (1584–1654): "Tis not the eating, nor 'tis not the drinking that is to be blamed, but the excess".]

Effects of theophylline on the mouse foetus

Tucci, S. M. & Skalko, R. G. (1978). The teratogenic effects of theophylline in mice. *Toxicology Lett.* **1**, 337.

Theophylline is a methylated xanthine. Like caffeine and theobromine, which also belong to this group of compounds, it is known to occur in beverages such as coffee, tea, cocoa and cola drinks. In addition, its clinical use is extensive. Compounds of this type increase the activity of liver processing enzymes in rats (*Cited in F.C.T.* 1976, **14**, 513) and theophylline itself has been shown to induce breaks in the chromosomes of HeLa cells (Ostertag, *Mutation Res.* 1966, **3**, 249) and of human lymphocytes in culture (Weinstein *et al. ibid* 1975, **31**, 57).

The study cited above on the teratogenic action of theophylline in mice extended earlier work by Fujii & Nishimura (*Okajimas Folia anat. jap.* 1969, **46**, 167). Female mice were given a single ip injection of 100, 150 or 200 mg theophylline/kg on day 10, 11, 12 or 13 of gestation. Females from all groups were killed on day 17 and viable foetuses were removed and examined for external malformations and subsequently lor any evidence of cleft palate.

Mice given the highest dose of theophylline showed an increased number of resorptions. Although there were no malformations in the litters of the untreated control mice, there was a dose-related incidence of structural anomalies in the treated groups. The predominant malformation was cleft palate but a variety of limb deformities (polydactyly, ectrodactyly, syndactyly and micromelia) also occurred, predominantly (83%) on the left side. The latter observation and the presence of many haematomas in surviving foetuses supported the earlier findings (Fujii & Nishimura, *loc. cit.*). Both the limb anomalies and cleft palate showed a peak incidence after treatment on day 11, with cleft palate affecting 63% of survivors in the group given 200 mg theophylline/kg on that day.

The mechanism underlying these effects has not been established, but the study showed theophylline to be a potent embryotoxin in the mouse. On this basis, the authors recommend against the indiscriminate use of the material, especially during susceptible periods of human pregnancy.

Pre-neoplastic changes in the liver

Neal, G. E. & Butler, W. H. (1978). A comparison of the changes induced in rat liver by feeding low levels of aflatoxin B_1 or an azo dye. *Br. J. Cancer* **37**, 55.

The induction of cancer by chemical carcinogens is probably due to the covalent binding of the carcinogen, or one of its metabolities, to the DNA present in cells of the target tissue. A study reported last year on the interplay of cytotoxicity and carcinogenicity showed that cells cultured from the livers of rats that had survived a 6-wk period of treatment with aflatoxin B_1-contaminated diet had developed a resistance to the acute, toxic action of aflatoxin B_1 (*Cited in F.C.T.* 1978, **16**, 403).

A later study has examined further the changes provoked in the liver during the initial sub-carcinogenic feeding period. Rats were fed for 1–6 wk with low dietary levels of aflatoxin B_1 (4 ppm) or 2-methyl-4-dimethyl-aminoazobenzene (2-Me-DAB; 0·04%). These diets caused weight loss but no deaths, and animals returned to a standard diet gained weight and showed an improvement in general condition. Small groups of animals were killed at the end of each week and the livers were examined histologically and subjected to zonal rotor centrifugation to isolate and fractionate the nuclei.

In confirmation of earlier reports, it was found that while the feeding of such diets for 3 wk was sub-carcinogenic, the 6-wk treatment with either toxin resulted in a high incidence of hepatic carcinoma in rats maintained on a normal diet for 9 months after the termination of treatment. In the histological and nuclear-fractionation studies, little change was seen after 2 wk on either toxin but, after 3 wk, tissue necrosis, bile-duct proliferation and the loss of tetraploid hepatocyte nuclei were observed, followed by a compensating proliferation of diploid hepatocytes. Changes seen in the colour of the nuclear pellet after 2-Me-DAB had been fed for 3–4 wk suggested that necrosis occurred in susceptible hepatocytes whose nuclei contained bound dye. These susceptible hepatocytes were subsequently replaced by a population whose nuclei exhibited little dye-binding.

Thus it appears that the feeding of either carcinogen induces the production of a population of hepatocytes resistant to the cytotoxicity of the inducing agent. Moreover, studies in which ingestion of one diet for 3 wk was followed by 3 wk on the other, showed that induction of resistance to the cytotoxicity of one carcinogen permits the development of hyperplastic growth in the presence of the other, an observation that could be of general relevance to the question of chemical hepatocarcinogenesis.

METHODS FOR ASSESSING TOXICITY

Trout eggs for carcinogen testing

Wales, J. H., Sinnhuber, R. O., Hendricks, J. D., Nixon, J. E. & Eisele, T. A. (1978). Aflatoxin B_1 induction of hepatocellular carcinoma in the embryos of rainbow trout (*Salmo gairdneri*). *J. natn. Cancer Inst.* **60**, 1133.

Aflatoxin B_1 (AFB_1), a toxic metabolite of the mould *Aspergillus flavus*, is one of the most potent chemical carcinogens known and has been shown to induce a 50% incidence of liver cancer when fed to rats at a dietary level of 0·1 ppm (Butler & Barnes, *Fd Cosmet. Toxicol.* 1968, **6**, 135). Similar effects have also been recorded in trout (Ayres *et al. J. natn. Cancer Inst.* 1971, **46**, 561). Although tumours have been induced in aquarium fish by the addition of carcinogens to the water (Stanton, *ibid* 1965, **34**, 117), no attempts to induce neoplasia in fish by treatment of the eggs with carcinogens appear to have been reported. The study cited above looks into the efficacy of this type of induction technique as a test system for carcinogens.

In the brood-stock Shasta rainbow trout used as the source of eggs, each female produces about 2000 eggs. To examine tumour incidence as a result of exposure to AFB_1, 14-day embryos were exposed to 0·5 ppm AFB_1 for 1 or 2 hr. After exposure, the eggs were allowed to hatch and the fish were maintained for a period of 1 yr, after which 100 were killed from each group for gross and histological examination of the liver. The most sensitive day of exposure was studied by exposing groups of eggs to 0·5 ppm AFB_1 for 1 hr between day 1 and day 23 after fertilization. In order to quantify the AFB_1 absorbed by the eggs, 14-day steelhead-trout eggs and 21-day rainbow-trout eggs were treated for 1 hr with 100 ml water containing 0·5 ppm [^{14}C]AFB_1. The amount of radioactivity remaining in the eggs as a function of time was determined from the time of treatment until after the eggs hatched.

In the tumour-incidence studies, the 2-hr treatment

proved lethal for many embryos, whereas the 1-hr treatment, while relatively non-toxic, was carcinogenic, producing a 42% incidence of trout with tumours. The incidence was low in fish from eggs treated before day 14, the time when the embryonic liver forms, but it increased steadily thereafter, reaching nearly 60% following exposure on day 23. However, the time of exposure had no significant effect on the numbers of tumours in individual livers. In view of the rapid growth of the liver at the time of exposure the number of tumours per liver was unexpectedly low, as was the fact that no evidence of neoplasia was detected until the fish were about 4 months old, a latent period similar to that in rainbow trout fed AFB_1 in the diet.

[^{14}C]AFB_1 was readily absorbed by the eggs but it was not conclusively established whether the finding that rainbow-trout eggs treated on day 21 absorbed more than twice as much [^{14}C]AFB_1 as steelhead eggs exposed on day 14 was due to the difference in strain or in the timing of treatment. Most of the ^{14}C remaining after 30 min was in the embryo and yolk sac bound to cellular constituents, but after 24 hr 85–90% of the absorbed radioactivity had been excreted or leached from the egg. Most of the rest remained in the egg until hatching. The quantity of AFB_1 initially absorbed (30 ng/egg) was 67 times less than the amount (2 μg/fish) ingested by fish exposed to 4 ppb for 1 yr in a typical feeding study. The trout embryo thus appears extremely sensitive to minute quantities of AFB_1 and the authors consider that this exposure method has potential as a test system for determining the carcinogenicity of minute quantities of suspect compounds.

Fd Cosmet. Toxicol. Vol. 17, pp. 319–320
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LETTER TO THE EDITOR

LYSINOALANINE UTILIZATION BY *ERWINIA CHRYSANTHEMI* AND *ESCHERICHIA COLI*

Sir,—Lysinoalanine is currently of interest as the agent responsible for the *pars recta* nephrocytomegaly caused by alkali-treated soya protein (Feron *et al.* in *Biochemical Aspects of New Protein Food*, Edited by J. Alder-Nissen *et al.*; Pergamon Press, New York, 1978; Gould & MacGregor, in *Protein Crosslinking, Nutritional and Medical Consequences*, Edited by M. Friedman; Plenum Press, New York, 1977, p. 29; Woodard & Short, *J. Nutr.* 1973, **103**, 569). The marked species differences in sensitivity to lysinoalanine-induced nephrocytomegaly (de Groot *et al. J. Nutr.* 1976, **106**, 1527) suggest that metabolism may be an important factor in nephrotoxicity. Finot *et al.* (In *Protein Crosslinking, Nutritional and Medical Consequences*, p. 51) have suggested that lysinoalanine is metabolized to CO_2 by gut bacteria, but no direct evidence was obtained. Since very little is known of the breakdown of lysinoalanine by living organisms and of its potential to be used in metabolism or incorporated into macromolecules, we have investigated whether lysinoalanine could be utilized by bacteria and whether it is extensively incorporated into the protein of an organism capable of using it.

We used both wild-type *Erwinia chrysanthemi* and a lysine-requiring mutant, *Erwinia chrysanthemi* 119 isolated by one of us (NJP). Overnight cultures were grown in complete Hunter's minimal medium (Cohen-Bazire *et al. J. cell. comp. Physiol.* 1957, **49**, 25). Complete medium contained, per 100 ml; 2·5 ml 0·1 N-NaH_2PO_4; 1 ml Hunter's mineral base; 0·5 ml 20% glucose; 1 ml 10% $(NH_4)_2SO_4$. Cells were washed twice in 10 ml 0·07 M-phosphate buffer, pH 7·0, and were resuspended at approximately 4×10^8 cells/ml. The cell suspension was streaked radially either on agar plates containing complete Hunter's minimal medium (HC^+N^+), or on the same medium lacking either carbon (HC^-N^+) or nitrogen (HC^+N^-). Filter-sterilized 10% lysinoalanine or 10% lysine solution or sterile water (50 µl/plate in each case) were added to filter-paper discs at the centre of the plates. The plates were incubated at 30°C and observed for growth from day 1 to day 5. The table below

		Growth	
Medium	Additions	Lysine-requiring	Wild type
HC^+N^+	None	−	+ + + + + + +
	Lysine	+ + +	+ + + + + + +
	Lysinoalanine	+ +	+ + + + + + +
HC^-N^+	None	−	±
	Lysine	+ +	+ +
	Lysinoalanine	+ +	+ +
HC^+N^-	None	−	±
	Lysine	+ +	+ +
	Lysinoalanine	+ + +	+ + +

summarizes the growth observed after incubation for 2 days, the number of + symbols representing the relative density of growth in the streaks on a subjective scale. Lysinoalanine was as good a carbon or nitrogen source for both strains as was lysine. In the lysine-requiring strain, lysinoalanine was an effective substitute for lysine.

Similar experiments were performed with *Escherichia coli* strain no. 3292, which requires lysine, histidine and tryptophan. Whereas there was no growth on minimal medium supplemented with histidine, tryptophan and glucose (M9 medium; J. H. Miller, *Experiments in Mol. Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 1972), typical growth was observed when either lysine or lysinoalanine was placed at one end of a streak as a 10% solution in water or as a solid. Lysinoalanine could, therefore, satisfy the lysine requirement of this organism. When glucose was omitted, lysinoalanine no longer supported growth, indicating that lysinoalanine was not used as a carbon source. Likewise, neither lysine nor alanine supported growth in the absence of glucose.

To investigate the possibility of lysinoalanine incorporation into the bacterial protein, the lysine-requiring mutant of *E. chrysanthemi* was grown in liquid Hunter's medium plus 0·1% lysinoalanine or lysine. After reaching the stationary phase, cells were harvested and washed four times in phosphate buffer. The resulting pellet was freeze-dried and analysed for lysinoalanine both before and

after acid hydrolysis, as described elsewhere (Karayiannis *et al. Fd Cosmet. Toxicol.* 1979, **17,** in press). Analysis of unhydrolysed cells grown on lysinoalanine served as a control for the possible carryover of lysinoalanine from the growth medium. The amino acid profiles of the cells grown on lysine and lysinoalanine were compared. Lysinoalanine was not incorporated as such into the microbial protein to any appreciable extent. Thus, the cells break down the lysinoalanine and use it as a source of lysine. A small quantity of lysinoalanine (4 μmol/g dry cells) was found, however, in the acid hydrolysed cells grown on lysinoalanine, whereas no lysinoalanine was found in the unhydrolysed sample. It was not clear whether there was a very limited incorporation of lysinoalanine into protein, or whether the amount found was simply tightly bound to some cell constituent.

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Fd Cosmet Toxicol Vol 17, p 321
Pergamon Press Ltd 1979 Printed in Great Britain

MEETING ANNOUNCEMENT

TOXICITY—MECHANISMS AND EVALUATION

The Second International Congress on Toxicology, to be held in Belgium on 7–11 July 1980, will be concerned with "Mechanisms of Toxicity and Hazard Evaluation". The preliminary announcement about this sequel to the very successful congress held in Toronto in 1977, names the Brussels Congress Center as the venue and outlines the preliminary timetable, which will include symposia on mechanisms of neurotoxicity (Co-Chairmen: B. Holmstedt and P. S. Spencer), short-term tests for predicting long-term effects (Chairman: L. Golberg), early changes in chemical carcinogenesis (Chairman: W. N. Aldridge), long-term exposure to occupational intoxicants (Chairman: M. Ikeda), clinical toxicology (Chairman: L. T. Prescott) and legislative, scientific and socio-economic considerations underlying the toxicological testing of new chemicals (Chairman: S. L. Friess).

The language of the Congress will be English. Requests for further information and for participation forms should be addressed to The Secretariat, SdR Associated, 16 Avenue des Abeilles, B-1050 Brussels, Belgium.

CANCER RESEARCH

The 5th Meeting of the European Association for Cancer Research will be held in Vienna on 9–12 September 1979. Two symposia will be held in parallel throughout the three working days; one will be concerned with the cell surface and the other with the cell nucleus in relation to cancer. The President of the Meeting will be Prof. Dr. H. Wrba, and the Congress Centre will be in Oberlaa, a southern district of Vienna.

Further information may be obtained from Dr. K. Letnansky, Organizing Secretary, 5th Meeting of the EACR, c/o Institute for Cancer Research, Borschkegasse 8a, A-1090 Vienna.

BIOTRANSFORMATION OF FOREIGN COMPOUNDS

Charles University, Prague, will be the centre for an international conference on "Industrial and Environmental Xenobiotics: Biotransformation and Kinetics" on 27–31 May 1980. The conference is being organized jointly by the Permanent Commission and International Association on Occupational Health, the Czechoslovak Medical Society J. Ev. Purkyne Medical Faculty of Hygiene, and the Institute of Hygiene and Epidemiology.

Among the main topics for discussion will be rate-determining factors in xenobiotic metabolism, distribution and excretion (particularly in relation to organic solvents, metals and plastics monomers), interactions among xenobiotics and the role of metabolic activation in tissue toxicity and its consequences for exposure tests.

Requests for further information about the conference or about the presentation of papers should be addressed to Dr. Ivan Gut, Conference Secretary, Czechoslovak Medical Society, Vitezného února 31, 120 26 Praha 2, Czechoslovakia.

Fd Cosmet. Toxicol. Vol. 17, p. 323
Pergamon Press Ltd 1979. Printed in Great Britain

FORTHCOMING PAPERS

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

Determination of benzo[*a*]pyrene in smoked, cooked and toasted food products. By C. Lintas, M. C. De Matthaeis and F. Merli.

Mutagenicity of soy bean sauce. By J. Y. Lin, H.-I. Wang and Y.-C. Yeh.

Studies on the interaction of methomyl and ethanol in rats. By M. Antal, M. Bedó, G. Constantinovits, K. Nagy and J. Szépvölgyi.

Further studies on the carcinogenicity of a food additive, AF-2, in hamsters. By M. Kinebuchi, T. Kawachi, N. Matsukura and T. Sugimura.

Teratology studies in mice exposed to municipal drinking-water concentrates during organogenesis. By R. Kavlock, N. Chernoff, B. Carver and F. Kopfler.

Contamination from skin-painting test chemicals. By E. B. Sansone and A. M. Losikoff.

Toxicity of novel sesquiterpenoids from the stressed sweet potato (*Ipomoea batatas*). By B. J. Wilson and L. T. Burka. (Short paper)

Possible use of dietary surveys to assess intake of food additives. By M. M. Disselduff, G. P. Try and W. T. C. Berry. (Short review)

Fd Cosmet. Toxicol. Vol. 17, p. 324
Pergamon Press Ltd 1979. Printed in Great Britain

CORRIGENDA

Volume 17 (1979)

p. 107, Table 1 and p. 108, Table 3: NDELA formed should be expressed in μg not μg/litre.

[*Contents continued*]

Aims and Scope

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

Instructions to Authors

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

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

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

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

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

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

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

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

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

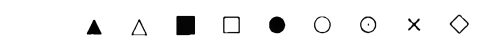
e.g. (McLaughlin, Bidstrup & Konstam, 1963); (McLaughlin *et al.* 1963).

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

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

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

Illustrations and Diagrams. These should be kept to a *minimum* and they should be numbered and marked on the back with the author's name. Legends accompanying illustrations should be typewritten on a *separate* sheet. Diagrams and graphs must be drawn in Indian ink on good quality paper or tracing linen. The following standard symbols should be used on line drawings since they are easily available to the printer:



Photographs and photomicrographs should be submitted unmounted and on glossy paper. When colour plates are to be printed, payment for blockmaking is the responsibility of the author.

Tables and Figures. These should be constructed so as to be intelligible without reference to the text, each table and column being provided with a heading. The same information should not be reproduced in both tables and figures.

Chemical Nomenclature. The fundamental principles of organic and inorganic chemical nomenclature are laid down in the I.U.P.A.C. 1957 Rules (Butterworths Scientific Publications, London, 1958, 1959). These are given in *Handbook for Chemical Society Authors* (1961), pp. 16–163.

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

Page Proofs. These will be sent to the first-named author for correction.

Reprints. Reprints can be ordered on the form accompanying proofs.

Frequency. The Journal will be published bi-monthly.

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