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Research Section

REDUCTION OF SULPHONATED WATER-SOLUBLE AZO DYES BY MICRO-ORGANISMS FROM HUMAN FAECES

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(*Received 29 November 1979*)

Abstract—The anaerobic reduction of four currently used sulphonated azo dyes, amaranth, sunset yellow, new coccine and tartrazine, has been studied by incubating bacterial suspensions isolated from human faeces and from the intestinal contents of rats with 50 μM concentrations of the dyes in phosphate buffer, pH 7·4, using nitrogen as the gaseous phase. Human faecal flora from five male volunteers reduced the four dyes at mean rates of 38·4, 25·1, 18·2 and 6·5 nmol/mg protein/hr, respectively. For each dye, there was little difference in the reduction rates effected by the bacterial suspensions from the different individuals in spite of a considerable divergence in age, daily diets and living circumstances. These reductions proceeded at an approximately constant rate until the colours had faded almost completely. The rates of amaranth and sunset yellow reduction by rat-gut flora were four to five times higher than those by human faecal flora, but there was no significant difference between the two types of bacterial suspension in the reduction rates for new coccine and tartrazine.

INTRODUCTION

Water-soluble sulphonated azo dyes are used extensively as colourings for foods, drugs, and cosmetics. However, only four such dyes are currently used in Japan as food colourings. These are amaranth (trisodium salt of 1-(4-sulpho-1-naphthylazo)-2-naphthol-3,6-disulphonic acid; US FD & C Red No. 2), sunset yellow (disodium salt of 1-(4-sulphophenylazo)-2-naphthol-6-sulphonic acid; FD & C Yellow No. 6), new coccine (trisodium salt of 1-(4-sulpho-1-naphthylazo)-2-naphthol-6,8-disulphonic acid; Ponceau 4R) and tartrazine (trisodium salt of 3-carboxy-5-hydroxy-1-*p*-sulphophenyl-4-*p*-sulphophenylazopyrazole; FD & C Yellow No. 5). Several other azo dyes that were at one time used in foods, such as Ponceau 3R, have been banned from use as food additives in the past two decades, because they have been associated with toxic effects, such as liver injury and tumour formation (Grice, Mannell & Allmark, 1961; Hansen, Davis, Fitzhugh & Nelson, 1963). Azo dyes are degraded to amines by the intestinal flora both *in vivo* (Jones, Ryan & Wright, 1964; Radomski & Mellinger, 1962) and *in vitro* (Roxon, Ryan & Wright, 1966 & 1967a,b; Scheline & Longberg, 1965) and the amines may themselves be toxic or form toxic metabolites in the body after being absorbed from the intestine (Daniel, 1962; Radomski & Mellinger, 1962), as has been demonstrated with a variety of aromatic amines (Clayson & Garner, 1976). This suggests that the toxicity of an azo dye depends to a large extent on the rate at which bacterial systems reduce the azo linkage.

Animals used for testing the toxicity of azo dyes are mainly rats, mice and rabbits. The gut flora in each of these species is known to differ from that in man (Bornside & Cohn, 1965). This suggests that the true toxicity of an azo dye to man may not be deduced from animal tests unless the difference in the rates of azo reduction by human and animal gut flora is taken into account. The reduction of water-soluble azo dyes by bacterial suspensions from human faeces has not yet been reported, although reduction by some predominant strains of microbes in the human gut was recently demonstrated (Chung, Fulk & Egan, 1978). This paper deals with a comparative study of the anaerobic reduction of amaranth, sunset yellow, new coccine and tartrazine by human faecal flora and flora from the rat gut.

EXPERIMENTAL

Materials. Amaranth, new coccine, sunset yellow and tartrazine were obtained from Tokushu Chemicals Co., Tokyo. Nutrient broth was purchased from Difco Laboratories, Detroit, MI, USA. All other reagents used were of analytical grade.

Animals and test subjects. Three male Wistar rats weighing about 250 g were maintained on a commercial diet obtained from Nippon Crea Co., Tokyo, and

housed in clean stainless-steel cages for more than 1 wk before being used for the experiments. In the human experiments faeces were collected from five male volunteers aged 24–42 yr.

Bacterial growth. All of the contents of the large intestine of a rat or about 3 g fresh human faeces taken from the last tenth of the excretion were transferred under sterile conditions and dispersed in 150 ml sterile nutrient broth containing 0·9% sodium chloride. After culturing under nitrogen at 37°C for 15 hr, the broth was filtered through glass wool to remove undispersed material. An aliquot of the filtrate was transferred into 200 ml of the same nutrient broth, and incubated at 37°C for 30 hr under nitrogen with shaking (60 oscillations/min). The bacterial growth curve was obtained by measuring the turbidity by absorptiometry.

Preparation of cell suspensions. The late log-phase cells were separated from the nutrient broth by centrifuging at 5000 **g** for 20 min and were washed once with an equal volume of sterile water. The bacterial pellets were suspended in water to give a concentration of bacterial proteins of approximately 1·0 and 2·5 mg protein/ml in the case of the rat and human samples, respectively, and were used immediately for azo reduction.

Azo-reduction incubations. For the incubations modified Thunberg tubes were used, the bottom of each of these being connected to a 40-ml Erlenmeyer flask. In each flask was placed 2 ml 0·4 M-phosphate buffer, pH 7·4, 1 ml 100 mM-glucose solution and 1 ml of the cell suspension, and in the upper bent arm of the Thunberg tube, 1 ml of a 250 μM solution of an azo dye was placed. After complete evacuation of the air from the tube, followed by the introduction of nitrogen, the flask was pre-incubated at 37°C for 30 min. Incubations were started by adding the dye solutions and were carried out at 37°C.

Measurement of azo dyes. At the end of each incubation, 5 ml 6% trichloroacetic acid was added to the reaction mixture. The clear supernatant obtained by centrifuging at 5000 **g** for 5 min was used for spectrophotometric determinations. The absorption maxima of the dyes in the supernatants were at 520 nm for amaranth, 510 nm for new coccine, 485 nm for sunset yellow and 420 nm for tartrazine. Azo reductase activities were expressed as nmol azo dye removed/mg bacterial protein. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) after alkaline hydrolysis of the bacterial cells.

RESULTS

When amaranth was incubated with faecal bacteria from the five male volunteers, decolorization of the incubation mixtures proceeded rapidly; the colour faded within 200 min, with reduction rates ranging from 20·7 to 48·9 nmol/mg protein/hr (Fig. 1a). The rat-gut flora decolorized the dye more rapidly, the reduction rates being in the range of 100–210 nmol/mg protein/hr. The reduction of amaranth by human and rat-gut flora proceeded linearly until the colour had faded almost completely. Reduction of sunset yellow by the bacterial suspensions showed a similar trend, the reduction proceeding much more rapidly with the flora from the rat (Fig. 1b). On the other hand, with new coccine (Fig. 1c) and tartrazine (Fig. 1d) there was no significant difference between the rates of reduction by the human and rat flora. The lowest reduction rates by the flora from both sources were observed with tartrazine. The mean rates of reduction of amaranth and sunset yellow by rat bacteria were, respectively, four and five times as fast as by human bacteria (Table 1).

For each of the dyes, only slight differences in reduction rates were observed between the individual samples of faecal bacteria from the five volunteers (Fig. 1; Table 1). With both the rat and human samples of bacteria, the rates of reduction were always in the decreasing order amaranth, sunset yellow, new coccine and tartrazine (Table 2). Rates for new coccine and tartrazine expressed relative to those for amaranth were much higher with human than with rat flora because of the higher rate of amaranth reduction by cell suspensions from the rat intestinal contents.

DISCUSSION

When plotted against incubation times, the reduction rates of sulphonated water-soluble azo dyes by microflora from both human faeces and the rat gut were linear over a remarkably long period, lasting until the dyes had almost disappeared, particularly in the case of amaranth. It is of interest that the reductive cleavage of all of the other dyes studied also followed zero-order kinetics. In this respect, the reactions effected by these microflora are very different from typical enzyme reactions, the rates of which generally become lower as the concentrations of the substrates are reduced. Similar results have been obtained by Larsen, Meyer & Scheline (1976) in the

Table 1. *Comparison of azo dye reduction rates by bacterial suspensions from human faeces and from the contents of the large intestine of rats*

Azo dye	Rate of azo dye reduction (nmol degraded/mg protein/hr) by bacteria from: Man	Rat	Man/Rat × 100
Amaranth	38·4 ± 4·8**	155·3 ± 36·2	24·7
Sunset yellow	25·1 ± 2·8**	114·3 ± 22·3	22·0
New coccine	18·2 ± 3·3	24·2 ± 4·9	75·2
Tartrazine	6·5 ± 1·6	7·7 ± 2·6	84·4

The results are expressed as means ± SEM for bacterial suspensions for five men or three rats. Those marked with asterisks (** $P < 0{\cdot}01$ by Student's *t* test) differ significantly from the corresponding value for the rat.

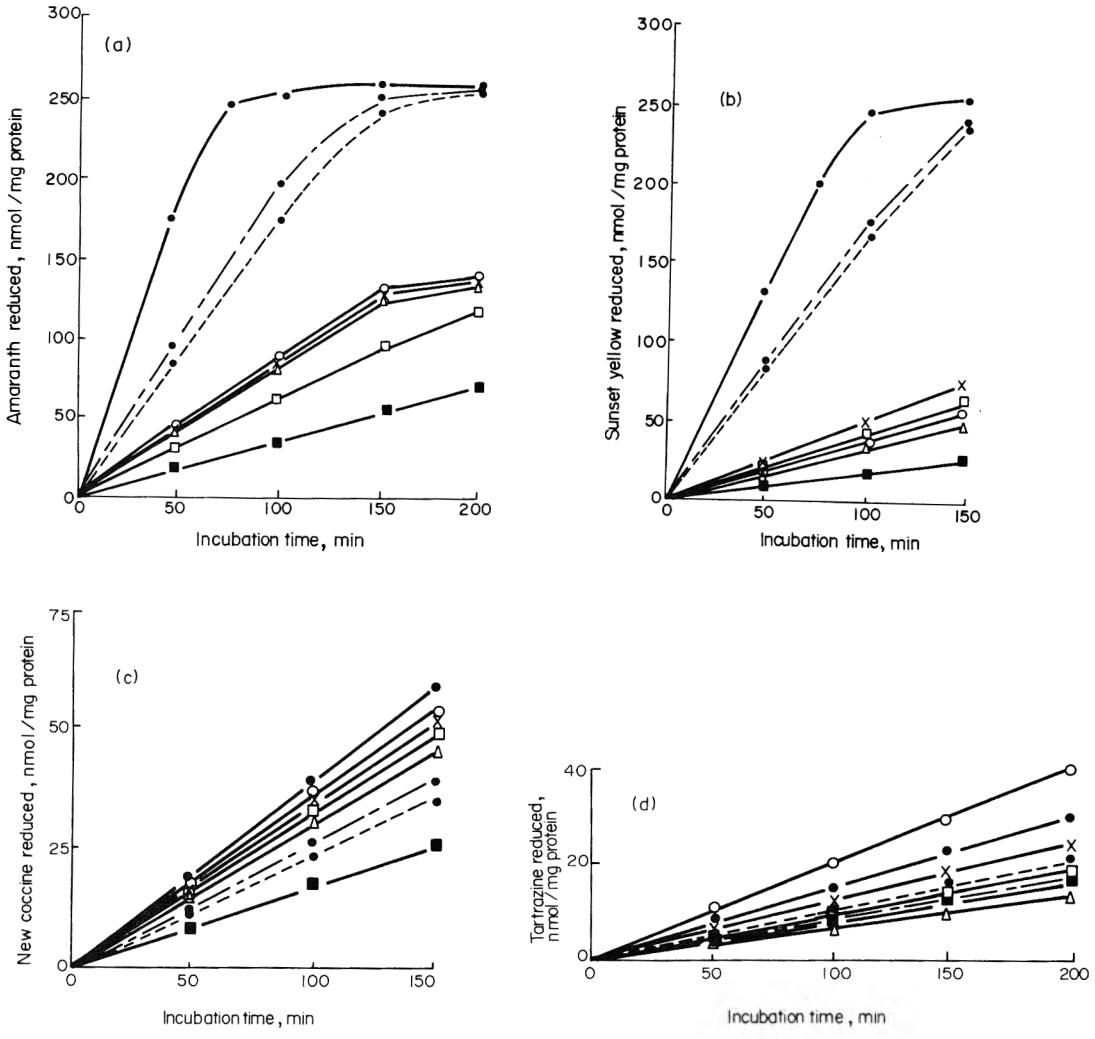

Fig. 1. Anaerobic reduction of (a) amaranth, (b) sunset yellow, (c) new coccine and (d) tartrazine by bacterial suspensions from human faeces (from five individuals: ○, ×, △, □ and ■) and from the contents of the large intestine of three rats (—●, – –● and — —●). Each dye (50 μM) was incubated anaerobically at 37°C with cell suspensions (1·0 and 2·5 mg protein for rat and human studies, respectively) in 160 mM potassium phosphate buffer, pH 7·4, containing 20 mM glucose. Azo reductase activities were expressed in terms of the disappearance of the substrate as determined by direct photometry at (a) 520 nm, (b) 485 nm, (c) 510 nm and (d) 420 nm.

Table 2. *Reduction rates of sunset yellow, new coccine and tartrazine in relation to those of amaranth in incubations using bacterial suspensions from human faeces and from the contents of the large intestine of rats*

Bacterial source		Amaranth	Sunset yellow	New coccine	Tartrazine
Rat:	1	1·0	0·72	0·11	0·04
	2	1·0	0·80	0·25	0·08
	3	1·0	0·70	0·12	0·03
	Mean ± SEM ...	1·0	0·74 ± 0·03	0·16 ± 0·05	0·05 ± 0·02
Man:	1	1·0	0·61	0·34	0·08
	2	1·0	0·71	0·69	0·15
	3	1·0	0·70	0·31	0·19
	4	1·0	0·70	0·47	0·17
	5	1·0	0·59	0·52	0·29
	Mean ± SEM ...	1·0	0·65 ± 0·04	0·47 ± 0·07*	0·17 ± 0·03*

Means marked with an asterisk differ significantly (*$P < 0.05$ by Student's t test) from the corresponding value for the rat experiments.

reduction of Fast Red E, amaranth, new coccine and Ponceau 6R by whole-cell extracts from rat faecal contents, although they offered no explanation for the finding. A prolonged linearity in the consumption of substrate, therefore, is very likely to be characteristic of the reduction of water-soluble azo dyes by anaerobic microflora from any source.

In their susceptibility to azo cleavage by human faecal and rat intestinal flora, the azo dyes used in this investigation fell into two distinct groups. Amaranth and sunset yellow were reduced more rapidly by flora from the rat than from man, while no significant difference was observed between the flora from the two sources in their reduction of new coccine and tartrazine. This suggests the possibility that in extrapolating to man toxicity data obtained in the rat, the potential human toxicity of some azo dyes may be overestimated, since many azo dyes are known to exert toxicity as a result of microbial reduction to aromatic amines followed by metabolic activation in the hepatocytes after absorption from the intestinal site. For example, Orange II (Bonser, Bradshaw, Clayson & Jull, 1956) undergoes reductive cleavage by intestinal bacteria to form 1-amino-2-naphthol, which can induce bladder tumours.

As the composition of the human intestinal flora is known to vary with age (Lerche & Reuter, 1961), daily diet (Petuely & Lindner, 1962; Vassena & Brignoli, 1962), health conditions (Sokolova, 1965) and other factors (Knothe & Mieth, 1961), it was assumed that the reduction rates observed with faecal flora from the five volunteers would be scattered over a wide range, but the individual differences demonstrated with each of the four dyes investigated in this study were unexpectedly small.

The fact that the gut flora from both the rat intestine and from human faeces reduced the four water-soluble azo dyes in the same rate order suggests a relationship between the structure of an azo dye and its susceptibility to microbial reduction. In this connection, Jones *et al.* (1964) suggested that their observation that rat-gut flora reduced tartrazine more slowly than amaranth or sunset yellow may have been due to the formation of a stable keto-hydrazone tautomer stabilizer by strong intramolecular hydrogen bonding in the tartrazine molecule. Larsen *et al.* (1976) also tried to demonstrate a relationship, using four water-soluble azo dyes differing in the number of sulphonate groups, and suggested that the reduction rate of an azo dye by rat-gut flora might depend on the number of sulphonate groups rather than on their positions in the dye molecule. They assumed that bacterial reduction would occur more readily in azo dye molecules in which the electron density at the azo linkage was lowered by substitution with a sulphonate group especially at the para or ortho position. However, microbial reduction rates were actually lower in the azo dyes with more sulphonate groups. Larson *et al.* (1976) suggested that this discrepancy was probably due to the different abilities of these dyes to penetrate the bacterial cell wall. Their suggestion about the structural influence on the microbial reduction of sulphonated azo dyes, however, does not fit in with our results, for amaranth with three sulphonate groups was reduced more readily than the disulphonated dye, sunset yellow, and even more readily than new coccine, which is trisulphonated. It would be of great interest to establish the true relationship between structure and the reduction rate of azo dyes by gut flora. A further study relating to this problem is in progress.

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THE EFFECT OF SODIUM SACCHARIN IN THE DIET ON CAECAL MICROFLORA

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(*Received 3 December 1979*)

Abstract—Total aerobic and anaerobic microbial populations of the caecum were compared in male rats fed 0 or 7·5% sodium saccharin for 10 days. The weights of caecal tissue and caecal contents were higher in the saccharin-fed animals than in the controls. The presence of a high saccharin content, about 90 mg/ml, in the caecal contents did not alter the total numbers of anaerobic microbes, but resulted in the deletion of a specific anaerobic microbe which, when isolated from control samples, was shown to be sensitive to saccharin *in vitro*. Saccharin ingestion resulted in an increase in the numbers of aerobic microbes. Finally, it was demonstrated that saccharin ($\geq$ 50 mg/ml of medium) could reduce the amount of ammonia produced from urea by *Proteus vulgaris* (ATCC 13315). These observations suggest a possible mechanism for the changes in urine previously noted in rats ingesting diets containing saccharin (Anderson, *Fd Cosmet. Toxicol.* 1979, **17**, 195).

INTRODUCTION

In a previous report (Anderson, 1979) it was hypothesized that ingestion of high doses of sodium saccharin may alter the intestinal microflora. This hypothesis was based on the following observations made during a 4-wk study in which dietary sodium saccharin was fed at levels of 1, 3, 5 and 7·5%: (1) animals fed diets containing 5 or 7·5% sodium saccharin showed transient diarrhoea followed by the voiding of stools with a high water content; (2) there was a dose-dependent reduction in urinary ammonia content, and it was speculated that this might have been due to a reduction in microbial urease activity in the intestine; (3) the concentrations of saccharin attained in the faecal water (> 20 mg/ml) had been shown to be toxic to microbial species used in mutagenesis assays (Office of Technology Assessment, 1977); (4) animals ingesting saccharin were observed to have a marked decrease in the odour of their faeces.

This hypothesis has been tested by direct assay of the flora of the caecal contents obtained from male rats fed diet containing either 7·5% sodium saccharin or no saccharin for 10 days.

EXPERIMENTAL

Materials. Sodium saccharin was obtained from Sherwin Williams Co., Cleveland, OH (Lot no. 1648) and cellulose (Celluflour) from Chicago Dietetic Supply House, Chicago, IL.

Animals and treatment. Weanling male Charles River rats (from Charles River Breeding Laboratories, Wilmington, MA) were fed Purina Laboratory Chow (Ralston Purina Co., St. Louis, MO) containing either 7·5% sodium saccharin or 7·5% cellulose, from weaning (animal weight 55 $\pm$ 3 g) for 10 days. The animals were housed in cages with wire bottoms and were allowed diet and distilled water *ad lib.* The saccharin-fed group contained seven animals and the control group five animals. The animals were weighed on days 4, 7 and 10 of the study. Feed consumption was measured but the data were not used to calculate feed efficiencies because of excessive diet-scattering by the saccharin-fed animals. On day 10 the animals were anaesthetized with ether and the intestine was ligated on either side of the caecum. The caecum was removed and weighed and the contents were dispersed in 10 ml cold sterile 0·1% peptone broth. The samples were shaken on an orbital shaker for 10 min and then placed in an ice bath. The caecum was removed from the peptone broth, drained, blotted and weighed.

Studies of caecal contents. The caecal contents were further diluted by addition of 90 ml sterile peptone broth. This dilution was designated 10^{-2}. Three additional 1/100 dilutions were made in sterile distilled water. The numbers of organisms in the samples were determined using the Spiral Plate Maker (Briner, Wunder, Blair, Parran, Blaney & Jordan, 1978; Gilchrist, Campbell, Donnelly, Peeler & Delaney, 1977) using 150-mm plastics Petri plates. The Spiral Plate Maker was obtained from J. E. Campbell, 1618 Dell Terrace, Cincinnati, OH (present supplier: Spiral Systems Marketing, Bethesda, MD). The aerobic organisms were grown on Brain Heart Infusion (BHI) Agar (BBL, Cockeysville, MD) and the anaerobic organisms were grown on Schaedlers Agar (BBL). The BHI Agar was incubated for 24 hr at 37°C and the Schaedlers Agar was incubated in anaerobic chambers for 5 days at 37°C. The colony-forming units were counted using an automatic colony counter (Spiral Systems Marketing). Since no attempt was made to exclude air from the sample during preparation, the numbers of anaerobic organisms recovered may have been somewhat reduced because of their sensitivity to oxygen.

The urease assay was performed according to the method of Stuart, van Stratum & Rustigian (1945). *Proteus vulgaris* (ATCC 13315), grown for 18 hr in Trypticase Soy Broth (BBL), was used as the test organism. Urease activity was measured by the colour change of the pH indicator, 0 activity being no

Table 1. *Growth and changes in caecal mass, content and flora in male rats fed 7·5% sodium saccharin in the diet for 10 days*

Parameter	Mean values (±SEM) Control group	Saccharin-fed group
No. of rats/group	5	7
Weight gain* (g)	62 ± 2	44 ± 3
Caecal weights (g/100 g body weight)		
Tissue	0·82 ± 0·04	1·19 ± 0·03
Contents	2·4 ± 0·22	4·3 ± 0·5
Saccharin in caecal contents (mg/g contents)	0	88 ± 15
Caecal flora		
Total (log counts/caecum): aerobes	7·5 ± 0·5	9·0 ± 0·2
anaerobes	8·8 ± 0·2	8·8 ± 0·2
Concn (log counts/g contents: aerobes	7·0 ± 0·4	8·2 ± 0·3
anaerobes	8·3 ± 0·2	8·1 ± 0·2
Anaerobes/aerobes	1·20 ± 0·09	0·99 ± 0·03

*Weight gain (from initial body weight of 55 ± 3 g) during *ad lib.* ingestion of control or saccharin diet for 10 days.

colour change and 4+ indicating maximum colour or activity.

Samples of the diluted caecal contents were assayed for their saccharin content by a previously published technique (Anderson, 1979).

RESULTS AND DISCUSSION

The gross appearance of the stools from the saccharin-fed animals was quite different from that of the controls during the first 7 days of the study. The stools voided by these rats were not discrete pellets, but spread on the collection papers and clung to the wire bottom of the cages. In the 7–10-day period, the stools became discrete pellets, but were very soft compared to the samples from control animals. On day 8, new collection papers were placed under the animals and fresh stool samples from control and saccharin-fed animals were placed in separate vials. Several experienced animal laboratory technicians were asked to evaluate the odour of the two stool samples. All identified the faeces from the saccharin-fed animals as being generally atypical of rat faeces and less odoriferous.

Table 1 shows the effect of 10-day ingestion of a diet containing 7·5% sodium saccharin on growth, caecal-tissue weight, caecal-content weight and the concentration of saccharin in the caecal contents. Saccharin ingestion reduced weight gain by about 30%. The caecal tissue (expressed in g/100 g body weight) was increased by 45% and the weight of caecal contents (g/100 g body weight) was increased by 80%.

The total populations of aerobic and anaerobic flora per caecum and the population densities (colony-forming units/g caecal contents) are also presented in Table 1. The high level of saccharin in the caecal contents was associated with an increase in the numbers of the aerobic population but no change in the numbers of the anaerobic population. Thus, the ratio of anaerobes/aerobes was lower in the saccharin-fed animals than the controls.

Visual assessment of plates showed that a specific type of anaerobic colony present in each of the samples from control animals was absent from all of the samples from saccharin-fed animals. Isolation of this microbe and assessment of its saccharin sensitivity showed that the addition of >50 mg sodium saccharin/ml of Trypticase Soy Broth caused a decrease in the growth of this specific unidentified caecal anaerobe isolated from control samples, whereas other isolates were not sensitive to 100 mg saccharin/ml.

Since it had been noted in a previous saccharin feeding study that sodium saccharin ingestion was associated with a reduction in urinary ammonia and an increased concentration of urea (Anderson, 1979), the effect of doses of sodium saccharin on the urease activity of a known urease-active microbe (*Proteus vulgaris* ATCC 13315) was determined. Figure 1 demonstrates that 50 or 100 mg sodium saccharin/ml of medium inhibited the urease activity. The inhibition of urease activity by saccharin appeared not to be a direct effect on the enzyme but to be due to an inhibition of microbial growth, since long-term incubations (72 hr) showed that saccharin reduced the numbers of organisms present in the culture tubes.

The results of this assessment of the effects of sodium saccharin on caecal microbial populations are not consistent with the hypothesis that saccharin-fed

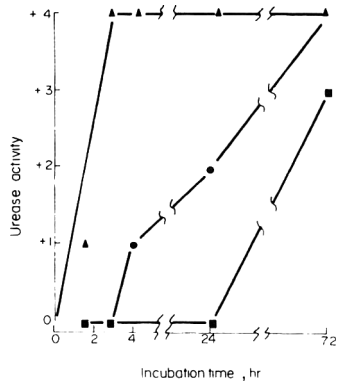

Fig. 1. Effect of sodium saccharin concentration (0–10 mg/ml, ▲; *c.* 50 mg/ml, ●; *c.* 100 mg/ml, ■) on the urease activity of *Proteus vulgaris* (ATCC 13315).

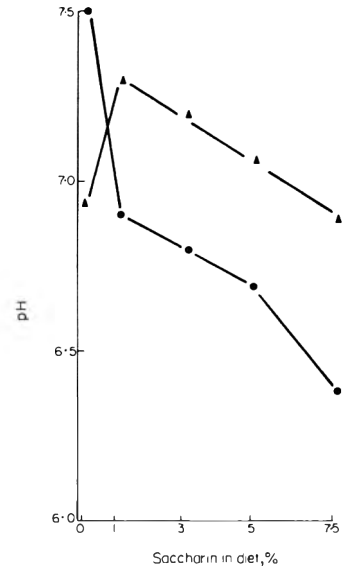

Fig. 2. Effect of sodium saccharin concentration in the diet on the pH of an aqueous slurry of dried faeces (1 g/20 ml distilled water; ▲) and on the pH of urine (●). The samples of faeces and urine were from a study reported earlier (Anderson, 1979).

animals are comparable to germ-free animals (Anderson, 1979), since large numbers of both aerobic and anaerobic organisms were isolated from the caeca of the saccharin-fed animals. The results do show, however, that 7·5% sodium saccharin increases the mass and the volume of the caecum, perhaps because of an osmotic effect of the high levels of saccharin present (approximately 90 mg/ml of caecal contents). Further, saccharin does have some effects on the caecal populations, as shown in the shift in the anaerobes/aerobes ratio and the specific deletion of a saccharin-sensitive anaerobe. In addition, the possibility that saccharin may be reducing the intestinal production of ammonia from urea is supported by the *in vitro* effect of saccharin on ammonia production by *P. vulgaris* (Fig. 1). Finally, this contention is also strengthened by a comparison of the pH of vacuum-dried stools in aqueous suspension and of urines from animals fed various doses of sodium saccharin in another study (Fig. 2).

The significance of the demonstrated effects of saccharin ingestion on caecal contents and ammonia production in relation to the overall toxicity and, especially, to the bladder cancer associated with the feeding of high doses of saccharin is certainly not obvious. The results presented do, however, provide a rationale for the shifts in acid/base balance induced by the ingestion of the acidic sodium saccharin (a 9% solution has a pH of 6·04). There is an increase in faecal pH corresponding to the decrease in urinary pH at the lower doses (Fig. 2) but at the higher doses the net effect is an acid shift, the pH of the stool suspension being the same as that of the control sample while the urine pH is below that of the control. The observation that saccharin can inhibit urease activity also provides a possible mechanism for the reduced urinary ammonia and increased urinary urea output previously reported (Anderson, 1979) for rats fed saccharin.

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SUBCHRONIC STUDIES IN RATS FED OCTENYL SUCCINATE-MODIFIED FOOD STARCH

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Abstract—Fischer 344 rats were fed octenyl succinate-modified food starch in a semi-purified diet from conception until they were killed 30 or 90 days after weaning. Complete autopsies and histopathological evaluations showed that growth and haematology were unaffected, but that liver, kidney and caecal weights tended to increase with increasing concentrations of dietary octenyl succinate starch. There were no consistent changes in serum chemistry values that could be associated with octenyl succinate starch intake. Female rats had higher concentrations of urinary magnesium and calcium than did male rats, and these higher mineral concentrations correlated with an increased incidence of renal cortico-medullary mineralization. The increase in mineralization of the cortico-medullary junction occurred in both control and in octenyl succinate starch-treated female rats. Pelvic nephrocalcinosis was not observed in any of the rats. It is concluded that no adverse effects associated with feeding octenyl succinate starch occurred in rats under the conditions of this study.

INTRODUCTION

Octenyl succinate-modified food starch (OS starch) is used as an emulsion stabilizer for various food items, including flavourings, salad dressings, vitamin preparations, and oil and fat products. No studies on this modified starch have been published, although unpublished data on a similar starch (starch sodium succinate) were reviewed by the Joint FAO/WHO Expert Committee on Food Additives (1974). Starch sodium succinate or unmodified starch was fed at a level of 70% in the diet to male and female rats for 10 wk and no significant differences between the dietary groups were observed in growth rates, feed conversion efficiency, or haemoglobin levels. In a calorific-value study (Joint FAO/WHO Expert Committee on Food Additives, 1974), groups of ten male rats were fed a basal diet supplemented with unmodified starch, succinate-modified starch or sucrose for 4 wk; the calorific values of the modified and unmodified starches were identical.

In the study reported here, rats were fed diets that contained 6, 12 or 30% OS modified starch or a control, unmodified, starch. The experimental rats were chosen from the second litters of mothers that had been fed one of the starch diets continuously from weaning and throughout gestation and lactation.

EXPERIMENTAL

Starch sodium octenyl succinate (OS starch) was prepared by treating granular, acid-hydrolysed starch with alkali and not more than 3% octenyl succinic anhydride, the residual as sodium octenyl succinate being 0·33%. When treated in this manner, this starch has a low degree of substitution (0·018). The control starch used was an unmodified corn starch.

Fischer 344 rats (50 males, 140 females; 56 days old) were obtained from Charles River Breeding Laboratories, Wilmington, MA. They were housed individually in solid-bottomed, polyethylene cages in temperature-controlled quarters (72 ± 4°F) with 12-hr light/dark cycles. The test diets were made up weekly and refrigerated. The rats were fed the diet in powdered form in a glass jar and were given fresh supplies of the feed three times per week.

The diet used was based on the requirements for laboratory animals published by the National Academy of Sciences/National Research Council (1978). It contained 30% starch, 20% vitamin-free casein, 17·5% dextrose, 17·5% sucrose, 5·0% alpha cell fibre, 5·0% corn oil plus vitamin K_1, 3·5% mineral mix no. 5, 1·0% vitamin mix no. 29, 0·3% DL-methionine and 0·2% choline chloride. The mineral mix contained (in g): $CaHPO_4$, 500; $K_3C_6H_5O_7.H_2O$, 220; $MgSO_4.7H_2O$, 116; NaCl, 74; K_2SO_4, 52; ferric citrate, 6·0; $MnCO_3$, 3·5; zinc carbonate, 1·6; chromium potassium sulphate, 0·55; cupric carbonate, 0·3; KIO_3, 0·01; Na_2SeO_3, 0·01. The mineral mix was made up to 1 kg with 26 g sucrose. The vitamin mix contained (in g): tocopheryl acetate, 20 g (500 IU/g); cyanocobalamin (0·1% trituration with mannitol), 5 g; retinyl palmitate, 3·2 g (250,000 IU/g); nicotinic acid, 3 g; calcium pantothenate, 1·6 g; pyridoxine, 0·7 g; riboflavin, 0·6 g; thiamine HCl, 0·6 g; vitamin D_3 (cholecalciferol), 0·25 g (500 IU/g); folic acid, 0·2 g; *d*-biotin, 0·02 g. The vitamin mix was made up to 1 kg with 964·8 g of sucrose. Vitamin K_1 was added to the corn oil (10 mg vitamin K_1/kg corn oil) supplied in the diet (50 g corn oil/kg diet).

The rats were fed OS starch at dietary levels of 0, 6, 12 or 30% (0, 3, 6 or 15 g/kg/day, respectively), the starch content of the diet being made up to 30% with the control pregelatinized, unmodified corn starch. When the animals reached breeding age they were mated and the females were fed their respective diets throughout gestation and lactation. The litters were adjusted to eight pups per litter by random selection. At weaning, two males and two females

Table 1. *Relative weights of liver, kidney and caecum of rats fed octenyl succinate starch or unmodified corn starch*

Dietary level (%) of octenyl succinate starch	No. and sex of rats	Relative weight (% of body weight) of		
		Liver	Kidney	Caecum
		30 days after weaning		
0	10 M	5·10 ± 0·17	0·82 ± 0·02	0·63 ± 0·07
	10 F	4·45 ± 0·21	1·00 ± 0·03	0·61 ± 0·08
30	10 M	5·61 ± 0·16	0·96 ± 0·03*	0·82 ± 0·06
	10 F	5·08 ± 0·15*	1·07 ± 0·04	1·03 ± 0·04*
		90 days after weaning		
0	50 M	3·54 ± 0·09	0·74 ± 0·01	0·31 ± 0·01
	50 F	3·18 ± 0·06	0·83 ± 0·01	0·34 ± 0·02
6	50 M	3·58 ± 0·08	0·78 ± 0·01	0·35 ± 0·02
	50 F	3·39 ± 0·06	0·87 ± 0·01	0·40 ± 0·02
12	50 M	3·53 ± 0·09	0·80 ± 0·01*	0·30 ± 0·02
	50 F	3·52 ± 0·07*	0·91 ± 0·01*	0·39 ± 0·02
30	52 M	3·72 ± 0·08	0·83 ± 0·02*	0·30 ± 0·02
	48 F	3·62 ± 0·07*	0·94 ± 0·02*	0·50 ± 0·02*

Values are means ± SEM for the number of animals indicated and those marked with an asterisk differ significantly (*$P < 0·05$) from the corresponding male or female control group. Control rats were fed unmodified corn starch at a dietary level of 30%.

were randomly selected from the second litter of each of the dams. These animals were divided into groups and were continued on the test diets corresponding to those of their dams for 30 or 90 days after weaning. There were approximately 60 males and 60 females in the control group and in the group fed 30% OS starch, and 50 males and 50 females in the groups fed 6 and 12% OS starch. Twenty rats fed 30% OS starch and 20 fed the control diet were killed 30 days after weaning; the remainder of the rats were killed 90 days after weaning.

At autopsy, the animals were examined grossly. Selected organs (liver, kidney, caecum, heart, spleen, thymus, brain and testes or uterus) were weighed and these and all other major organs and tissues were taken for histological examination. Haematological tests (red cell, white cell, haematocrit, haemoglobin, total protein and differential white cell) were carried out on blood samples. Serum samples were analysed for sodium, potassium, chloride, glucose, blood urea nitrogen, magnesium, alkaline phosphatase, serum glutamic–oxalacetic transaminase, serum glutamic–pyruvic transaminase, calcium, phosphorus, total protein and albumin. Urine samples were analysed by standard methods for pH, total protein, glucose, ketones, occult blood, sodium, potassium, creatine, calcium and magnesium.

RESULTS

Growth curves showed no treatment-related effects in any of the dietary groups. Organ weights as percentages of body weights are listed in Table 1. Liver weights of females increased with increased concentration of OS starch. Kidney weights of both males and females also increased with increased OS starch concentration. There was a significant increase in caecum weight in females fed 30% OS starch for 30 or 90 days. No differences were found between the OS starch-fed and control rats in the relative weights of heart, spleen, thymus, brain, testes or uterus.

The haematology of animals killed 90 days after weaning revealed no differences between the groups in any of the parameters studied. There were no consistent changes in serum-chemistry values that could be associated with OS starch intake.

Urine analyses (Table 2) demonstrated that female rats had higher concentrations of urinary magnesium

Table 2. *Results of analyses of urine from rats fed octenyl succinate starch and unmodified corn starch for 90 days after weaning*

Dietary level (%) of octenyl succinate starch	No. and sex of rats	Urinary pH	Urinary levels of				
			Sodium (mmol/litre)	Potassium (mmol/litre)	Creatine (mg/100 ml)	Calcium (mg/100 ml)	Magnesium (mg/100 ml)
0	50 M	6·17 ± 0·22	67·87 ± 14·16	140·27 ± 10·72	7·67 ± 0·57	6·6 ± 1·8	14·2 ± 2·8
	50 F	5·80 ± 0·14	54·80 ± 7·74	122·07 ± 42·33	6·07 ± 1·31	11·5 ± 1·1	23·3 ± 1·8
6	50 M	6·50 ± 0·22	46·40 ± 13·62	122·00 ± 5·94	6·94 ± 0·77	8·3 ± 2·6	8·2 ± 4·5
	50 F	6·00 ± 0·00	33·40 ± 7·20	76·20 ± 20·00	5·33 ± 1·28	11·1 ± 3·0	17·4 ± 5·3
12	50 M	6·00 ± 0·00	84·80 ± 28·26	150·20 ± 17·32	7·76 ± 0·68	9·4 ± 2·8	22·1 ± 2·0
	50 F	6·00 ± 0·00	62·60 ± 18·38	137·80 ± 15·14	7·13 ± 0·40	16·0 ± 0·0	29·8 ± 2·1
30	52 M	5·83 ± 0·12	37·40 ± 6·50	88·07 ± 6·96	6·62 ± 0·44	9·5 ± 1·3	13·6 ± 2·7
	48 F	6·33 ± 0·21	56·73 ± 7·32	97·53 ± 7·24	5·43 ± 0·57	14·3 ± 2·3	25·2 ± 5·0

Values are means ± SEM for the number of animals indicated. Control rats were fed unmodified corn starch at a dietary level of 30%.

Table 3. *Incidence of renal corticomedullary mineralization in rats fed unmodified corn starch or octenyl succinate starch at a dietary level of 30%*

Dietary group	Sex	No. of rats with lesions* (no. of rats in group)
30 days after weaning		
Unmodified corn starch	M	2 (10)
	F	8 (10)
Octenyl succinate starch	M	3 (10)
	F	8 (10)
90 days after weaning		
Unmodified corn starch	M	6 (50)
	F	49 (50)
Octenyl succinate starch	M	8 (52)
	F	48 (48)

*The severity of the observed lesions ranged from mild to moderate.

and calcium than did male rats, and this correlated with an increased incidence of renal cortico-medullary mineralization in the females (Table 3). These increases in mineral concentrations in the kidneys and urine were not reflected by parallel increases in serum calcium or magnesium levels. Table 3 lists the incidence of renal lesions observed by light microscopy. Renal lesions, primary cortico-medullary, were observed in some animals from all groups, and were more severe in female rats (Figs 1–3). However, because lesions occurred both in control groups and in groups fed OS starch, they were not treatment-related. None of the rats in any group had mineralization in the renal pelvis (pelvic nephrocalcinosis), a lesion reportedly associated with the feeding of some modified starches (de Groot, Til, Feron, Dreef-van der Meulen & Willems, 1974).

Minimal focal fatty change was observed in the livers of some rats from both the control and experimental groups. Various other minor pathological changes unrelated to starch administration were also observed.

DISCUSSION

No adverse effects were observed on the growth, relative organ weights, haematology, serum chemistry or histopathology of rats exposed to OS starch over their entire lifespan (*in utero*, via the mothers' milk and in the diet for up to 90 days after weaning).

de Groot *et al.* (1974) previously reported that focal hyperplasia of the renal papillary and pelvic epithelium accompanied by calcification of the underlying tissue occurred to a slightly higher degree and frequency in male rats fed certain modified food starches at a dietary level of 30% compared to the controls. Although cortico-medullary mineralization was observed in the present study, it was not related to OS starch intake. The incidence of renal lesions was higher in both the treated and the control females than it was in the corresponding males. The reason for this is not clear but it is presumed to be endocrine related.

In earlier studies (Buttolph & Newberne, 1979) we observed that when hamsters were fed selected modified food starches the amount of dietary magnesium was an important factor in determining the extent of renal mineralization.

It is interesting to note that in this study, the female rats excreted more magnesium and also had more renal lesions. The possible role of magnesium, and of mineral imbalances in general, in renal mineralization needs to be explored further. It is clear that a well-balanced diet is essential when a dietary compound is being evaluated in order to obtain valid results and to interpret them correctly. Evaluation and comparison of modified starch studies are difficult because difficult dietary concentrations of calcium, magnesium and phosphorus have been used by the various investigators.

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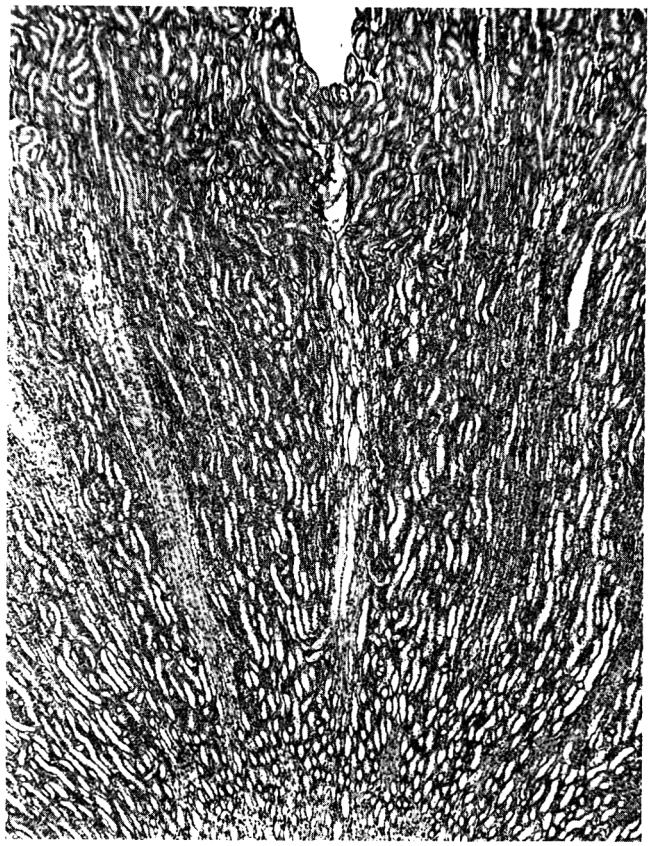

Fig. 1. Cortico-medullary junction of a normal rat kidney. Haematoxylin and eosin ×60.

Fig. 2. Mineralization of the cortico-medullary junction of a rat kidney. Haematoxylin and eosin ×60.

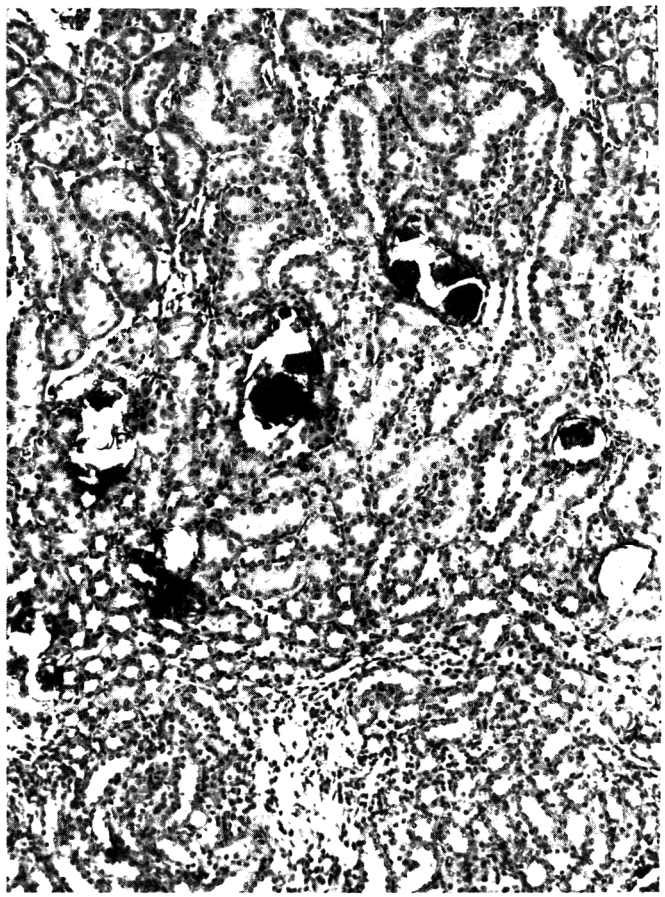

Fig. 3. Mineral deposits in tubules at the cortico-medullary junction of the kidney. Haematoxylin and eosin ×128.

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ABSORPTION, EXCRETION, METABOLISM AND CARDIOVASCULAR EFFECTS OF BEETROOT EXTRACT IN THE RAT

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Abstract—The uptake, metabolism and excretion of betanin from beetroot extract were studied in the rat after iv injection or peroral administration *in vivo*, and in the isolated perfused liver *in vitro*. When injected iv, betanin was almost completely recovered in the urine. When given orally only 3% was recovered in the urine and 3% in the faeces after 24 hr. In the isolated perfused liver very little betanin was found in the bile, and most of the added betanin was recovered from the perfusion fluid. In studies *in vitro* it was found that betanin was largely metabolized by the tissues lining the gastro-intestinal tract. It is concluded that orally-administered betanin is poorly absorbed, and that the majority of it is metabolized in the gut. Betanin injected iv had effects on the cardiovascular system, transiently increasing the blood pressure and heart rate. It also increased the magnitude of the contractions of an isolated portal vein, *in vitro*. Both the *in vivo* and the *in vitro* effects could be partially blocked by specific adrenergic and cholinergic blockers, but the mechanism of the cardiovascular effects of betanin remains to be determined.

INTRODUCTION

Colourings in food are either naturally occurring pigments or artificial dyes. Since the number of artificial colourings is limited, and the safety of some has been questioned, there is a need to further explore the use of natural pigments as food colourings. As long ago as the middle of the nineteenth century, the red-violet pigments of the beetroot have been used as colouring agents. Of these pigments, the most well known is betanin (Fig. 1), which belongs to the group of betacyanins. Betanin and its epimer isobetanin form 95% of the betacyanins in the red-beet (Nilsson, 1970). The pathway of the biogenesis of betanin is not yet understood. However, betanin is chemically related to some alkaloids, and is therefore sometimes called a chromoalkaloid (Mabry, Wyler, Sassu, Mercier, Parikh & Dreiding, 1962; Wyler, Mabry & Dreiding, 1963), and it has been shown that betanidin, the aglycone of betanin, could be derived from L-dopa like some other alkaloids (Hörhammar, Wagner & Fritzsche, 1964; Minale, Piatelli & Nicolaus, 1965). Knowledge of the possible absorption, excretion, metabolism and physiological effects of betanin in mammals is practically nonexistent. The following study was therefore initiated to throw some light on these processes, using the albino rat as a model.

EXPERIMENTAL

Beetroot extract and betanin. Betanin was obtained as beetroot powder no. Eg-162 from Ringe & Kuhlman, Hamburg, Federal Republic of Germany. The powder contained about 1% betanin, mixed with about 1·5% nitrate, 10% citric acid, 10% ascorbic acid, and 5% lactose, the remainder being made up with calcium stearate. The powder was suspended in 0·1 M-phosphate buffer (pH 5·5) to give a concentration of 4·5 mM-betanin. The suspension was centrifuged at 2000 **g** for 10 min and the supernatant was sterilized by filtering it through a 0·45-μm filter (Millipore Ltd., Bedford, MA, USA). This stock solution, referred to below as betanin (its betanin content being determined by its absorption at 535 nm) was kept at 4°C and further diluted as required.

Chemicals and solutions. The following substances were used for studies on the cardiovascular effects of betanin: acetylcholine chloride (E. Merck AG, Darmstadt, Federal Republic of Germany) adrenalin (ACO; Solna, Sweden), propranolol (Inderal; ICI Ltd., Macclesfield, England), phentolamine (Regitine; CIBA Geigy Ltd., Basle, Switzerland) and atropine (Sigma Chemical Co., St. Louis, MO, USA). All the substances were dissolved in and further diluted in

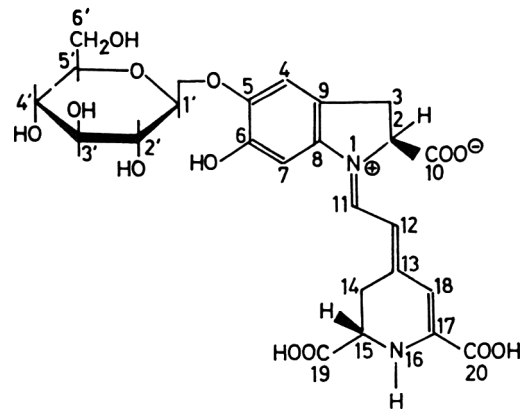

Fig. 1. Structure of betanin (isobetanin is the C-15 epimer).

0·1 M-phosphate buffer (pH 5·5). The physiological salt solution used in the *in vitro* experiments had the following composition (mM): Na, 137; K, 6; Ca, 2·6; Mg, 1·2; Cl, 134; HCO_3, 15·5; H_2PO_4, 1·2; glucose, 11. An O_2–CO_2 mixture (95:5, v/v) was continously bubbled through the solution. The temperature of the solution was 37°C and the pH was 7·4.

Animals and treatment. Sprague–Dawley rats of both sexes, weighing 200–300 g were used. They were SPF-bred and kept within strict hygienic barriers before the experiments. In the experiments *in vivo* the animals were given beetroot extract either by gavage or by iv injection. Blood samples were obtained by orbital puncture from conscious animals, and from the jugular vein in anaesthetized animals (Nembutal; Abbott Laboratories, Chicago, IL, USA; 50 mg/kg body weight, ip). Samples of urine and faeces were collected from animals kept in metabolism cages (Tecniplast, Milan, Italy) and given beetroot extract by gavage or by iv injection. A faecal supernatant was prepared by adding 0·1 M-phosphate buffer to the faeces, shaking vigorously for 2 min, centrifuging at 2000 **g** for 10 min and discarding the pellet.

Isolated liver perfusion was performed by the method of Wahlström and Blennow (1978). Beetroot extract was added to the perfusion fluid and bile and 'blood' samples were withdrawn at regular intervals.

Samples of bile, rat plasma, perfusion plasma, urine and faecal supernatant were treated with 6·5% perchloric acid (1:1, v/v) and centrifuged at 2000 **g** for 10 min. The supernatants were diluted in 0·1 M-phosphate buffer as required for spectrophotometric determination of betanin at 535 nm. The spectra of the samples were compared with those of a betanin standard solution.

In studies of the breakdown of betanin in the stomach and gut, pieces of stomach, small intestine and colon were dissected, cut into small pieces and incubated with 2·25 μmol betanin in either 0·9% NaCl or 0·9% NaCl containing 70% ethanol (to stop all metabolism). Stomach or gut contents were rinsed out with, and collected for incubation in, 0·9% NaCl with or without ethanol. Incubation was carried out at 37°C for 24 hr with continuous shaking.

For studies of the physiological effects of beetroot extract *in vitro* the spontaneously active portal vein was used. The vein was dissected, mounted in an organ bath and connected to a pen recorder (Devices MX212, Devices Ltd., Welwyn Garden City, England) by means of an isometric transducer (Grass FTO3, Grass Co., Quincy, MA, USA) and mechanical activity was recorded. The physiological effects of beetroot extract *in vivo* were studied in anaesthetized animals. Tracheotomy was performed and the jugular vein was cannulated for injections. Arterial blood pressure was measured in the left carotid artery by means of a pressure transducer (Statham P23DC, Devices Ltd.) and respiratory activity was measured using a thermistor placed in the trachea. Blood pressure and heart and respiratory activity were continuously recorded and displayed on a polygraph (Devices M19).

RESULTS AND DISCUSSION

Absorption, metabolism and elimination of betanin

The stability of betanin in biological samples was tested by adding known amounts of betanin to blood, urine and faeces *in vitro* and measuring the extractable amount of the pigment. It was found that in all cases the total recovery was 70–75% of the added dose. All the data given below have been corrected for this degree of recovery.

When 4·5 μmol betanin was injected iv it was found from elimination curves that the mean (n = 4) half-life of betanin in plasma was 32 min. The urinary excretion of betanin was 88·0 ± 6·7% (mean ± 1SD; n = 3) of the injected dose after 4 hr (Table 1). The urine was coloured red after less than 3 min, indicating that excretion took place rapidly.

After the oral administration of 4·5 μmol betanin the pigment could not be detected in the blood but about 3% had been recovered in the faeces and about 3% had been excreted in the urine after 24 hr (Table 1). These data suggest that there was very little absorption of betanin from the gastro-intestinal tract. The low recovery of betanin in urine after oral administration might have been due to the elimination of betanin via the bile and/or enterohepatic circulation. However, in isolated-liver-perfusion studies it was shown that biliary excretion was not a major route of elimination. When betanin was added to the perfusion fluid (9 μmol/100 ml), it was found that the 'blood' level of betanin was practically constant during the experiment and that over 3 hr only 1·1 ± 0·4% (mean ± 1SD; n = 4) of the added betanin was eliminated in the bile (Table 1). This was in contrast to a half-life of betanin in plasma of 32 min following iv injection. It may thus be concluded that the majority of absorbed betanin is excreted through the kidneys. The fact that the 'blood' betanin level remained constant during experiments with the isolated perfused

Table 1. *Recovery of betanin in blood, urine and faeces after iv injection or oral administration to conscious animals or after isolated liver perfusion*

Type of experiment (no. of animals)	Duration (hr)	Recovery (% of administered dose) in				
		Bile	Urine	Blood	Faeces	Total recovery
Injection, iv (3)	4	—	88·0 ± 6·7	—	—	88·0 ± 6·7
Oral administration (8)	24	—	2·7 ± 1·2	—	3·1 ± 5·3	4·8 ± 5·9
Oral administration (5)	3·5, 24	—	—	ND	—	ND
Perfused isolated liver (4)	3	1·1 ± 0·4	—	83·7 ± 12·7	—	84.7 ± 12·9

ND = Not detectable

Values are means ± 1SD.

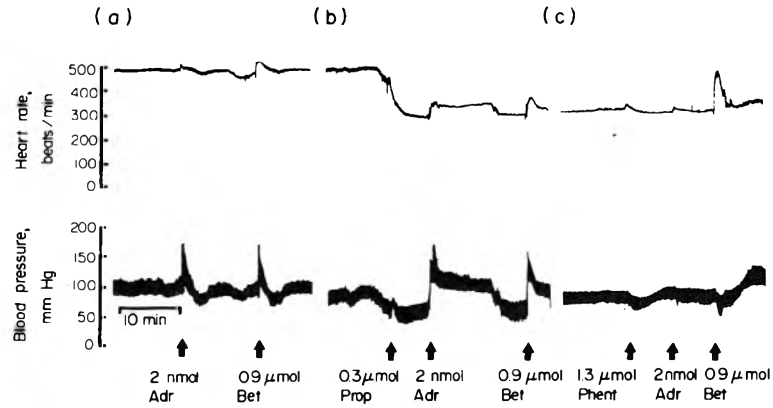

Fig. 2. Cardiovascular effects of betanin in an anaesthetized rat; (a) iv injections of adrenalin (Adr) and betanin (Bet); (b) iv injections of adrenalin and betanin after iv injection of propranolol (Prop); (c) iv injections of adrenalin and betanin after iv injections with phentolamine (Phent). The substances were injected at the times indicated by the arrows.

liver also indicated that the extent of metabolism of betanin in the liver is very small. When betanin was added to the isolated perfused liver there was a small decrease in the 'blood' flow through the liver and a concomitant decrease in the bile flow, both effects lasting about 30 min after the addition of betanin. After about 1 hr the flows had returned to the normal values. These effects may have been due to a vasoconstricting effect of betanin, and further evidence for such an effect was obtained in the studies on the cardiovascular effects of betanin (see below).

Since about 95% of orally-administered betanin was not recovered in either the urine or the faeces, the degradation of betanin either by intestinal bacteria or by the walls of the gastro-intestinal tract was suspected. Therefore, 2·25 μmol betanin was added to finely chopped pieces of stomach, intestine or their contents in a suspension of saline or ethanolic saline. It was found that suspensions in saline of the stomach wall, of the small intestine and of the colon metabolized about 75, 35 and 60% of the added betanin respectively. On the other hand, almost no metabolism of betanin occurred in suspensions of intestinal or caecal contents. There were great variations between the individual tissue samples in their capacity to metabolize betanin. It seems likely, however, that the poor absorption of orally-administered betanin is due to the extensive metabolism of the pigment in the walls of the gastro-intestinal tract.

Cardiovascular effects of betanin

When betanin was injected iv into anaesthetized rats, it had pronounced cardiovascular effects. The arterial blood pressure and the heart rate were transiently increased (Fig. 2a). The effect of 0·9 μmol betanin was about equivalent to that of 2 nmol adrenalin given iv. Previous iv injection of propranolol (0·3 μmol) did not block the cardiovascular effects of betanin (Fig. 2b). On the other hand, after iv injection of 1·3 μmol phentolamine the effect of betanin on blood pressure was slightly reversed, but there was a pronounced increase in the heart rate (Fig. 2c).

Betanin also increased the spontaneous mechanical activity of the rat portal vein *in vitro* (Fig. 3a). The contraction amplitude was greatly increased but the frequency of the contractions was almost unchanged after the administration of 0·5 μmol betanin in normal physiological salt solution. This excitatory effect of betanin was similar to that of adrenalin and acetylcholine (Johansson, Jonsson, Axelsson & Wahlström, 1967). The previous addition of atropine (7 μmol) or phentolamine (0·5 μmol) to the organ bath blocked the effect of betanin on the contraction amplitude (Fig. 3b and 3c). On the other hand, pretreatment with 1·3 μmol propranolol changed the effect of betanin so that the frequency of contractions was almost doubled, while the contraction amplitude was unaltered (Fig. 3d). Similar results were obtained in Mg^{2+}-free salt solution after β-blocking with propranolol at only a quarter of the concentration of that used in the normal salt solution. Since the betanin solution contained other substances besides betanin, these were tested separately on the spontaneously-active rat portal vein. At the concentrations at which they were present in the betanin solution, none of these substances showed any effects similar to those of betanin. In fact, citric acid and ascorbic acid *decreased* the spontaneous mechanical activity of the rat portal vein.

The effects of betanin on cardiovascular parameters and on the spontaneous mechanical activity of the rat portal vein seem to be mediated via adrenergic and/or muscarinic receptor sites, since the effects can be influenced by known specific blocking agents. The adrenalin-like excitatory effect of betanin might be due to an indirect action on catecholamine release and uptake mechanisms. At the present stage this is only a very tentative hypothesis. It is known that other naturally-occurring pigments, such as the anthocyanosides of Vaccinium species, can have effects on the cardiovascular system *in vivo* and *in vitro* (Lietti, Cristoni & Picci, 1976). Workers in our laboratory have previously found that extracts from *Vaccinium myrtillus* and *V. uliginosum* have excitatory effects on the spontaneous mechanical activity of the rat portal vein (B. Wahlström and C. Krantz, unpublished data, 1979). It has also been shown from determinations of blood flow in rabbit epidermis that anthocyanoside extracts from *Vaccinium* myrtillus have vaso-protective and anti-inflammatory proper-

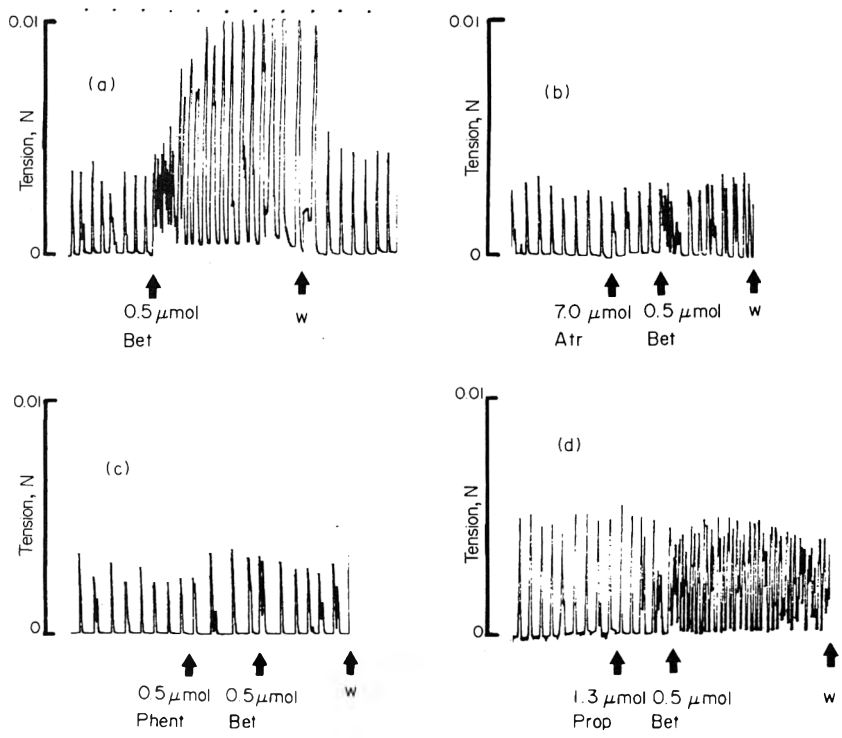

Fig. 3. The effect of betanin (Bet) on the spontaneous mechanical activity of a rat portal vein in physiological salt solution. The arrows indicate the point of addition or washout (W) of: (a) betanin; (b) atropine (Atr) and betanin; (c) phentolamine (Phent) and betanin; (d) propranolol (Prop) and betanin. The time scale is indicated by the dots in (a), the distance between two points representing 1 min.

ties (Lietti *et al.*). However, in view of the limited absorption of betanin from the gastro-intestinal tract, as shown in this study, we have not considered it worthwhile further investigating the mechanism of action of the cardiovascular effects of betanin.

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EFFECT OF FREE FATTY ACIDS ON AFLATOXIN PRODUCTION IN A SYNTHETIC MEDIUM

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Abstract—The effects of fatty acids on aflatoxin B_1 production by *Aspergillus parasiticus* were investigated *in vitro*. Incorporation of mixed fatty acids from groundnut oil into a liquid synthetic medium stimulated aflatoxin B_1 production. Three individual saturated fatty acids, myristic acid, palmitic acid and stearic acid stimulated aflatoxin B_1 synthesis while two unsaturated fatty acids, oleic acid and linoleic acid tended to inhibit toxin synthesis. Lauric acid greatly inhibited the growth of *A. parasiticus*. The net amount of aflatoxin production in a food grain may therefore be expected to depend on the ratio of saturated to unsaturated fatty acids present.

INTRODUCTION

The contamination of food by aflatoxin-producing fungi such as *Aspergillus flavus* and *A. parasiticus* is common in tropical countries. Considering the hepatotoxic and carcinogenic potential of aflatoxins, steps must be taken to prevent aflatoxin contamination of the food consumed both by animals and by man. Several methods have been suggested to prevent fungal infection and aflatoxin contamination of food grains (Goldblatt & Dollear, 1977). One approach that seems promising involves the identification and development of varieties of food crops that are resistant to fungal invasion and/or aflatoxin production. Such resistance to aflatoxin production has been observed in some varieties of maize (Laprade & Manwiller, 1976; Nagarajan & Bhat, 1972), groundnut (Mixon & Rogers, 1973; Rao & Tulpule, 1967) and sorghum (Anandam, 1970). However, the stability of the promising genetic characteristics must be established before this approach is recommended for agricultural applications. A knowledge of the factors responsible for the inhibition and/or stimulation of toxin production should strengthen our understanding of this approach.

Although much is known about factors such as the relative humidity, temperature, gaseous environment and nutrients required for the growth of Aspergilli and for aflatoxin production, very little information is available on the effects of neutral fats and fatty acids which are known to influence aflatoxin production. Jemmali & Guilbot (1974) suggested that lipids may have a role in the synthesis of aflatoxins and that the saponifiable fraction from wheat germ lipids could stimulate aflatoxin synthesis. Later, Ba, Jemmali & Drapron (1977) showed that toxigenic fungi had higher lipolytic activity than did the non-toxigenic strains indicating a relationship between this activity and the biosynthesis of aflatoxins.

Recent observations from our laboratory have shown that increased aflatoxin production in wheat as a consequence of irradiation is accompanied by a concomitant increase in fatty acid levels in the grain (Priyadarshini & Tulpule, 1979). The levels of free fatty acids in a grain may, therefore, determine the amount of aflatoxin produced to some extent. In view of these observations, an investigation was undertaken of the effects of several fatty acids that are normally present in food grains on aflatoxin production by a highly toxigenic strain of *A. parasiticus* in a chemically-defined liquid medium.

EXPERIMENTAL

A 4-day-old subculture of *A. parasiticus* (NRRL 2999) maintained on potato dextrose agar slants was used and a uniform spore suspension containing approximately 8×10^5 spores/ml was prepared in sterile distilled water. Aliquots (1 ml) were inoculated into each of the flasks containing 50 ml of the liquid synthetic medium described by Adye & Mateles (1964) with or without added fatty acids.

The mixed fatty acid (MFA) fraction was prepared from groundnut oil by saponification with alcoholic KOH followed by acidification. The individual fatty acids studied were lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0) and stearic acid (C18:0), all of which are saturated fatty acids and oleic acid (C18:1) and linoleic acid (C18:2) both of which are unsaturated. Each of the fatty acids was incorporated into 50 ml of the liquid medium (5 mM) and three drops of the emulsifier Tween-80 were added. The fat-fortified medium was sterilized at 15 psi for 15 min before inoculation with spore suspension and incubation at 28°C for 7 days. Each fatty acid was studied in duplicate in three separate experiments giving a total of six observations. At the end of the incubation period, the mycelium was separated from the medium, thoroughly washed with water and dried to constant weight. The washings were added to the medium and aflatoxin B_1 was extracted and estimated according to the method of Stoloff, Nesheim, Yin, Rodricks, Stack & Campbell (1971), using a Densicord Densitometer (Photovolt Corp., New York, USA) for quantitative determination.

Table 1. *Effect of fatty acids and natural fats on* in vitro *production of aflatoxin B_1 by* A. parasiticus

Fatty acid or oil added to medium*	Aflatoxin B_1 production (μg/50 ml medium)	Percentage increase or decrease in toxin production compared with control value	Fungal growth (mg)	Aflatoxin B_1 production (μg/100 mg fungal weight)
Control	165 ± 1·0		503 ± 2·6	32·8
Saturated fatty acids				
Lauric acid (C12:0)	ND	—	41·4 ± 4·20	—
Myristic acid (C14:0)	288 ± 9·5	+74	593 ± 29·8	48·6
Palmitic acid (C16:0)	348 ± 2·0	+110·9	582 ± 6·9	59·8
Stearic acid (C18:0)	364 ± 12·0	+120·6	579 ± 28·5	62·8
Unsaturated fatty acids				
Oleic acid (C18:1)	131 ± 15·0	−20·6	636 ± 5·4	20·6
Linoleic acid (C18:2)	ND	—	446 ± 53·2	—
Coconut oil	410 ± 6·0	+148·4	533 ± 1·7	76·9
Safflower oil	99 ± 8·0	−40·0	485 ± 0·5	20·4

*Each of the fatty acids was incorporated in the incubation medium at the 5 mM level; in the case of coconut or safflower oil, 100 mg of oil was added to each 50 ml sample of the medium.
Values in columns 2 and 4 are mean ± SEM for a total of six observations. ND = Not detectable.

In further experiments, either 40 mg of MFA prepared from groundnut oil or 100 mg each of coconut oil or safflower oil were incorporated into the liquid medium. These experiments were also repeated three times in duplicate.

RESULTS AND DISCUSSION

The effect of the MFA fraction from groundnut oil on aflatoxin B_1 production by *A. parasiticus* was studied *in vitro*. The results of a preliminary experiment indicated that the addition of MFA obtained from groundnut oil stimulated aflatoxin B_1 production (160 μg aflatoxin/50 ml medium) in a synthetic medium inoculated with a toxigenic strain of *A. parasiticus* compared with the control value (128 μg aflatoxin/50 ml medium) representing a 25% increase. This observation may have practical implications with respect to aflatoxin production in groundnut seeds having a high free fatty acid content.

The results for the individual fatty acids (Table 1) indicate that saturated fatty acids such as myristic acid (C14:0), palmitic acid (C16:0) and stearic acid (C18:0) stimulated aflatoxin B_1 synthesis whereas the unsaturated fatty acids, oleic acid (C18:1) and linoleic acid (C18:2) tended to inhibit toxin production when compared with the control value. In the case of saturated fatty acids, the increase in toxin production was proportional to the chain length of carbon atoms in the fatty acid. Although the fungal growth was slightly higher with these fatty acids, this was not related to the changes in toxin production; even when production was expressed per unit fungal weight, it was significantly greater with saturated fatty acids. In contrast, oleic acid which supported higher mycelial growth by about 27% compared with the control, inhibited aflatoxin B_1 production by about 20%. Linoleic acid on the other hand, did not affect mycelial growth but inhibited aflatoxin B_1 production almost completely. Lauric acid (C12:0) markedly inhibited the growth of the mycelium, showing an antifungal effect (41·6 mg fungal growth compared with the control value of 503 mg). No toxin was produced when lauric acid was added to the medium.

These results suggest that in addition to the absolute amount of fatty acids present, the ratio of saturated to unsaturated fatty acids may have a significant influence on aflatoxin production. The results of further studies support this hypothesis. The incorporation of 100 mg of either coconut oil or safflower oil into the medium produced markedly different results (Table 1). When coconut oil (90 to 95% saturated fatty acids) was added the amount of toxin produced was 410 μg/50 ml medium compared with only 99 μg/50 ml when safflower oil (5% saturated fatty acids and 95% unsaturated fatty acids) was added.

The observation that the composition of the fatty acid mixture can influence the level of aflatoxin production has considerable practical significance. This finding is in keeping with the report of Jemmali & Guilbot (1974) that the saponifiable fraction from wheat-germ lipids is responsible for higher aflatoxin production. It is further supported by the observation that aflatoxin production is independent of fungal growth (Priyadarshini & Tulpule, 1978) and that increased toxin production is associated with increased free fatty acid levels as seen in irradiated wheat (Priyadarshini & Tulpule, 1979). The results for individual fatty acids have considerable implications since the saponifiable fractions of the lipids present in various foodstuffs differ in the fatty-acid compositions. The ratio of saturated to unsaturated fatty acids present may well determine the amount of toxin produced under natural conditions. Results of recent studies by Schultz & Leudecke (1977) have suggested that the degradation of aflatoxin may be more pronounced in a system which contains unsaturated fats due to the possible formation of peroxides. This possibility is supported by the present observations with coconut oil and safflower oil. Coconut oil which contains predominantly saturated fatty acids supported significantly increased aflatoxin production whereas safflower oil which has a very high proportion of unsaturated fatty acids tended to suppress toxin synthesis.

The mechanism by which unsaturated fatty acids inhibit toxin production is not understood. It is possible that peroxide intermediates of polyunsaturated fatty acids may react with and modify the chemical nature of proteins and enzymes involved in aflatoxin production and/or the peroxide may degrade the aflatoxin B_1 at a faster rate. Further studies are in progress to elucidate the mechanism by which fatty acids exert their action.

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AN INVESTIGATION OF THE GENETIC TOXICOLOGY OF IRRADIATED FOODSTUFFS USING SHORT-TERM TEST SYSTEMS. 1. DIGESTION *IN VITRO* AND THE TESTING OF DIGESTS IN THE *SALMONELLA TYPHIMURIUM* REVERSE MUTATION TEST

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Abstract—The genetic toxicology of irradiated foodstuffs has been investigated by the use of a battery of short-term tests for genetic damage. This paper discusses appropriate methods for the preparation of food samples for testing by techniques involving micro-organisms and mammalian cells in culture. A new method of sample preparation by enzymatic digestion *in vitro* is described and its use in the testing of three irradiated foodstuffs by the *Salmonella typhimurium* reverse mutation test is reported. The results of the mutation tests provide further evidence of the lack of genetic toxicity of irradiated foods.

INTRODUCTION

The usefulness of irradiation as a means of food preservation has been recognized for many years. The application of the process has been delayed, however, by the need to demonstrate that irradiation has no adverse effects on the nutritional properties or the wholesomeness of food. In particular, there has been concern over the possible genetic effects of consuming foodstuffs treated with such a powerful mutagenic agent as ionizing radiation.

Extensive animal feeding trials have failed to reveal any adverse effects of irradiated food. However, it is generally recognized that traditional animal studies, as normally carried out, are rather insensitive to genetic effects because of the relatively small numbers of animals that can be used. In the case of pure chemicals this problem can be overcome, partially at least, by giving very large doses of the test compound. With food treated by physical processes such as irradiation, this expedient cannot be applied. The dose that can be administered in the case of a foodstuff is limited by nutritional considerations and increasing the dose of radiation applied is not a valid procedure on radiation chemical grounds. Furthermore, the chemical changes induced in food by processing are too complex to make the testing of all the compounds produced a practical or rational proposition.

As alternatives to long-term animal studies, a wide variety of sensitive short-term tests for genetic damage have recently been enjoying great popularity. While some of these methods, such as the dominant lethal and cytogenetic tests, have been in use for a long time as adjuncts to animal feeding trials, others have been introduced only recently.

It is generally accepted that none of these tests is sufficiently reliable to be used on its own as a screen for genetic toxicity but that batteries of complementary techniques determining different genetic endpoints should be used. For this reason, the International Project in the Field of Food Irradiation has organized a research programme in which irradiated foodstuffs are tested simultaneously by the following methods:

(1) The *Salmonella typhimurium*/mammalian microsome mutagenicity test of Ames;
(2) Sister chromatid exchange and point mutation assays in cultured mammalian cells;
(3) The micronucleus, sister chromatid exchange and germ-cell cytogenetic tests in mammals;
(4) Bone-marrow cytogenetics and DNA repair induction in mammals;
(5) The sex-linked recessive lethal mutation test in *Drosophila melanogaster*.

Some of these tests have been used previously in the study of irradiated foods but the systematic application of a broad test battery is new in this field.

Initially, three foodstuffs were chosen for testing: dried dates, fish (cod) and chicken. This choice was

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based largely on the extent of current interest in the use of radiation processing of these foods, but the inclusion of a product consisting largely of sugar (dates) is important because it is well known that irradiated sugars can, under certain circumstances, cause genetic damage in a variety of organisms (Schubert, 1969).

This paper is the first in a series describing our work and deals primarily with tests using *Salmonella typhimurium*. Bacterial mutation tests have been widely used, not only for testing chemical compounds, but also for the detection of mutagenicity in complex substances, including foodstuffs. Irradiated products, such as mixed vegetables (Van Kooij, Leveling & Schubert, 1978), fish (Joner, Underdal & Lunde, 1978) and onions (Hattori, Mori, Kaneko & Matsuyama, 1979) have been tested and found to give negative results in bacterial systems. The effects of heat treatment, a process comparable in many ways to irradiation, are also under intensive investigation using the Ames technique and significant positive results have been obtained with various heated foodstuffs (Commoner, Vithayathil, Dolara, Nair, Madyastha & Cuca, 1978; Nagao, Honda, Seino, Yahigi & Sugimura, 1977).

However, *in vitro* mutation tests have been designed primarily for use with chemicals that can be incorporated easily into culture media. Most foodstuffs cannot be dealt with so simply. In the studies cited above, samples have been prepared either by extraction of foodstuffs with various solvents or by the use of natural juices. These procedures, although simple, are unsatisfactory for a number of reasons which will be discussed below. This paper describes a novel method for preparing food samples, involving enzymatic digestion *in vitro*. The method was developed in an attempt to answer some of the objections to the use of extracts. It has already been described in outline elsewhere (Phillips & Elias, 1978).

Samples of the three foodstuffs have been prepared by digestion and tested for mutagenicity. The results obtained with the Ames test will be reported here; those obtained with mammalian cell tests will form the subject of the second paper in the series.

EXPERIMENTAL

Food samples and irradiation. A sample of dried but otherwise untreated dates was kindly supplied by the Nuclear Research Institute, Baghdad, Iraq. Fresh cod and chicken were obtained from local sources. Several frozen chickens were tested and found to yield bactericidal extracts. This effect was not observed with locally reared 'free-range' birds, which were therefore used in experiments on irradiation. Each foodstuff was handled as if it were for human consumption. The radiation doses used were in each case the maximum likely to be used in practice: 0·5 kGy for dates, 2·2 kGy for cod and 7·0 kGy for chicken. Samples of food were packed in polyethylene bags and irradiated under ambient conditions with 10 MeV electrons from a linear electron accelerator. Control samples were handled identically but not irradiated. Cod and chicken were stored at 0°C for 1 wk before being cooked in a pressure cooker and prepared for testing. Dates were stored at 20°C for 1 wk and used uncooked.

Preparation of samples for testing. Weighed portions of fish, de-boned chicken and stoned dates were homogenized in distilled water with an Ultra-Turrax® T45 homogenizer at 10,000 rev/min for 1 min. The volume of each homogenate was adjusted to give a calculated content of about 75–100 mg dry weight/ml. One portion of each homogenate was allowed to stand at 4°C for 3 hr and was then centrifuged at 5000 **g** for 15 min. The supernatants were filtered under pressure through Diaflo® PM10 ultrafiltration membranes (molecular weight cut-off 10,000; Amicon GmbH, Witten/Ruhr).

From the remaining homogenates, 100-ml samples were adjusted to pH 1·8 with 30% hydrochloric acid (Suprapur®, Merck AG, Darmstadt), and 300 mg of three times crystallized swine pepsin (Carl Roth KG, Karlsruhe) was added to each. The mixtures were incubated with shaking for 3 hr at 40°C. The pH rose during this period from 1·8 to about 2·4. After 3 hr, 5 N-NaOH was added until pH 7·5 was reached and then 1 g crystalline swine pancreatin (Carl Roth KG) and 50 mg sodium taurocholate were added. Incubation was then continued for 5 hr, with periodic addition of NaOH to maintain pH 7·5. The resulting digests were centrifuged and filtered in the same way as the extracts.

Ultrafiltrates were tested for protease activity, dry-weight content, and the content of sugars, amino acids, glycerol and fatty acids, by standard methods. Before mutation testing, the solutions were sterilized by filtration

Bacterial mutation assay. Salmonella typhimurium strains TA1535, 1537, 1538, 98 and 100 were kindly supplied by Dr. J. G. van Kooij of the Institute for Atomic Science in Agriculture, Wageningen, The Netherlands. Manipulation of the tester strains and plate-incorporation assays were carried out exactly according to the methods described by Ames, McCann & Yamasaki (1975); 2–8 × 10^7 bacterial cells from an overnight culture and 0·1 ml test material, with or without 0·5 ml S-9 mix (0·1 mg S-9 fraction/ml of mix), were added to 0·05 M histidine–biotin-supplemented top agar, which was layered on minimal glucose agar. The plates were incubated for 48 hr at 37°C.

The S-9 fraction was obtained from three pooled livers of male Sprague–Dawley rats (180–200 g), that were injected ip with 500 mg Aroclor 1254/kg in corn oil (200 mg/ml) 5 days before they were killed by decapitation and allowed to bleed.

For optimum mutagenesis with a particular compound, the amount of S-9 per plate may be of significance. We therefore used three different concentrations: 20, 35 and 50 μl S-9 fraction/plate. As the number of histidine revertants was not affected, only the results obtained with 50 μl per plate are presented.

To confirm both the reversion properties of the Salmonella strains and the activity of the S-9 mix, routine spot tests were carried out with 2-aminofluorene and sodium azide.

RESULTS

Detailed results of the analysis of extracts and digests are not presented here. However, Table 1

Table 1. *Efficacy of extraction and digestion procedures as determined by estimations of dry weight*

Foodstuff	Weight* of dried residue: Total homogenate (mg/ml)	Extract ultrafiltrate: mg/ml	Extract ultrafiltrate: % total	Digest ultrafiltrate†: mg/ml	Digest ultrafiltrate†: % total
Dates	80·0	66·6	83·0	69·6	87·0
Fish	69·5	11·4	16·4	62·0	89·2
Chicken	83·2	9·2	11·0	53·0	63·7

*Typical results.
†Values corrected for substances added during digestion.

shows the results of dry-weight determinations, which give a good overall impression of the efficacy of the digestion procedure. It should be remembered that these figures relate to soluble material of molecular weight below about 10,000. The figures for digests have been corrected, using appropriate controls, for low-molecular-weight material derived from enzyme preparations, acid and alkali added during digestion.

As would be expected from the composition of dates, digestion has little effect on the water-soluble proportion of this foodstuff. Most of the dry matter in dates consists of water-soluble sugars of small molecular weight. In contrast, comparatively little material can be extracted from fish or chicken before enzymatic breakdown. The digestion of fish is extremely efficient but chicken is apparently broken down to a lesser extent, probably because of its high fat content.

Undigested residues of dates were shown microscopically to consist mainly of cell walls, whereas intact nuclei and connective tissues made up the bulk of undigested fish and chicken. A relatively large quantity of fat was retained on the filter when chicken digests were filtered. Irradiation had no detectable effect on the extractability or digestibility of these foodstuffs. No protease activity could be detected in any of the ultrafiltrates.

Table 2 shows the actual amounts of dissolved material to which the bacteria in the mutation tests were exposed and the dry and wet weights of each foodstuff which gave rise to these quantities of soluble substances.

The results of the bacterial mutagenicity tests are shown in Table 3. No increase in the number of revertants was observed with any sample in any strain.

DISCUSSION

Most foodstuffs are unsuitable for direct incorporation into *in vitro* test systems. In the testing of processed foods this problem has until now been circumvented by the use of extracts and juices. Although solvent extraction is a simple and valuable technique which has led to the identification of a number of interesting mutagens, for example in heat-treated foodstuffs, the method is open to a number of criticisms:

(1) Foodstuffs vary widely in composition and structure. The reactions induced in them by physical treatments are extremely complex and the products have not all been identified. It is therefore very difficult to devise a method of extracting all possible toxic compounds from all foods.
(2) Chemically altered macromolecules, which are inactive biologically until broken down into small molecules, may be formed during processing. Also, toxic small molecules may become bound to macromolecules and only be released on digestion. Although not concerned directly with toxicology, the observation of Boley, Crosby & Roper (1979) that the extractability of artificial colourings from food is greatly enhanced by enzymatic digestion serves to illustrate this point.
(3) Active mutagens detectable in extracts may be extremely labile under normal conditions of digestion. Mutagenicity of food extracts may therefore have little relevance to the effects of the food on an intact organism. Conversely, digestion could activate otherwise innocuous substances. The detection of mutagens in extracts of fish treated with nitrite

Table 2. *Quantities of material added to the plates in mutation tests and their equivalents in the weight of original foodstuff*

Foodstuff	Dry weight (mg*/plate): Extract	Dry weight (mg*/plate): Digest	Amount of foodstuff (mg) extracted to give 0·1 ml sample: Wet weight	Amount of foodstuff (mg) extracted to give 0·1 ml sample: Dry weight
Dates	6·7	6·9	10·0	8·0
Fish	1·1	6·2	40·6	6·9
Chicken	0·9	5·3	27·7	8·3

*In 0·1 ml.

Table 3. *Results of bacterial mutagenicity tests of date, fish and chicken samples using five strains of* S. typhimurium *incubated with or without S-9 mix*

Type of sample		No. of revertant colonies/plate									
		TA100		TA1535		TA98		TA1537		TA1538	
		−S-9	+S-9	−S-9	+S-9	−S-9	+S-9	−S-9	+S-9	−S-9	+S-9
Date samples											
Control		102	104	12	15	30	35	5	5	22	26
Unirradiated:	extract	108	110	15	13	43	40	6	8	22	22
	digest	127	105	15	14	43	45	4	7	23	27
Irradiated:	extract	113	116	12	13	34	46	4	6	19	23
	digest	121	122	15	14	33	42	6	6	19	29
Fish samples											
Control		93	102	21	18	18	28	7	9	12	14
Unirradiated:	extract	90	109	22	20	20	24	7	6	15	19
	digest	100	99	24	23	22	26	9	12	20	22
Irradiated:	extract	85	88	20	18	18	24	8	9	16	15
	digest	101	104	22	21	25	24	7	11	20	23
Chicken samples											
Control		120	118	20	18	18	25	7	8	20	24
Unirradiated:	extract	127	120	28	24	15	23	8	7	17	24
	digest	110	112	30	20	17	19	5	6	16	22
Irradiated:	extract	124	137	27	18	14	21	7	4	18	22
	digest	129	119	25	19	12	13	4	6	15	20

Values are mean for 5, 6–12 and 6–15 plates, for date, fish and chicken samples, respectively.

and incubated at low pH, simulating stomach conditions (Marquardt, Rufino & Weisburger, 1977), is an example of such an effect.

(4) Reaction between food constituents and solvents is possible and, in addition, solvent residues may persist in the samples.

Some of these points may apply equally well to the detection of mutagenic chemical contaminants and additives in food, particularly where interaction between the toxic compound and food components is possible.

In an attempt to answer some of these criticisms, we have investigated the possibility of using digestion, *in vitro*, to prepare samples for testing in bacterial and cell-culture systems. As far as possible we have avoided methods that could be regarded as grossly unphysiological. For example, ultrafiltration was chosen rather than heat treatment or chemical precipitation for the purpose of removing enzymes and undigested material.

The method described above is obviously not entirely comparable with *in vivo* digestion. Certain processes, such as digestion by enzymes of the intestinal mucosa and of the gut flora are totally absent and a satisfactory method of mimicking fat absorption by the gut has not been found. To solve the problem of fats it will probably be necessary to perform a subsidiary organic solvent extraction just before filtration. An entirely satisfactory method of digestion *in vitro* would be difficult to devise and probably too complex for routine use. Nevertheless, the method used achieves the primary objective of reducing the great bulk of a variety of solid foods to manageable solutions of materials of comparatively low molecular weight. In addition, the method, to some extent at least, takes account of the possible interactions of food constituents with one another and with digestive secretions during digestion. The role of digestion in modifying the effects of ingested toxic substances has in the past been rather neglected. The method described here may prove useful in examining this question.

The results of the mutagenicity tests on irradiated dates, fish and chicken provide further evidence of the safety of the irradiation process. However, as discussed by van Kooij *et al.* (1978), two major problems are associated with the use of the Ames test on samples prepared from food. Firstly, many substances present in food extracts or digests can interfere with the growth of the test bacteria, either by stimulation or inhibition, and thus affect the outcome of the test. Secondly, there is specific interference from histidine, the cellular requirement for which by the tester strains forms the basis of the test. Digests of protein-rich foods contain comparatively high concentrations of histidine. These two factors severely limit the quantity of material that can be incorporated in the test. Although a more extensive study may provide solutions to these problems, it must be admitted that at present the *Salmonella* mutation test still has limitations when used with foodstuffs.

The second paper in this series will describe the use of cultured mammalian cells as test organisms for the detection of genetic toxicity in the same digests of irradiated food as were used for the Ames tests.

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LIVER-ENZYME INDUCTION IN LINDANE- AND CAPTAN-TREATED RATS

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Abstract—The effects of lindane and of captan on several liver-enzyme activities involved in the detoxication/activation of foreign chemicals was studied in male rats. The parameters selected were acid phosphatase as a marker of the lysosomal system involved in the cellular response to intoxication, azoreductase and *p*-nitroanisole-*O*-demethylase as detoxication enzymes, and the binding of benzo[*a*]-pyrene (BP) and dimethylaminoazobenzene metabolites to DNA as indicators of metabolic activation. The continuous feeding of diet containing 120 ppm lindane for 4 wk induced *O*-demethylase and BP–DNA binding (between two- and threefold), without affecting the other enzymes. BP–DNA binding was induced by as little as 24 ppm lindane. When lindane was given ip (3 × 20 mg/kg/day), induction of *O*-demethylase and benzo[*a*]pyrene activation again occurred and there was also a decrease in acid phosphatase. A similar but more pronounced pattern was observed in rats treated with phenobarbital (3 × 80 mg/kg/day). Methylcholanthrene induced the three activities in the decreasing order: BP–DNA binding, *O*-demethylase and acid phosphatase. Ingestion of diet containing 10 or 50 ppm captan for 4 wk did not alter any enzymatic activities. Levels of 3000 or 15,000 ppm led to a significant (fourfold) induction in *O*-demethylase and BP–DNA binding, but did not affect acid phosphatase activity. Thus not only organochlorine pesticides but also others such as the phthalimide, captan, are potent inducers of the mixed-function oxidases. The route of administration, dose and duration of treatment are predominant factors in their effects on the balance of enzymes involved in the detoxication or activation of chemicals.

INTRODUCTION

The process of enzyme induction and its pharmacological implications have been studied extensively using two types of inducers, namely phenobarbital, which primarily induces cytochrome *P*-450, and methylcholanthrene, which promotes the synthesis of another type of cytochrome, cytochrome *P*-448 (Conney, 1967; Lu, Kuntzman, West & Conney, 1971).

Other classes of compounds, namely organochlorine pesticides, are also known to stimulate the activity of microsomal mixed-function oxidases (Conney, 1967; Pélissier, Manchon, Atteba & Albrecht, 1975). For example, lindane (γ-hexachlorocyclohexane) induces aminopyrine *N*-demethylation and aniline hydroxylation, both of which are related to the synthesis of cytochrome *P*-450 (Pélissier & Albrecht, 1976). In addition, from the pattern of benzo[*a*]pyrene metabolism in lindane-, phenobarbital- and methylcholanthrene-treated rats it is possible to demonstrate that lindane is a phenobarbital-like inducer (Mikol & Decloître, 1979). However, studies of the effect of environmental pollutants on enzyme activities have yielded puzzling data, including some reports of inhibition of drug-metabolizing enzymes, in captan-treated rats for example (Truhaut, Do Phuoc & Nguyen, 1974; Peeples & Dalvi, 1978). The increasing production and use of pesticides, which interact with other chemicals in the environment, may lead to potentiation of or antagonism to metabolic processes.

The work described here was concerned with the effects of an agricultural insecticide, lindane, and of a widely used fungicide, captan, on various liver-enzyme activities involved in detoxication and activation processes. The cleavage of the molecule of carcinogenic azo dyes into non-carcinogenic products by azoreductase, and the activity of *p*-nitroanisole-*O*-demethylase, leading to the formation of a phenol which can subsequently be converted to a water-soluble glucuronide and excreted, were considered as detoxication reactions. The metabolic activation of chemical carcinogens (Miller & Miller, 1966; Miller, 1970) was determined for two carcinogens, benzo[*a*]-pyrene (BP) and dimethylaminoazobenzene (DAB), by trapping the metabolites on calf thymus DNA in a microsomal *in vitro* system as already described (Meunier & Chauveau, 1970; Mikol & Decloître, 1979). Acid phosphatase was used as a marker for

lysosomal function in liver cells in order to estimate the interference with catabolic and autophagic processes in the liver resulting from lindane and/or captan treatment.

EXPERIMENTAL

Chemicals. Lindane (purity 98%) was purchased from Merck AG (Darmstadt, Federal Republic of Germany). Captan in its commercial form "Orthocid" was a gift from the Chevron Company, Paris, France; it was 83% pure and contained 17% kaolin. DAB ring-labelled with ^{14}C was synthesized in the laboratory as described earlier (Meunier & Chauveau, 1970) and was diluted with ethanol to a concentration of 418 μg/ml (specific activity 20·25 μCi/μmol). [7,10-^{14}C]BP was purchased from the Radiochemical Center (Amersham, Bucks., UK) and diluted with ethanol to a final concentration of 402 μg/ml (specific activity 5·1 μCi/μmol). Calf-thymus DNA (grade V), NADPH (grade III), NADP, glucose-6-phosphate, nicotinamide and glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Animals and treatment. Male Sprague–Dawley rats (4 wk old and supplied by Cesal, Orléans) were fed a semi-synthetic diet containing 23% casein, 4% lactalbumin, 10% lard, 53% starch, 2·5% brewers yeast, 5% saline and 2·5 vitamin mixture. Lindane was mixed with this standard diet at 24, 120 and 240 ppm and captan at 10, 50, 3000 and 15,000 ppm. Groups of rats were fed these seven diets for 4 wk, while control rats received untreated diet. Other groups of rats (weighing 250–270 g) were given daily ip injections of lindane (20 mg/kg), methylcholanthrene (20 mg/kg) or phenobarbital (80 mg/kg) over a 3-day period. All the rats were fasted overnight and then decapitated between 08.00 and 09.00 hr.

Determination of liver-enzyme activities. The liver was homogenized in 0·25 M-sucrose to provide 100 mg wet tissue/ml, and the homogenate was centrifuged at 8500 **g**. Post-mitochondrial supernatants were quick-frozen at −50°C for use within 1 month, no significant loss of activity being observed during such storage. Microsomes were obtained by centrifugation of fresh post-mitochondrial supernatant at 50,000 rev/min for 45 min in a Ti 50 Spinco rotor.

Acid phosphatase activity was determined in aliquots of total homogenate according to de Duve, Pressmann, Gianetto, Wattiaux & Appelmans (1955) using β-glycerophosphate as the substrate. Aliquots of the post-mitochondrial supernatant were used for determinations of *p*-nitroanisole-*O*-demethylase activity as described by Zannoni (1972) and of azoreductase activity by the colorimetric disappearance of DAB as already reported (Decloître, Martin & Chauveau, 1975).

The binding of [^{14}C]BP and [^{14}C]DAB metabolites to DNA was determined as previously described (Decloître *et al.* 1975; Mikol & Decloître, 1979). The metabolic activation of carcinogens was performed in a medium containing 4 mg calf thymus DNA in buffer, pH 7·4 (10 mM-NaCl–50 mM-phosphate–100 mM-EDTA, 1:0·5:0·5, by vol.) and 2·4 μmol NADPH. [^{14}C]BP (80 nmol) was metabolized by 0·5 ml of post-mitochondrial supernatant (50 mg wet tissue) and DAB (93 nmol) by 0·2 ml of fresh microsomal suspension (70 mg wet tissue in 0·25 M-sucrose). After 15 min at 37°C, the reaction was stopped with sodium dodecyl sulphate (3·5 μM). For 40 hr, at 20°C and 40,000 rev/min, the DNA was purified by centrifugation on a caesium chloride gradient using a Ti 50 Spinco rotor. The DNA was precipitated with 20% trichloroacetic acid and was washed twice with ethanol and four times with ether. It was then hydrolysed in N-HCl for 20 min at 100°C. The amount of DNA was determined by UV spectrometry at 260 nm (1 OD: 36 μg/ml DNA), and its radioactivity was measured with Instagel in an Intertechnique scintillation counter. The results were expressed as nmol carcinogen-bound metabolites/g DNA.

The protein concentration of subcellular fractions was determined according to Hartree (1972).

Analysis of results. Statistical analyses were performed by one-way variance analysis and groups were compared by the Fisher-Snedecor *F* test.

RESULTS

Effect of treatments on body and liver weights

Assuming a daily intake of diet up to 10 g/day/100 g body weight, the ingestion of the experimental diets containing 24, 120 or 240 ppm lindane, and 10, 50, 3000 or 15,000 ppm captan would theoretically result in maximum daily intakes equivalent to 2, 10 and 20% of the lindane LD_{50} and 0·01, 0·03, 2 and 10% of the captan LD_{50}. After the 4-wk treatment period, the 32 control rats were 9 wk old and had reached an average body weight of 253 ± 5 g. The oral treatment with lindane did not significantly affect body weight or liver weight at any dose level and a similar lack of effect was observed with the lowest doses of captan.

However, in the group of rats fed 15,000 ppm captan, the daily intake of diet was 47% less than that of the control group. Consequently, the body and liver weights of treated rats were, respectively, lower than those of the controls by 46 and 18% ($P < 0·01$). Another consequence was a decrease in the assumed dose of captan, which never exceeded 7% of the LD_{50}. No significant variation was observed in the body weights of rats treated ip for 3 days, but the liver weights of these rats showed significant increases, those of the methylcholanthrene-, phenobarbital- and lindane-treated rats being, respectively, 10·2 ± 0·21 g, 10·4 ± 0·36 and 9·1 ± 0·25 g compared with the mean control value of 7·9 ± 0·22 g.

Effect of lindane ingestion on liver-enzyme activities

The continuous ingestion of lindane for 4 wk at the 120 ppm level did not modify either acid phosphatase activity or the two pathways of DAB inactivation/activation (Table 1). The azoreductase activity which detoxifies DAB remained constant irrespective of the dose of lindane, as did the binding of *N*-hydroxylated DAB metabolites to DNA. The inducing effect of lindane was apparent only with *p*-nitroanisole-*O*-demethylase and BP metabolic activation. A threefold increase in demethylase activity was observed in the liver of rats fed 120 ppm lindane.

The formation of BP metabolites bound to DNA seemed a sensitive parameter which responded to as little as 24 ppm lindane with an increase of 77%

Table 1. *Effects of 4-wk oral administration of lindane on liver-enzyme induction in rats*

Dietary level of lindane (ppm)	Acid phosphatase (μmol Pi/hr/mg protein)	*p*-Nitroanisole-*O*-demethylase (μmol *p*-nitrophenol/hr/100 mg protein)	DAB-azoreductase (nmol reduced DAB/min/mg protein)	DAB–DNA binding†	BP–DNA binding‡
0	3·06 ± 0·07 (6)	1·51 ± 0·50 (3)	0·67 ± 0·10 (7)	5·67 ± 1·03 (7)	66 ± 4·0
24	ND	ND	0·74 ± 0·12 (4)	7·28 ± 0·71 (6)	117 ± 18·3* (6)
120	2·97 ± 0·26 (5)	4·33 ± 0·72** (8)	0·85 ± 0·06 (8)	6·95 ± 1·10 (8)	163 ± 14·5** (9)
240	ND	ND	0·75 ± 0·07 (5)	6·21 ± 0·78 (4)	161 ± 20·2** (8)

DAB = Dimethylaminoazobenzene BP = Benzo[*a*]pyrene ND = Not determined

†As nmol DAB bound per g DNA/15 min/0·2 ml microsomal suspension.

‡As nmol BP bound per g DNA/15 min/0·5 ml post-mitochondrial supernatant.

Values are means ± SEM for the numbers of rats indicated in parentheses. Those marked with asterisks differ significantly (Fisher-Snedecor *F* test) from the control: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$.

Table 2. *Effects of acute ip administration of lindane, phenobarbital or methylcholanthrene on liver-enzyme induction in rats*

Compound	Dose level† (mg/kg/day)	Acid phosphatase	*p*-Nitroanisole-*O*-demethylase	Benzo[*a*]pyrene–DNA binding
Lindane	20	0·86**	2·16**	2·27**
Phenobarbital	80	0·33**	4·58**	4·42**
Methylcholanthrene	20	1·44**	2·13**	6·70**

†Given by ip injection on three consecutive days.
‡As nmol BP bound per g DNA/15 min/0·5 ml post-mitochondrial supernatant.
The results are expressed as ratios of the control values which were 3·6 ± 0·17 (8), 3·8 ± 0·48 (9) and 84·9 ± 5·84 (5) for acid phosphatase, *p*-nitroanisole-*O*-demethylase and BP–DNA binding, respectively, the figures in parentheses being the number of rats/group. Figures marked with asterisks differ significantly (Fisher-Snedecor *F* test) from the control: $^{**}P < 0.01$.

(Table 1) and showed a dose-related increase at 120 ppm, which added 147% to the control value, but 240 ppm lindane did not induce any further significant increase in BP activation.

Acute studies of liver enzyme induction

To compare the influence of the route of administration on the process of liver-enzyme induction, 20 mg lindane/kg/day was given to rats by ip injection. This was repeated on three consecutive days and induced a slight but significant decrease in acid phosphatase activity (−14%; Table 2), which did not occur when lindane was administered orally. On the other hand, acute ip doses of lindane enhanced *p*-nitroanisole-*O*-demethylase activity and BP activation by 116 and 127% respectively (Table 2). These effects were similar to those obtained with 120 ppm lindane in the diet.

As phenobarbital and methylcholanthrene are traditionally used to induce these enzyme systems, the extent of induction or inhibition of enzyme activities in lindane-treated rats was compared with that following phenobarbital or methylcholanthrene treatment (Table 2). The acid phosphatase activity was reduced more by phenobarbital pretreatment than by lindane, but was enhanced by methylcholanthrene. *p*-Nitroanisole-*O*-demethylase and BP-metabolite binding to DNA showed similar patterns of induction in lindane- and phenobarbital-treated rats, with twofold and fourfold increases, respectively. Pretreatment with methylcholanthrene induced a sevenfold increase in BP-metabolite binding to DNA but only a twofold increase in *p*-nitroanisole-*O*-demethylase.

Effect of captan ingestion on liver enzymes

The lowest dietary levels of captan (10 and 50 ppm) fed for 4 wk did not affect the enzyme activities measured (Table 3). Higher levels (3000 and 15,000 ppm captan) caused no change in the acid phosphatase activity, but both levels induced *p*-nitroanisole-*O*-demethylase activity causing approximately two- and fourfold increases respectively (Table 3).

The most significant effect of the captan diets was an induction of BP metabolic activation which increased BP-metabolite binding to DNA. A twofold increase of such metabolites resulted from the ingestion of 3000 ppm captan for 4 wk and a fourfold increase was obtained with 15,000 ppm (Table 3).

DISCUSSION

Effects of lindane

Ingestion of 24, 120 and 240 ppm lindane in the diet was equivalent to a maximum daily intake of about 2, 10 and 20% of the LD_{50}. Although high, these levels did not produce any effect on the growth of rats. The ingestion of lindane for 4 wk did not alter acid phosphatase activity whereas ip administration of lindane for 3 days, as well as that of phenobarbital, decreased the activity of this enzyme. On the other hand, the ip injections of methylcholanthrene increased the acid phosphatase activity, as was previously reported by Berg & Christoffersen (1974). According to Glaumann, Arborgh & Lideborg (1977), who showed an early decrease in the specific activities of lysosomal enzymes, 1 day after seven daily injections of phenobarbital, the reduction of acid phosphatase activity could reflect a decreased rate of synthesis or an increased rate of degradation of lysosomal enzymes. These events may contribute to the hypertrophy of endoplasmic reticulum and increased amounts of drug-metabolizing enzymes associated with phenobarbital treatment. A similar mechanism could be considered to explain the effect of acute ip administration of lindane; in liver-cell cultures treated with $2{\cdot}5 \times 10^{-5}$ M-lindane for 48 hr, a 20% inhibition of acid phosphatase was seen together with a marked induction of δ-aminolaevulinate synthase, the rate-limiting enzyme of haem biosynthesis (Roux, Bescol-Liversac, Guillam & Fournier, 1976). However, the lack of a significant variation in acid phosphatase activity in rats fed lindane for 4 wk suggests that a long-term induction of the microsomal drug-metabolizing system does not change the level of the lysosomal enzyme involved in intracellular catabolism, although early changes were noted after acute treatment.

Two enzyme activities were found to be extremely susceptible to induction by lindane. These were *p*-nitroanisole-*O*-demethylase and the enzyme responsible for the binding of BP metabolites to DNA. When the acute administration of lindane (3 × 20 mg/kg) was compared with an intake of 120 ppm for 4 wk, a similar rate of enzymatic induction was found for both groups, the activity being roughly two to three times that of the control group.

On the basis of *O*-demethylase induction of BP metabolic activation by acute administration of phe-

Table 3. *Effect of 4-wk oral administration of captan on liver-enzyme induction in rats*

Dietary level of captan (ppm)	Acid phosphatase (μmol Pi/hr/mg protein)	*p*-Nitroanisole-*O*-demethylase (μmol *p*-nitrophenol/hr/100 mg protein)	DAB-azoreductase (nmol reduced DAB/min/mg protein)	DAB–DNA binding†	BP–DNA binding‡
0	3·06 ± 0·07 (6)	2·8 ± 0·10 (3)	0·58 ± 0·07 (6)	3·83 ± 0·31 (6)	96 ± 9·6 (6)
10	ND	ND	0·66 ± 0·09 (6)	3·01 ± 0·37 (6)	96 ± 24·9 (6)
50	ND	ND	0·80 ± 0·09 (6)	4·61 ± 0·69	105 ± 7·4 (7)
3000	2·93 ± 0·26 (3)	4·4 ± 0·62 (4)	ND	ND	187 ± 11·8** (5)
15,000	2·87 ± 0·20 (3)	12·5 ± 0·61** (4)	ND	ND	398 ± 17·9** (5)

DAB = Dimethylaminoazobenzene BP = Benzo[*a*]pyrene ND = Not determined

†As nmol DAB bound per g DNA/15 min/0·2 ml microsomal suspension.
‡As nmol BP bound per g DNA/15 min/0·5 ml post-mitochondrial supernatant.
Values are means ± SEM for the numbers of rats indicated in parentheses. Those marked with asterisks differ significantly (Fisher-Snedecor *F* test) from the control: $^{**}P < 0{\cdot}01$.

nobarbital or lindane, it can be said that the latter exerts a comparable action to that of phenobarbital, but to a less marked extent under the given conditions. The effect of methylcholanthrene is different. This compound induced BP-metabolite binding to DNA to a much greater degree than phenobarbital but its induction of *O*-demethylase was relatively weak, being similar to that of lindane. Such data extend previous reports relating to the inducing effect of lindane on mixed function oxidases (Chadwick, Cranmer & Peoples, 1971; Conney, 1967; Pélissier & Albrecht, 1976) and to the phenobarbital-like effect of lindane (Mikol & Decloître, 1979).

The lack of sensitivity of DAB metabolic-activation/inactivation processes to induction by lindane may have been a consequence of either an inappropriate route and level of administration or a resistance to induction in the enzymes involved in DAB metabolism. The microsomal DAB azoreductase has already been reported to be resistant to induction by either phenobarbital (Decloître *et al.*, 1975) or lindane (Pélissier *et al.*, 1975). The activation of DAB through *N*-hydroxylation implied the existence of a cytochrome *P*-450-dependent pathway and a non-dependent one (Kadlubar, Miller & Miller, 1976). The synthesis of cytochrome *P*-450 in lindane-treated rats would not be efficient enough to have an effect upon the cytochrome *P*-450-dependent pathway.

Effects of captan

Although the oral LD_{50} of captan is extremely high for rats (about 15,000 mg/kg), the ingestion of 15,000 ppm captan in the diet delayed growth mainly because of a drastically reduced appetite during the first days of treatment.

Very low levels of captan in the diet (10 and 50 ppm, resulting in a maximum daily ingestion of 1 and 5 mg/kg) did not induce any of the enzyme activities that were measured. However, two of these activities, *O*-demethylase and BP activation, were induced significantly when the level of captan in the diet was 3000 or 15,000 ppm. These inductions were not associated with any change in acid phosphatase activity. In contrast, ip injection of captan has been reported to inhibit drug-metabolizing enzymes (Peeples & Dalvi, 1978; Truhaut *et al.*, 1974). This observation may be related to the very high toxicity of ip injections of captan (we have already observed a 100% death rate after a second ip injection of 20 mg captan/kg in weanling rats) but another possible explanation is that the two routes of administration are associated with different mechanisms of action. This possibility is suggested by the widely differing LD_{50} values (ip < 20 mg/kg; oral 15,000 mg/kg) as well as by the present results.

In terms of the LD_{50} values, dietary levels of 120 ppm lindane and 15,000 ppm captan are of comparable toxicity (10% of the LD_{50}), but captan is the more potent inducer. The enzyme-inducing activities of these levels of captan and lindane are, respectively, fourfold and less than threefold for both *p*-nitroanisole-*O*-demethylase and BP activation.

These data suggest that not only organochlorine insecticides, such as lindane, but also other classes of pesticides, like the phthalimide captan, are potent inducers of mixed-function oxidases. The detoxication

process effected by *O*-demethylase and the metabolic activation of BP through monooxygenase and epoxide hydratase activity were particularly sensitive to induction by lindane and captan. The route of administration, as well as the duration of treatment, appear to be predominant factors in the balance of enzyme activities determining the final appearance of toxic metabolites of a chemical. The dose-response relationship that seems to exist should be analysed carefully in assessments of the risk of pesticides.

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ABSORPTION AND METABOLISM OF THREE PHTHALATE DIESTERS BY THE RAT SMALL INTESTINE

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Abstract—The *in vitro* absorption of three phthalate diesters (dimethyl, di-*n*-butyl and di-(2-ethylhexyl) phthalates) and their corresponding monoesters was studied using an everted gut-sac preparation from the rat small intestine. All phthalate monoesters were absorbed in significantly greater quantity than their corresponding diesters. Esterases within the mucosal epithelium actively hydrolysed the phthalate diesters during absorption; 81·2% of the dimethyl, 95·5% of the di-*n*-butyl and 100% of the di-(2-ethylhexyl) esters were hydrolysed to the monoester. When the esterase activity of the mucosa was reduced by intragastric treatment of the rats with the organophosphate *S,S,S*-tributylphosphorotrithioate (8 mg/kg body weight), the absorption of di-*n*-butyl phthalate was significantly reduced. In contrast, the absorption of mono-*n*-butyl phthalate was unaffected by esterase inhibition. Intestinal esterases may play a key role in the absorption and metabolism of phthalate diesters.

INTRODUCTION

Phthalate diesters are carboxyl esters of *o*-benzenedicarboxylic acid. The phthalate diesters are used commercially in many ways, but their major use is as a plasticizing agent in the formulation of products based on vinyl chloride polymers (Graham, 1973). Their widespread distribution in the environment has been documented by several investigators (Corcoran, 1973; Giam, Chan, Neff & Atlas, 1978; Hites, 1973; Stalling, Hogan & Johnson, 1973).

Previous studies on the metabolism of phthalate diesters in rats have demonstrated that partial hydrolysis of the diester to form a monoester plus alcohol is a common step in their metabolism (Albro & Moore, 1974; Albro, Thomas & Fishbein, 1973; Lake, Phillips, Linnell & Gangolli, 1977; Rowland, Cottrell & Phillips, 1977). Various enzymes associated with the small intestine have been investigated for their role in this hydrolysis (Albro, Corbett & Latimer, 1976; Daniel & Bratt, 1974; Lake *et al.* 1977; Rowland, 1974).

The purpose of this study was to examine the *in vitro* intestinal absorption of three phthalate diesters (dimethyl, dibutyl and di-(2-ethylhexyl) phthalate) and their corresponding monoesters using the isolated perfused intestine, in order to estimate the importance of intestinal esterases in the *in vivo* absorption of phthalate diesters.

EXPERIMENTAL

Chemicals. Mono-*n*-butyl phthalate (MBP) and di-*n*-butyl phthalate (DBP) were obtained from Eastman Kodak Company (Rochester, NY). Monomethyl phthalate (MMP), dimethyl phthalate (DMP) and di-(2-ethylhexyl) phthalate (DEHP) were obtained from Pfaltz and Bauer, Inc. (Stamford, CT). MBP and MMP were extracted into chloroform to remove phthalic acid contamination and were recrystallized before use. [*Carbonyl*-^{14}C]phthalic anhydride was obtained from ICN Pharmaceuticals, Inc. (Cleveland, OH) and also through a generous gift from Dr. Steve Halladay of Dynapol (Palo Alto, CA). Mono-(2-ethylhexyl) phthalate (MEHP) and all [*carbonyl*-^{14}C]phthalate esters were synthesized by standard methods (Morrison & Boyd, 1966). The prepared MEHP migrated as a single spot and was well separated from phthalic acid and DEHP standards by thin-layer chromatography (Quanta gram plates, LQGOF, Pierce Chemical Company, Rockford, IL) using the solvent dichloromethane–ethanol–acetic acid, 80:20:1, by vol.). The purity of the [*carbonyl*-^{14}C]phthalate esters prepared was 98% or greater, as determined by thin-layer chromatography on the materials described above. *S,S,S*-Tributylphosphorotrithioate (DEF; technical) was obtained from Chemagro Agricultural Division, Mobay Chemical Corporation, Kansas City, MO.

Animals. Male Sprague–Dawley rats (300–400 g) from the National Institute of Health, Bethesda, MD, were used in all experiments. Room temperature (25°C) and a 12 hr light-dark cycle were controlled automatically. Rats were housed in hanging cages and were allowed food and water *ad lib.* The animals were fasted for 24 hr prior to the start of any experiment.

Distribution ratio determinations. The distribution ratio of each phthalate ester was determined using a dichloromethane–Krebs bicarbonate buffer (pH 7·4) system. Both the dichloromethane and buffer were saturated with the appropriate aqueous or organic phase before use. All esters were dissolved in the organic phase. Concentrations of 5–20 μmol/ml were used for the monoesters. Concentrations of 70–175 μmol/ml were used for the diesters. Equal volumes of both phases were used and agitated for 24 hr at 24 ± 1°C in order to allow sufficient time for equilibrium to occur. After equilibrium, the concentration of phthalate in the aqueous layer was determined by measuring the absorbance of the solution at 280 nm on a dual-beam spectrophotometer (Acta CIII, Beckman Instrument Co,. Fullerton, CA). A

standard curve was constructed to relate absorbance to concentration for each phthalate ester used. All determinations were done in triplicate.

Mucosal esterase activity. Test animals received 8 mg DEF/kg intragastrically, while under light ether anaesthesia. The DEF was dissolved in propylene glycol at 8 mg/ml. Groups of animals were killed by decapitation 6, 12 and 24 hr after dosing. The small intestine was severed at the pyloric valve and the first 45 cm of the intestine was removed. Its lumen was flushed with 50 ml saline. The intestine was then opened longitudinally and the mucosal surface was scraped with a glass slide. The resulting mucosal scrapings were placed into a chilled test tube with 10 ml ice-cold 0·25 M-sucrose and homogenized with a Polytron PCU-2 (Brinkman Instruments, Inc., Westbury, NY) for 7 sec at power setting No. 7. The resulting homogenate was centrifuged at 3000 **g** for 10 min at 0–4°C and the supernatant between the upper mucous layer and the lower pellet containing cell debris was pipetted into a chilled test tube. The protein content of the resulting homogenate was determined by the biuret method (Kachmar, 1970).

The esterase activity of the mucosal homogenates was studied by a modification of the method of Albro & Thomas (1973) for micellar substrates. The incubation mixture contained mucosal homogenate (2 mg protein/ml), sodium cholate (60 mM), DBP (6 mM and 0·1 M-Tris buffer (pH 8·2) to give 4 ml total volume. This mixture was incubated at 37°C for 60 min. Enzymatic hydrolysis was stopped by addition of 1 ml 4 N-HCl. The MBP and DBP was extracted into 5 ml ethyl acetate after the aqueous phase had been saturated with NaCl. A 1-ml aliquot of the ethyl acetate was mixed with 4 ml methanol containing 1·25 mM-DMP.

High-performance liquid chromatography was used to separate and quantify the MBP formed by the enzymatic hydrolysis of DBP. A reverse-phase column (Spectra-Physics, Santa Clara, CA; Spherisorb ODS, 10 μm, RPG 44) and a water–methanol mobile phase containing 10 mM-tetrabutylammonium bromide as an ion-pairing agent were used. Gradient elution from 50 to 70% methanol over 10 min (10-min delay) was used to separate the phthalate species. DMP was used as the internal standard. Peak area was measured by an AutoLab Computing Integrator (Spectra-Physics).

Everted-gut preparation. Animals were dosed intragastrically with 8 mg DEF/kg 6 hr before they were killed. The DEF was dissolved in propylene glycol at 8 mg/ml. All animals were killed by asphyxiation with ether, as this has been reported to help to preserve the structural integrity of the mucosal epithelium in the everted-gut preparation (Levine, McNary, Kornguth & LeBlanc, 1970). The everted-gut technique described by Blanchard & Straussner (1977) was used for this investigation.

The solutions used in the perfusing apparatus were modified Krebs bicarbonate buffers (pH 7·4). The serosal buffer contained KCl (5 mM), KH_2PO_4 (1 mM), $NaHCO_3$ (26 mM) and NaCl (122 mM). The mucosal buffer was identical except that it contained only 97 mM-NaCl plus 25 mM-sodium cholate. The mucosal buffer included 5 mM of the appropriate phthalate ester. The monoester forms were completely dissolved by constant stirring and gentle heating. Solubilization of the diester forms required vigorous mixing with the buffer on a Polytron PCU-2 (Brinkman Instruments, Inc.) at power setting No. 7 for 45 sec. No separation of the phthalate diesters from the emulsion was observed during the course of the experiment.

Mucosal incubation solution (100 ml) was added to each of two tubes suspended in a 37°C constant-temperature water-bath. Next, the appropriate radiolabelled tracer was added to each solution. Approximately 1·0 μCi of the diester form and 0·5 μCi of the monoester form was used. The tracer was mixed by bubbling O_2–CO_2, 95:5, through the solution. Just before the everted-gut segments were added, a 50-μl sample of the mucosal buffer was removed and the specific activity of the solution was determined. The mucosal buffer was aerated continuously with O_2–CO_2, 95:5, throughout the 1-hr collection period.

The serosal perfusing solution was aerated with O_2–CO_2, 95:5, for 45 min and then placed in 50-ml syringes on a constant infusion pump (Model 975, Harvard Apparatus Company, Millis, MA). This solution was pumped through preheating coils before it entered the perfusing apparatus. Zero time was established when serosal buffer filled the gut segment and began to pass out through the sample collection point. Thereafter, at 10-min intervals, 5 ml of serosal buffer was pumped through the segments at approximately 4 ml/min.

To determine the amount and type of phthalate ester present in the samples, a selective extraction procedure was used. After the sample had been acidified to pH 1·0 with concentrated HCl it was extracted three times with 5-ml aliquots of ethyl acetate. The ethyl acetate extracts were combined and evaporated to dryness under a stream of nitrogen. To the residue, 3 ml 0·4 M-Na_2CO_3 was added with an equal volume of an organic solvent. Cyclohexane was used when butyl or 2-ethylhexyl phthalates were being extracted, and diethyl ether was used when methyl phthalates were being extracted. The aqueous phase was extracted three times. The combined organic solvent was evaporated to dryness under nitrogen and the amount of ^{14}C present was determined by scintillation counting. The aqueous phase was transferred to a 20-ml glass scintillation vial and neutralized with concentrated HCl and the amount of ^{14}C present was determined by liquid scintillation counting. The diesters were extracted into the organic phase while the monoesters or more polar metabolites remained in the aqueous phase.

A Beckman LS-3133P (Beckman Instrument Co.) was used for scintillation counting, 10 ml of Handifluor liquid scintillation medium (Mallinkrodt Chemical Co., St. Louis, MO) being added to all samples counted.

Statistical comparisons were made using Student's t test at a confidence level of $P < 0{\cdot}05$ (Runyon & Haber, 1972).

RESULTS

Each phthalate monoester tested was significantly less lipophilic than the corresponding diester (Table 1) as measured by the distribution ratio between dichloromethane and Krebs buffer (pH 7·4). As the

Table 1. *Distribution ratio of phthalate esters*

Ester	Distribution ratio*
Monomethyl phthalate	0·11 ± 0·02
Mono-*n*-butyl phthalate	0·22 ± 0·05
Mono-(2-ethylhexyl) phthalate	1·62 ± 0·20
Dimethyl phthalate	412 ± 55
Di-*n*-butyl phthalate	832 ± 11
Di-(2-ethylhexyl) phthalate	1130 ± 35

*Determined in dichloromethane–Krebs bicarbonate buffer (pH 7·4).

length of the ester side chain increased from 1 to 4 to 8 carbon atoms, both the mono- and diester phthalates became more lipophilic. Intragastric administration of 8 mg DEF/kg significantly reduced the esterase activity of mucosal-cell homogenates toward DBP (Fig. 1). Maximum esterase inhibition was 77%, 12 hr after dosing. Enzyme activity was still over 50% below control levels 24 hr after DEF administration.

All the phthalate monoesters were absorbed by the everted gut sac in greater quantity than the corresponding diesters (Fig. 2). This difference in absorption became more pronounced as the length of the ester side-chain increased. Very little diester crossed the intestinal mucosa intact; 81·2% of the DMP and 95·5% of the DBP was hydrolysed to monoester before it reached the serosal perfusing solution. All of the 2-ethylhexyl phthalate entering the serosal buffer was in the monoester form.

Inhibition of mucosal esterases by 8 mg DEF/kg did not significantly alter the flux of MBP across the everted gut, but esterase inhibition significantly decreased total DBP absorption (Fig. 3). Approximately the same amount of intact diester entered the serosal buffer, but 35% less monoester was formed and absorbed by the everted gut preparation.

DISCUSSION

These experiments indicate that the hydrolysis of phthalate diesters to monoesters controls the intestinal absorption of these compounds. In these everted-gut experiments, phthalate monoesters were the primary species to cross the intestine and inhibition of esterase activity by DEF significantly reduced the total amount of phthalate diester that moved across the intestinal mucosa.

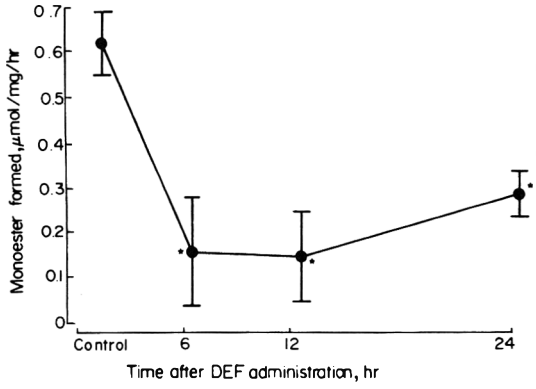

Fig. 1. The effect of *S,S,S*-tributylphosphorotrithioate (DEF), given intragastrically (8 mg/kg body weight), on the hydrolysis of di-*n*-butyl phthalate by rat intestinal epithelial homogenates. Data points represent the results (means ± SEM) of three animals. An asterisk indicates a statistically significant difference from the control value: $*P < 0·05$.

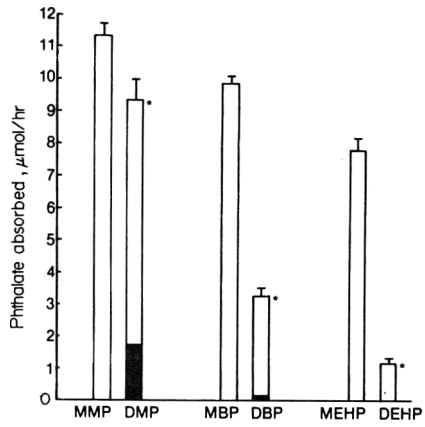

Fig. 2. A comparison of phthalate ester flux across the everted gut into the serosal perfusing solution, when 500 μmol mono- or diester phthalate was placed in the mucosal incubation solution. Comparisons include monomethyl phthalate (MMP) *v.* dimethyl phthalate (DMP), mono-*n*-butyl phthalate (MBP) *v.* di-*n*-butyl phthalate (DBP), and mono-(2-ethylhexyl) phthalate (MEHP) *v.* di-(2-ethylhexyl) phthalate (DEHP). The data represent the results (means ± SEM) of four experiments. An asterisk indicates a statistically significant difference between monoester (□) and diester (■) flux: $*P < 0·05$.

These results appear to be contrary to the pH-partition hypothesis of absorption. The distribution ratio measurements showed that the phthalate monoesters were much less lipophilic than the diesters, and they are probably ionized at pH 7·4 since the pKa of a phthalate monoester is about 3·0. Since, according to the pH-partition hypothesis, the amount of phthalate absorbed by the everted gut should correlate with the distribution ratio, one would have predicted greater absorption of diesters. The observed results suggest that a hydrophilic barrier to intestinal absorption is present. Such a barrier has been proposed by Houston, Upshall & Bridges (1974) and by Dietschy, Sallee & Wilson (1971). This hydrophilic barrier, or unstirred water layer, has been invoked to explain patterns of intestinal absorption of fatty acids (Wilson, Sallee & Dietschy, 1971) and may explain the results of this experiment. The ionic charge of the monoester would increase its penetration into the unstirred water layer and ultimately would result in a greater concentration of monoester at the mucosal surface for absorption.

Several investigators have found enzymes that are associated with the intestinal tract and are capable of hydrolysing phthalate diesters. Albro *et al.* (1976) purified from rat pancreas an enzyme that they called non-specific lipase. This enzyme was very efficient in hydrolysing one of the ester bonds of phthalate diesters. Daniel & Bratt (1974) showed that lipase (EC 3.1.13) from hog pancreas rapidly hydrolysed DEHP

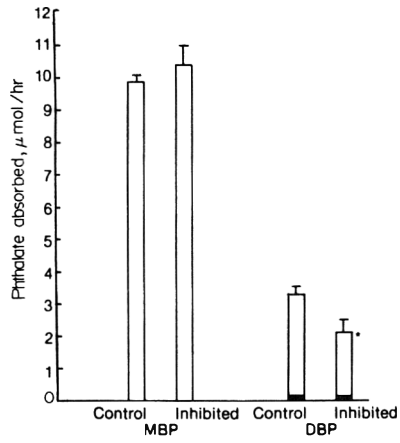

Fig. 3. The effect of esterase inhibition on mono-*n*-butyl phthalate (MBP) and di-*n*-butyl phthalate (DBP) absorption by the everted-gut preparation. Animals in the inhibited group received *S,S,S*-tributylphosphorotrithioate (DEF), intragastrically (8 mg/kg) 6 hr before testing. The data represent the results (means ± SEM) of four experiments; monoester (□); diester (■). An asterisk indicates a statistically significant difference in absorption between control and inhibited groups: $^*P < 0.05$.

to the monoester. Rowland *et al.* (1977) have demonstrated that the contents of the rat small intestine are capable of hydrolysing many phthalate diesters. However, intracellular esterases of the intestinal epithelium also hydrolyse phthalate diesters. Lake *et al.* (1977) showed that intestinal homogenates from several species were capable of hydrolysing phthalate diesters. The results of our study, in which luminal contents were absent, also support a potentially important role for intestinal mucosal esterases in facilitating the absorption of phthalate diesters.

Oral administration of 8 mg DEF/kg was shown to be effective in reducing intestinal epithelial esterase activity. This was demonstrated by its ability to inhibit esterase activity in mucosal cell homogenates for more than 24 hr and by its reduction of monoester formation in the everted gut segments. Silver & Murphy (1978) showed that 4 mg DEF/kg given by ip injection effectively inhibited liver esterases for 18 hr, but they did not examine its effect on intestinal esterases.

The permeability of the everted gut segments to intact phthalate diester is similar to the quantity of phthalate diester excreted in the urine, as reported by Albro & Moore (1974) and Albro *et al.* (1973). These authors found that 8% of orally administered DMP was recovered as intact diester while only 0·4 and 0·0% of orally administered DBP and DEHP, respectively, were recovered intact. These results suggest that the intestinal epithelium limits the amount of phthalate diester which is absorbed intact by the rat.

The flux of DBP across the everted gut was greatly influenced by the level of intestinal esterase activity. Intracellular hydrolysis of DBP to MBP appeared to control the rate of entry into serosal perfusing solution. Modifying the rate of hydrolysis, by pretreating with DEF, decreased the formation and therefore the absorption of MBP without affecting the absorption of intact DBP. Thus the level of epithelial esterase activity is the rate-limiting step in the net absorption of DBP by the everted-gut preparation.

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ACCUMULATION OF POLYCHLORINATED DIBENZOFURANS IN THE LIVERS OF MONKEYS AND RATS

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Abstract—A mixture of polychlorinated dibenzofurans (PCDFs) was analysed by gas chromatography, mass fragmentography and high-pressure liquid chromatography. The mixture consisted of 14% 1,2,7,8-tetra-chlorodibenzofuran (1,2,7,8-tetra-CDF), 35% 2,3,7,8-tetra-, 1% 1,2,4,7,8-penta-, 49% 1,2,3,7,8-penta-, 1% 2,3,4,7,8-penta- and 1% hexa-CDF. The mixture was administered orally to monkeys and ip to rats. The livers of the animals were analysed for the individual PCDFs. It was found that the accumulative properties of the different PCDFs varied. 2,3,4,7,8-Penta-CDF was the most highly accumulative in the livers of both species.

INTRODUCTION

Polychlorinated dibenzofurans (PCDFs) are highly toxic compounds (Bauer, Schulz & Spiegelberg, 1961; Vos, Koeman, van der Maas, ten Noever de Brauw & de Vos, 1970). These compounds were detected at levels similar to those of polychlorinated biphenyls (PCBs) in the livers of patients with Yusho, PCB poisoning that occurred in Japan in 1968 (Kuroki & Masuda, 1978; Nagayama, Masuda & Kuratsune, 1977). PCDFs are therefore assumed to be one of the causes of the symptoms of Yusho. Furthermore, PCDFs are possible environmental contaminants associated with PCBs, because commercial PCB formulations are known to be contaminated with PCDFs (Bowes, Mulvihill, Simoneit, Burlingame & Risebrough, 1975; Nagayama, Kuratsune & Masuda, 1976) and PCDFs have been found in the fly ash of some municipal incinerators (Buser, Bosshardt, Rappe & Lindahl, 1978). The biological activities of PCDFs of different structures have been reported to be very different from one another (Poland, Glover & Kende, 1976; Saeki, Ozawa & Yoshimura, 1977). However, the fate of the different PCDFs *in vivo* has not been examined except in a few studies in rats and mice (Morita & Oishi, 1977; Nagayama, Tokudome, Kuratsune & Masuda, 1980). Therefore we investigated the accumulation of different PCDFs in the livers and adipose tissue of monkeys and rats after the administration of a mixture of PCDFs. This paper describes the determination of PCDFs in the PCDF mixture and in the animal tissues using gas chromatography and mass fragmentography.

EXPERIMENTAL

Chemicals. A mixture of PCDFs was prepared by the chlorination of dibenzofuran (Nishizumi, Kuratsune & Masuda, 1975). 1,2,3,7,8-Penta-chlorodibenzofuran (1,2,3,7,8-penta-CDF) was synthesized from 3,4,5-trichlorophenol and 2,4,5-trichloronitrobenzene by the method of Kende, Wade, DeCamp, Ridge & Poland (1974), and was found to have m.p. 225–227°C and a proton magnetic resonance in $CDCl_3$ of δ 8·40 s, 7·71 s and 7·65 s (FT NMR, JNM-FX 100; JEOL, Tokyo). 1,2,7,8-Tetra-CDF was similarly synthesized from 3,4-dichlorophenol and 2,4,5-trichloronitrobenzene and was then chromatographed on a silica-gel column eluted with *n*-hexane in order to separate it from by-products. It had m.p. 209–210°C and a proton magnetic resonance in $CDCl_3$ of δ 8·44 s, 7·71 s, 7·57 d (J = 8·8 Hz) and 7·43 d (J = 8·8 Hz). Other authentic PCDF isomers were obtained by the syntheses described in the previous papers (Kuroki & Masuda, 1978; Rappe, Buser, Kuroki & Masuda, 1979) or were kindly donated by Dr. Pomerantz, Food and Drug Administration, Washington, DC or Dr. Kende, University of Rochester, Rochester, NY, USA. The purities of these PCDF isomers were found to be more than 99% by the gas chromatographic (GC) and GC–mass spectrometric methods described below. Kanechlor 400 was supplied by Kanegafuchi Chemical Industry Co., Osaka. Basic alumina for column chromatography was obtained from Woelm Pharma, Eschwege, Federal Republic of Germany. All of the other chemicals and reagents were of the purest grades available.

Experimental procedure. Adult rhesus monkeys, weighing 4–6 kg were purchased from Kyudo Co. Ltd., Tosu. They were individually housed in stainless-steel cages and were given water and monkey chow (Oriental Kobo Co., Chiba, Japan) *ad lib.* Kanechlor 400 and the PCDF mixture were dissolved in salad oil and were injected into bananas (each weighing about 50 g). One male and two female monkeys were each given daily for 26–32 days a banana containing doses of 0·25 or 0·5 mg Kanechlor 400/kg and 1·25 or 2·5 μg

PCDF mixture/kg. The monkeys were killed at the end of the treatment period and the livers and adipose tissue were removed for analysis.

Young male Wistar rats, 4-wk old and weighing about 100 g, were purchased from Kyudo Co. Ltd. They were given water and rat chow (Nippon Clea Co. Ltd., Tokyo) *ad lib.* The mixture of PCDFs was dissolved in corn oil and four rats were each given a single ip dose of 10 mg PCDFs/kg. The rats were kept in a stainless-steel cage without bedding for 5 days and then killed. The liver was dissected out and analysed for PCDFs.

Clean-up procedures. The method of cleaning up the samples for the analyses of PCBs and PCDFs was essentially the same as that described previously (Kuroki & Masuda, 1978; Nagayama *et al.* 1977). The tissue samples from monkeys were saponified with 1 N-KOH–ethanol solution, extracted with *n*-hexane, fractionated on a silica-gel column, and then chromatographed on a column of alumina eluted successively with *n*-hexane–methylene chloride (49:1, v/v) and *n*-hexane–methylene chloride (4:1, v/v). The former eluate was analysed for PCBs by GC. The latter eluate was analysed for PCDFs by GC and GC-mass fragmentography (see below).

The liver samples from rats were extracted with chloroform–methanol (2:1, v/v) by the method of Folch, Lees & Sloane Stanley (1957) and the extracts were chromatographed on a silica-gel column eluted with *n*-hexane. The eluate was analysed for PCDFs by GC.

Instrumental analysis. The analyses of PCBs were carried out on a gas chromatograph (GC-4BM; Shimadzu, Kyoto) fitted with an electron-capture detector (Nickel 63) and a glass column (3 mm × 2 m) packed with Chromosorb W AW-DMCS (60–80 mesh) coated with 2·5% SE-30 (Nishio Ind. Co., Tokyo). The temperatures of the inlet, column and detector were maintained at 200, 170 and 230°C, respectively. Pure nitrogen (99·999%) was used as the carrier gas at a flow rate of 30 ml/min. The PCBs were quantified by comparing the total heights of the GC peaks with those of Kanechlor 400.

For the analysis of PCDFs the gas chromatograph (Shimadzu GC-5A) used was fitted with an electron-capture detector (Nickel 63) and a glass column (3 mm × 5 m) packed with 1·5% Apiezon L (Wako Pure Chemical Co., Osaka) on Chromosorb W AW-DMCS (60–80 mesh) or 1·5% OV-17 (Nishio Ind. Co.) on Chromosorb W AW-DMCS (80–100 mesh). The temperatures of the inlet, column and detector were 250, 240 and 260°C, respectively. The carrier gas used was 99·999% pure nitrogen at a flow rate of 30 ml/min. Individual PCDFs were quantified by comparing their GC-peak areas with those of authentic compounds.

Mass fragmentography was carried out using a gas chromatograph–mass spectrometer (JGC-20K-JMS-D-100; JEOL, Tokyo), fitted with a glass column (2 mm × 2 m) packed with Chromosorb W AW-DMCS (80–100 mesh) coated with 3% Apiezon L. The temperatures of the inlet, column, enricher and ionizing chamber were 240, 235, 250 and 150°C, respectively. The ionizing energy was 25 eV and the ionizing current was 300 μA. The high-pressure liquid chromatograph (HPLC) (Model ALC/GPC-204; Waters Associates, Inc., Milford, MA, USA) was fitted with a 6000A pump (Waters Associates, Inc.) and a column (4 mm × 30 cm) packed with reverse-phase μBondapak C_{18} (Waters Associates, Inc.). The column was eluted with methanol–water (9:1, v/v), and the absorbance of the eluates at 254 nm was determined.

RESULTS

The composition of PCDF mixture

The gas chromatography of the PCDF mixture was carried out on columns packed with Chromosorb W AW-DMCS coated with either Apiezon L or OV-17 and the retention times of the individual PCDFs were compared with those of the authentic compounds (Table 1). As shown in Fig. 1, on the Apiezon L column, peaks A, B, C, D and E of the PCDF mixture had the same retention times as those of 1,2,7,8-tetra-, 2,3,7,8-tetra-, 1,2,4,7,8-penta-, 1,2,3,7,8-penta- and 2,3,4,7,8-penta-CDFs, respectively. Coincident retention times of PCDFs in the mixture with those of these five authentic compounds were also observed on the OV-17 column. Mass fragmentography indicated that a hexa-CDF (represented by peak F) was present in the PCDF mixture (Fig. 2). However, the GC-retention time of the hexa-CDF did not correspond with that of 1,2,3,4,7,8-hexa-, 1,2,3,6,7,8-hexa- or 2,3,4,6,7,8-hexa-CDF (Table 1) on either the Apiezon L or the OV-17 column. The identities of the five components of the PCDF mixture that had been identified by GC (Fig. 1) were confirmed by HPLC: the HPLC-retention times of the five components of the mixture were identical with those of the five authentic PCDFs. The proportions of the six PCDFs in the mixture were determined by quantitative GC on an Apiezon-L column, the hexa-CDF being tentatively quantified by comparing its peak areas with those of 1,2,3,4,7,8-hexa-CDF. The mixture consisted of 14% 1,2,7,8-tetra-, 35% 2,3,7,8-tetra-, 1% 1,2,4,7,8-penta-, 49% 1,2,3,7,8-penta, 1% 2,3,4,7,8-penta- and 1% hexa-CDFs; the amounts of other PCDFs present were negligible.

Table 1. *Gas chromatographic data for authentic samples of PCDF isomers*

PCDF	Relative retention time* on a column packed with Chromosorb W AW-DMCS coated with	
	Apiezon L	OV-17
2,3,6,8-Tetra-CDF	0·90	0·89
1,2,7,8-Tetra-CDF	0·90	0·96
2,3,7,8-Tetra-CDF	1·00	1·00
1,2,4,7,8-Penta-CDF	1·47	1·35
1,2,6,7,8-Penta-CDF	1·61	1·66
1,2,3,7,8-Penta-CDF	1·65	1·55
1,2,3,6,7-Penta-CDF	1·67	1·63
2,3,4,6,7-Penta-CDF	1·70	1·86
2,3,4,7,8-Penta-CDF	1·82	1·78
1,2,3,4,7,8-Hexa-CDF	2·96	2·62
1,2,3,6,7,8-Hexa-CDF	2·98	2·66
2,3,4,6,7,8-Hexa-CDF	3·17	3·02

*The retention times are relative to those of 2,3,7,8-tetrachlorodibenzofuran (2,3,7,8-tetra-CDF) which were 18·7 and 18·4 min on the Apiezon L and OV-17 columns, respectively. Details of the gas chromatographic method are given in the text.

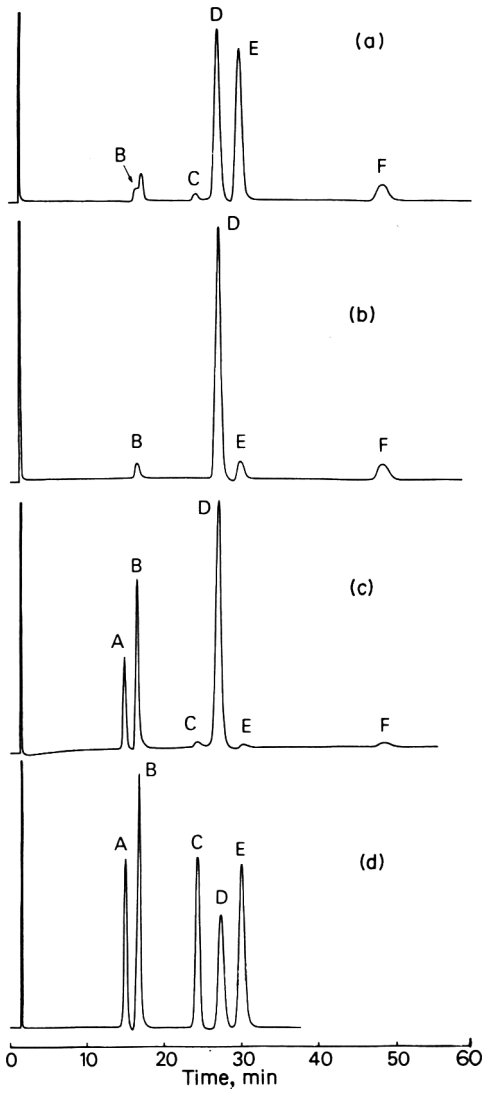

Fig. 1. Gas chromatograms of PCDFs in (a) the liver of a monkey (no. 1 in Table 2), (b) the liver of a rat (no. 3 in Table 3), (c) the synthesized PCDF mixture and (d) a mixture of authentic PCDFs. Peaks A, B, C, D, E and F correspond, respectively, to 1,2,7,8-tetrachlorodibenzofuran (1,2,7,8-tetra-CDF), 2,3,7,8-tetra-CDF, 1,2,4,7,8-penta-CDF, 1,2,3,7,8-penta-CDF, 2,3,4,7,8-penta-CDF and hexa-CDF.

Analysis of PCDFs in animal tissues

Gas chromatograms of the PCDF fractions obtained from the livers of the monkey and rat are shown in Fig. 1. The retention times of four peaks (B, C, D and E) coincided with those of 2,3,7,8-tetra-, 1,2,4,7,8-penta-, 1,2,3,7,8-penta- and 2,3,4,7,8-penta-CDF, respectively, on both the Apiezon L and the OV-17 columns, but the identity of peak F was not determined by GC. The PCDF fractions from the adipose tissue and liver of the monkeys were also determined by mass fragmentography (Fig. 2). Four peaks (B, C, D and E) of the mass fragmentograms corresponded well with those of the authentic samples of 2,3,7,8-tetra-, 1,2,4,7,8-penta-, 1,2,3,7,8-penta and 2,3,4,7,8-penta-CDF, respectively. Peak A, corresponding to 1,2,7,8-tetra-CDF, was observed only in the PCDF fraction from the adipose tissue of monkey

Table 2. *Levels of PCBs and PCDFs in the livers and adipose tissue of monkeys given oral doses of PCBs and PCDFs for up to 32 days*

Monkey no. (sex)	Duration of treatment (days)	Total intake of PCBs (Kanechlor 400), mg	Total intake of PCDFs, μg	Tissue (weight, g)	Levels* of PCBs	Levels* of PCDF of type... A	B	C	D	E	F	Total	PCDFs:PCBs ratio, × 1000
1 (M)	32	86·0	430	Liver (117)	1·7 (0·2)	ND	0·3 (0·02)	0·2 (0·4)	2·7 (0·2)	1·9 (5·2)	0·3 (0·8)	5·4 (0·15)	3·2
				Adipose	8·6	ND	ND	ND	0·7	0·2	ND	0·9	0·1
2 (F)	29	88·1	440·5	Liver (148)	0·8 (0·1)	ND	0·9 (0·09)	0·1 (0·3)	2·7 (0·2)	0·5 (1·7)	0·04 (0·1)	4·24 (0·14)	5·3
				Adipose	25	0·4	8·5	0·4	16	1·0	ND	26·3	1·1
3 (F)	26	30·3	151·3	Liver (140)	0·04 (0·02)	ND	ND	ND	1·0 (0·2)	0·1 (0·8)	ND	1·1 (0·10)	27·5
				Adipose	1·2	ND	ND	ND	0·2	ND	ND	0·2	0·2

ND = Not detected PCDFs = Polychlorinated dibenzofurans PCBs = Polychlorinated biphenyls A = 1,2,7,8-Tetra-chlorodibenzofurans (1,2,7,8-tetra-CDF)
B = 2,3,7,8-Tetra-CDF C = 1,2,4,7,8-Penta-DF D = 1,2,3,7,8-Penta-CDF E = 2,3,4,7,8-Penta-CDF F = Hexa-CDF

*Values are for PCDF concentration (ppb), for PCB concentration (ppm) and, in brackets, for the concentration of PCBs and each PCDF in the liver expressed as a percentage of the total intake.

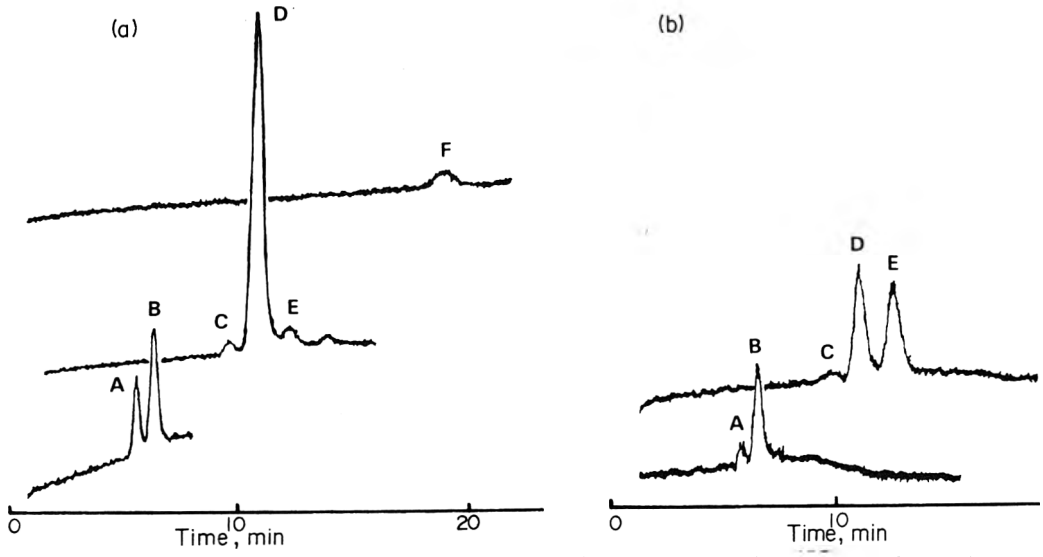

Fig. 2. Mass fragmentogram of (a) the synthesized PCDF mixture (peaks A and B at *m/e* 306, C, D and E at *m/e* 340 and F at *m/e* 374) and (b) the PCDF fractions from the liver of monkey 2 (peaks A and B; *m/e* 306) and from the liver of monkey 1 (peaks C, D and E; *m/e* 340). Peaks, A, B, C, D, E and F correspond, respectively, to 1,2,7,8-tetra-chlorodibenzofuran (1,2,7,8-tetra-CDF), 2,3,7,8-tetra-CDF, 1,2,4,7,8-penta-CDF, 1,2,3,7,8-penta-CDF, 2,3,4,7,8-penta-CDF and hexa-CDF.

2. The amounts of PCDFs extracted from the tissues of the monkeys were insufficient to determine hexa-CDF by mass fragmentography.

The concentrations of PCBs and PCDFs in the livers and adipose tissue of the monkeys that were fed the Kanechlor 400 and PCDFs for 26–32 days are shown in Table 2. The concentrations of PCBs and PCDFs in the tissues varied between the three animals. However, the concentrations of PCBs were much higher than those of PCDFs in both the livers and the adipose tissue of the monkeys, mainly because the concentration of PCBs administered was about 200 times greater than that of the PCDFs. The concentrations of the individual PCDFs found in the tissues varied (Table 2), partly because of the differences in the proportions of the individual PCDFs in the PCDF mixture and partly because of variations in the degree of accumulation of the different PCDFs. The levels of the individual PCDFs in the liver were expressed as a percentage of the total intake in order to assess the accumulative properties of the different PCDFs (Table 2). 2,3,7,8-Tetra-CDF was more accumulative than 1,2,7,8-tetra-CDF; 0·02 and 0·09% 2,3,7,8-tetra-CDF was found in the livers of monkeys 1 and 2, respectively, while no 1,2,7,8-tetra-CDF was detected in either animal. Neither of the tetra-CDFs was detected in the liver of monkey 3. The three penta-CDFs tested were more readily retained in the livers than were the tetra-CDFs. Of the penta-CDFs, 2,3,4,7,8-penta-CDF was the most accumulative, being found at levels of 0·8–5·2%, while the levels of the other penta-CDFs were less than 0·4%. The retention of the hexa-CDF was similar to those of the penta-CDFs in the livers of monkeys 1 and 2 but no hexa-CDF was detected in monkey 3.

The concentrations and percentage retention of PCDFs in the livers of rats given a single ip dose of PCDFs and killed 5 days later are shown in Table 3. There was less variation in the levels of PCDFs among the rats than there was among the monkeys. About 11% of the total PCDFs injected were accumulated in the livers of rats. The percentages were much higher than those in the monkeys (0·10–0·15%), probably because of the different methods of administration. Of the six PCDFs, 1,2,7,8-tetra- and 1,2,4,7,8-penta-CDF were the least accumulative in

Table 3. *Levels of PCDFs in the livers of rats killed 5 days after a single ip dose of 1 mg PCDFs*

Rat no.	Hepatic level* of PCDF of type....						
	A	B	C	D	E	F	Total
1	ND	0·6 (1·0)	ND	15·5 (18)	1·4 (81)	2·6 (109)	20·1 (12)
2	ND	0·8 (1·5)	ND	12·5 (17)	1·2 (80)	2·2 (104)	16·7 (11)
3	ND	1·8 (2·5)	ND	20·2 (21)	1·6 (82)	2·9 (108)	26·5 (13)
4	ND	0·3 (0·6)	ND	9·8 (14)	1·1 (76)	2·1 (107)	13·3 (9)

PCDFs = Polychlorinated dibenzofurans A = 1,2,7,8-Tetra-chlorodibenzofuran (1,2,7,8-tetra-CDF)
B = 2,3,7,8-Tetra-CDF C = 1,2,4,7,8-Penta-CDF D = 1,2,3,7,8-Penta-CDF
E = 2,3,4,7,8-Penta-CDF F = Hexa-CDF

*The values are for PCDF concentration (ppm) and, in brackets, for the concentration of each PCDF in the liver expressed as a percentage of the total intake.

the livers of rats, and were not detected in the livers of any of the rats (at detection limits of 0·05 and 0·7% for 1,2,7,8-tetra- and 1,2,4,7,8-penta-CDF, respectively). In rats, as in monkeys, 2,3,7,8-tetra-CDF was more accumulative (0·6–2·5%) than was 1,2,7,8-tetra-CDF, and 1,2,3,7,8-penta-CDF showed an intermediate degree of retention (14–21%). 2,3,4,7,8-Penta-CDF and hexa-CDF were the most accumulative of the PCDFs in rats, about 80 and 100%, respectively, of the amounts injected being concentrated in the liver. High retention of 2,3,4,7,8-penta-CDF was also observed in the livers of monkeys.

DISCUSSION

By the chlorination of dibenzofuran, Gray, Dipinto & Solomon (1976) obtained 1,2,7,8-tetra- and 2,3,7,8-tetra-CDF as the major products. Our PCDF mixture was also produced by the chlorination of dibenzofuran and mainly consisted of 1,2,7,8-tetra-, 2,3,7,8-tetra and 1,2,3,7,8-penta-CDF (Fig. 1). The PCDF mixture was probably synthesized by the chlorination of dibenzofuran to 1,2,7,8-tetra- and 2,3,7,8-tetra-CDFs and the subsequent chlorination of some of the tetra-CDFs to 1,2,3,7,8-penta-CDF.

Since the individual PCDFs in the mixture could be identified and then quantified at ng levels, the accumulation of the individual PCDFs in the livers of monkeys and rats was investigated. It was found that the accumulation in the livers of PCDFs of different structures varied. 2,3,4,7,8-Penta-CDF was one of the PCDFs most readily accumulated in the livers of both monkeys and rats, even though it was administered to the two species by different routes. The high accumulation of 2,3,4,7,8-penta-CDF has previously been observed in the livers of patients with Yusho (Kuroki & Masuda, 1978; Rappe, Buser, Kuroki & Masuda, 1979). In addition to its high retention in the liver, 2,3,4,7,8-penta-CDF has been shown to have a strong binding affinity for hepatic cytosol protein in mice and to be a powerful inducer of hepatic aryl hydrocarbon hydroxylase in chick embryos (Poland *et al.* 1976). Therefore, in order to understand the symptoms of Yusho, it is important to continue the investigations of the accumulative properties of this PCDF isomer in the liver and of its toxicology in different species.

The large differences between the levels of accumulation of 2,3,4,7,8-penta-CDF in the livers of the monkeys and rats (0·8–5·2 and 76–82%, respectively) is not considered to be a species difference but to be due to the different routes of administration—relatively long-term oral administration at low levels to monkeys but a single ip injection at a comparatively high level to rats. Evidence for this is derived from the results of our earlier animal experiments on mice (Nagayama *et al.* 1980), in which it was found that more than 80% of the PCDFs administered were eliminated when the compound was given orally to mice, and that more than 95% PCDFs that were absorbed were concentrated in the liver.

Poland *et al.* (1976) found that like 2,3,4,7,8-penta-CDF, 2,3,7,8-tetra-CDF and 1,2,3,7,8-penta-CDF also had strong binding affinities for the hepatic cytosol of mice *in vitro* and that the affinities of the three PCDFs were of similar magnitudes. In our animal experiments these three PCDFs showed relatively high accumulation in the livers of monkeys and rats. However, their levels of accumulation in the livers were markedly different, increasing in the order of 2,3,7,8-tetra-CDF, 1,2,3,7,8-penta-CDF and 2,3,4,7,8-penta-CDF (Tables 2 & 3). The reason for this difference is not clear at present. In order to elucidate the relationship between the chemical structures of the PCDFs and their biological activities further animal experiments using other PCDF isomers are necessary.

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ANILINE: EVIDENCE FOR AN ENTEROGASTRIC CYCLE IN THE RAT

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Abstract—The autoradiographic distribution of ^{14}C-aniline hydrochloride was studied in male Fischer 344 rats following iv administration of 3, 30 or 100 mg/kg. The highest initial concentrations of radioactivity associated with ^{14}C-aniline or its metabolites were found in the blood, liver, kidney, bladder and gastro-intestinal tract. At the 100 mg/kg dose, the spleen was the only organ that did not show a time-dependent decrease in radioactivity over 24 hr. After 0·5 and 6 hr, radioactivity was concentrated in the stomach and small intestine but there was comparatively little activity in the colon. Analysis of the stomach and jejunum for aniline and its metabolites using gas chromatography–mass spectroscopy showed that aniline was predominant in the gastric contents (aniline:acetanilide, 7·3:1) and that acetanilide was the principal compound in the jejunal contents (acetanilide:aniline, 15:1). Trace amounts of *o*- and *p*-aminophenol and of acetaminophen were also detected. High levels of acetanilide in the small intestine were probably due to the metabolism of aniline in the mucosa of the gut. It was concluded that after iv injection aniline is rapidly concentrated in the stomach but that aniline and acetanilide are reabsorbed in the small intestine, thus completing an enterogastric cycle. Therefore the gastro-intestinal tract appears to be a major site for the metabolism of aniline in the rat.

INTRODUCTION

Aniline is the simplest of the aromatic amino compounds and is important as an intermediate in the manufacture of dyes, pharmaceuticals, plastics, photographic chemicals and explosives. Aniline has long been associated with methaemoglobinaemia in man and experimental animals and more recently has been shown to be tumorigenic in rats (National Cancer Institute, 1978). Following oral administration in the rat 96% of ^{14}C-aniline-associated activity is recovered in the urine, principally as the sulphate of *N*-acetyl-*p*-aminophenol (Kao, Faulkner & Bridges, 1978). Aniline is a weak organic base (pK_a 4·6) which is concentrated in the stomach following parenteral administration (Brodie & Hogben, 1957); nevertheless, only 2% of a single dose of the compound or its metabolites can be recovered in the faeces of rats even after oral administration (Bus, Rickert, Norton & Gibson, 1978; Kao *et al.*, 1978). Using whole-body autoradiography and gas chromatography–mass spectrometry we investigated the disposition of aniline hydrochloride (aniline HCl) and its principal metabolites in the stomach and intestine of the rat following iv administration.

EXPERIMENTAL

Radiolabelled aniline (78·1 mCi/g; New England Nuclear, Boston, MA) was dissolved in ethanol, evaporated under nitrogen and redissolved in sterile saline for injection. Aniline HCl (Eastman Chemicals, Rochester, NY) was added to provide the appropriate specific activity.

Male Fischer 344 rats, weighing 250–260 g were obtained from Charles River Breeding Laboratories, Wilmington, MA and were fed Wayne Lab Blox (Allied Mills, Chicago, IL). The animals used for the autoradiographic studies were given ^{14}C-aniline HCl (100 μCi) via the tail vein, at doses of 3, 30 or 100 mg/kg, and 0·5, 6 or 24 hr later the rats were anaesthetized under sodium pentobarbital anaesthesia, shaved, embedded in 1·5% carboxymethylcellulose and frozen in liquid nitrogen. Whole-body sagittal sections (50 μm thickness) were obtained using a PMV 2250 cryomicrotome (LKB Instruments, Inc., Rockville, MD). Sections were cut and freeze dried at −25°C and autoradiographic films were made using Kodak NS-2T film (Eastman Kodak Co., Rochester, (NY) according to the method of Ullberg & Appelgren (1969).

The rats used for analyses by gas chromatography–mass spectrometry (GC–MS) were given aniline HCl (100 mg/kg) via the tail vein and were killed by cervical dislocation 0·5 hr after treatment. The contents and mucosal scrapings of the stomach and of the distal end of the jejunum were collected for the analysis of metabolites. The samples were analysed for aniline and metabolites by the procedure of Bus *et al.* (1978). The method is based upon the extraction of the samples in alkaline aqueous solution using diethyl ether, followed by derivatization with trifluoroacetic anhydride. The analyses were carried out on a Finnigan Model 402B GC–MS system (Finnigan Corp., Sunnyvale, CA). Gas chromatographic separations were obtained using a 6 ft (2 mm ID) glass column packed with 3% SP2401DB on 100/120 Supelcoport (Supelco Co., Bellefonte, PA). The column oven was maintained at 130°C for 0·5 min after injection, then programmed to 150°C at a rate of 6°C/min and held for 1 min. The injector and glass separator were main-

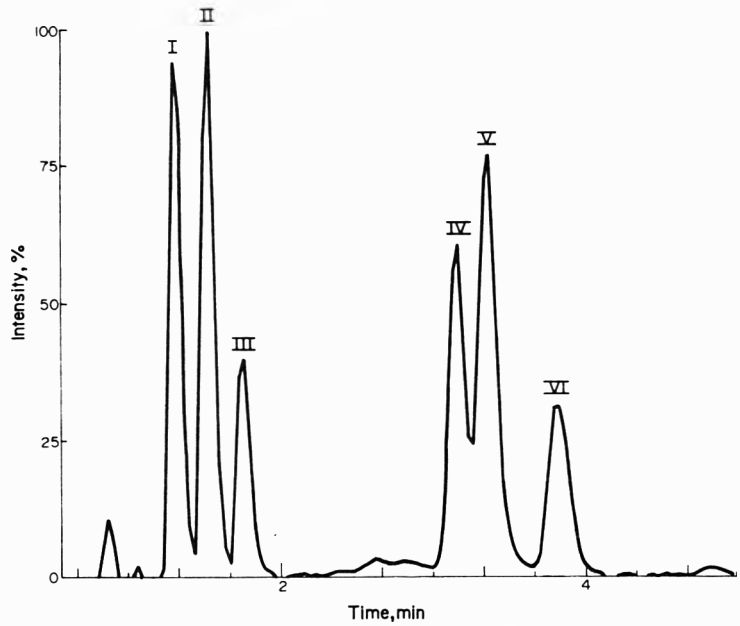

Fig. 1. Total ion chromatogram obtained by gas chromatography–mass spectrometry (GC–MS) for authentic standards of: aniline (I); *o*-aminophenol (II); acetanilide (III); *m*-aminophenol (IV); *p*-aminophenol (V); acetaminophen (VI). GC–MS was used to obtain the metabolite ratios reported in the text.

tained at 200°C and 235°C, respectively. Helium was used as a carrier with a flow rate of 20 ml/min. Figure 1 shows a chromatogram of authentic samples of the six principal metabolites for which analysis was carried out. The relative concentration of aniline and acetanilide (the only metabolite found in significant concentration) was determined for each sample. The relative concentrations were obtained from mass chromatograms of m/z = 189 (parent ion and fragment ion for acetylated aniline and acetanilide, respectively) by correcting for differences in the relative abundance of this ion for both compounds. No attempt was made to determine the absolute concentrations of these compounds by GC–MS.

RESULTS

Radioactivity associated with ^{14}C-aniline was distributed throughout the body following iv administration of a single dose. At all doses, after 0·5 and 6 hr the highest levels of radioactivity were associated with the blood, liver, kidney, bladder and gastro-intestinal tract (Figs 2–4). The spleen of animals given the 100 mg/kg dose was the only organ that did not show a time-dependent decrease in ^{14}C-aniline-associated radioactivity, the level of activity in the red pulp remaining relatively constant between 0·5 and 24 hr after administration (Fig. 3).

Parenterally-administered aniline was rapidly mobilized from the blood and concentrated in the stomach where radioactivity was sharply delimited to the lower 75% of the organ (Figs 2 & 4), coincident with the point of transition from oesophageal to gastric mucosa in the rat. Analysis of metabolites in the gastric contents 0·5 hr after administration showed that aniline was the predominant form present, the aniline:acetanilide ratio being 7·3:1. Trace amounts of *o*- and *p*-aminophenol were also detected. In contrast to the gastric contents, the gastric mucosa contained primarily acetanilide, which was present in a ratio of 14:1 over aniline. Acetanilide was also the principal metabolite found in the jejunal mucosa and contents, the ratio of acetanilide to aniline being 14:1 and 15:1, respectively. Trace amounts of acetaminophen were detected in the jejunal mucosa and contents in addition to trace amounts of *o*- and *p*-aminophenol.

Radioactivity associated with the gastro-intestinal contents was concentrated in the stomach and small intestine with relatively little associated with the contents of the caecum, colon or rectum (Figs 2–5). A dose-dependent increase in radioactivity was associated with the caecum and colon soon after treatment, but this appeared to be confined to the mucosa and not to be due to concentration in the intestinal contents (Fig. 5).

DISCUSSION

The persistence of ^{14}C-aniline-associated radioactivity in the red pulp of the spleen showed a threshold, occurring only in animals receiving the 100 mg/kg dose. In animals receiving the lower doses there was a time-dependent decrease in radioactivity associated with the spleen. This phenomenon may be relevant in understanding the tumorigenicity of aniline in the spleen that was observed in a study involving the chronic exposure of rats and mice to high concentrations of the compound (National Cancer Institute, 1978). A high incidence of haemangiosarcomas and fibrosarcomas of the spleen, particularly in male rats, was reported in this study. The involvement of the vascular endothelium and fibroblasts in these neoplastic lesions, together with the retention in the spleen of aniline-associated radioactivity at the high dose reported here, suggests that the retention of ani-

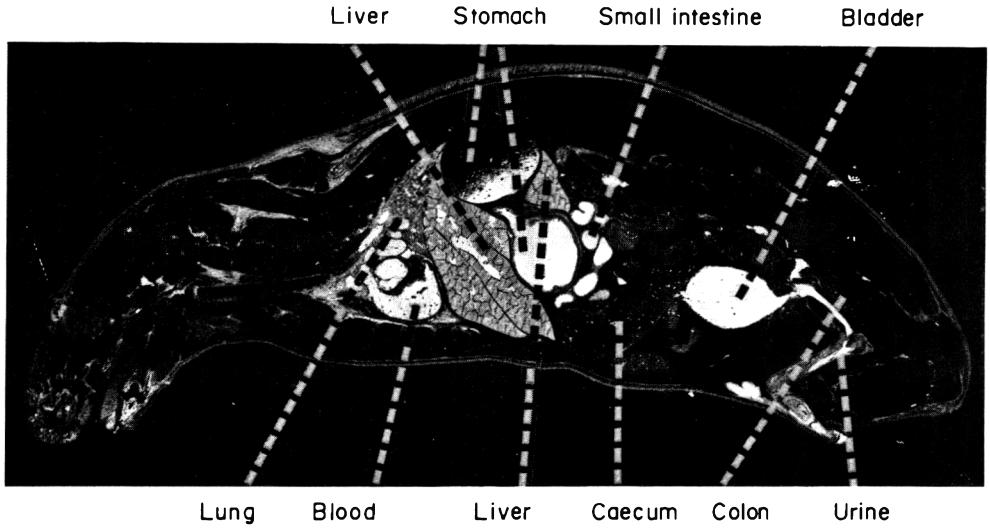

Fig. 2. Whole-body autoradiogram of a rat 6 hr after iv administration of 100 μCi ^{14}C-aniline HCl (100 mg/kg). Light areas indicate substantial accumulation of ^{14}C activity. × 1.

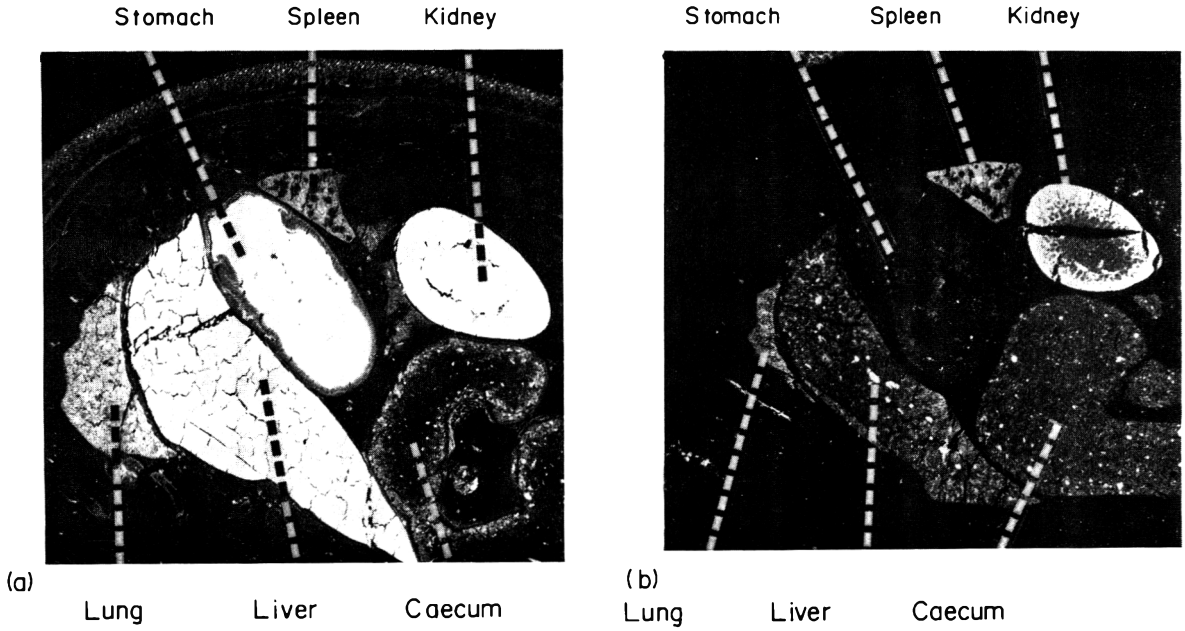

Fig. 3. Detail of a whole-body autoradiogram of a rat (a) 0·5 hr and (b) 24 hr after receiving 100 μCi ^{14}C-aniline HCl (100 mg/kg). × 2.

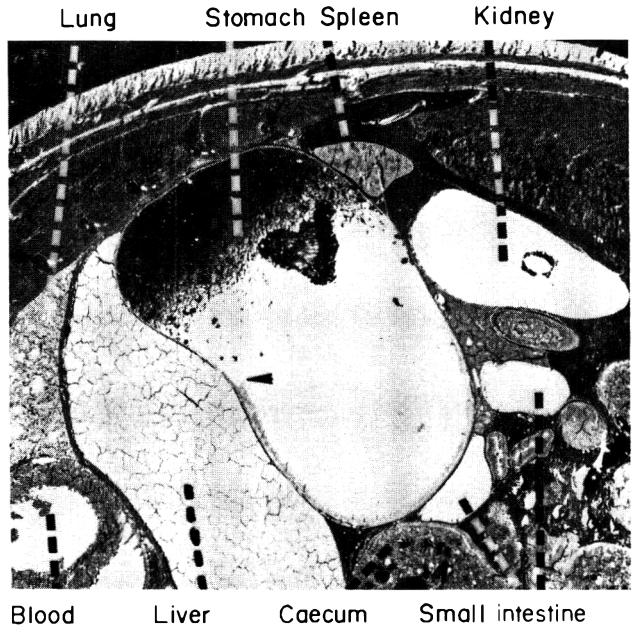

Fig. 4. Detail of whole-body autoradiogram of a rat 6 hr after receiving 100 μCi ^{14}C-aniline HCl (100 mg/kg). The arrowhead indicates the transition from oesophageal to gastric mucosa. × 2.

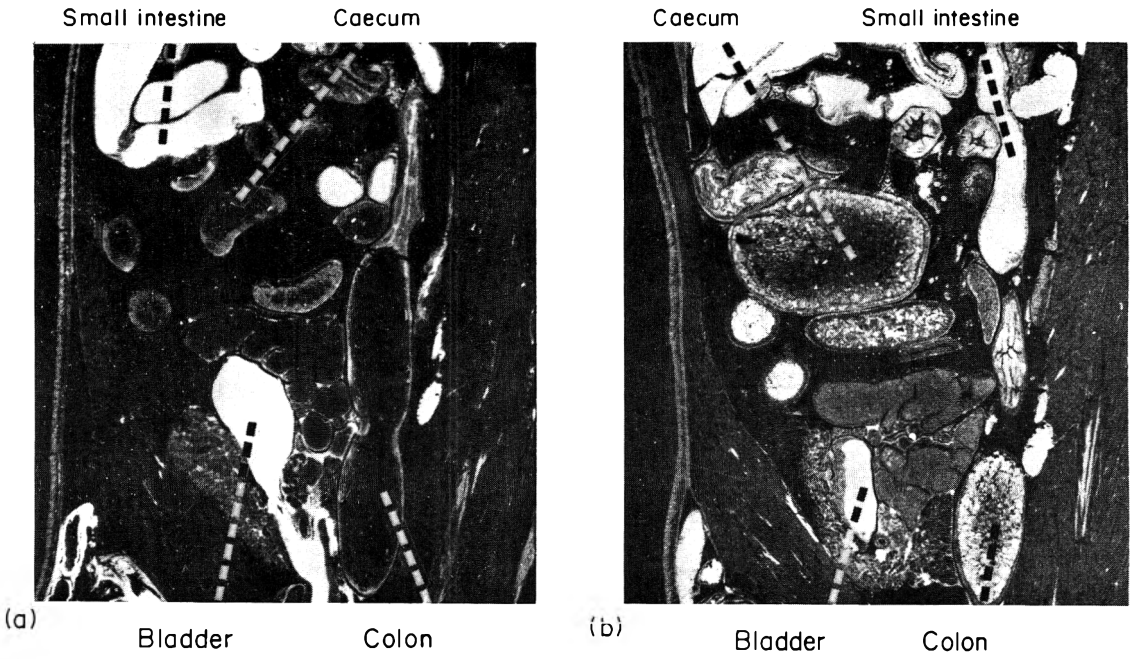

Fig. 5. Detail of whole-body autoradiogram of a rat 0·5 hr after receiving 100 μCi of ^{14}C-aniline HCl in a dose of (a) 3 mg/kg and (b) 100 mg/kg. × 2.

line or its metabolites in the spleen may play a role in the pathophysiology of this disease. Accumulation and covalent binding of ^{14}C-aniline-HCl-associated radioactivity in the spleen has recently been reported (Bus & Sun, 1979).

The rapid concentration of aniline in the stomach is consistent with the partition of an organic base between the plasma and gastric compartments, the gastric mucosa permitting the passage of only the nonionized form from the plasma (pH 7·2) to the stomach (pH 1·0). Elimination in the bile is not an important route of aniline excretion in the rat, biliary excretion principally as *p*-aminophenol and its glucuronide, accounting for less than 6% of a dose of ^{14}C-aniline 3 hr after administration (Abou-el-Makarem, Millburn, Smith & Williams, 1967). Similar results would be expected for acetanilide (mol wt 135) on the basis of the molecular-weight threshold (mol wt 350 $\pm$ 50) for biliary excretion in the rat (Smith, 1973). Therefore, presumably aniline is metabolized to acetanilide in the gastro-intestinal mucosa. Supportive of this conclusion are the findings of Shirkey, Kao, Fry & Bridges (1979) who examined the capability of epithelial cells isolated from the small intestine of the rat to metabolize aniline and found acetylation to be the major pathway for aniline metabolism in these cells.

The marked decrease in radioactivity associated with the contents of the large intestine in conjunction with the previously-reported lack of any significant faecal excretion of aniline or its metabolites (Kao *et al.*, 1978; Bus *et al.*, 1978) effectively make the case for an enterogastric cycle for aniline in the rat. Although aniline is rapidly secreted into the stomach, the higher pH of the small intestine (pH 6–7) and its large mucosal surface area favour essentially-complete reabsorption of both aniline (pK_a 4·6) and acetanilide (pK_a 4·3). The ability of intestinal cells to acetylate aniline and the effective enterogastric cycle for aniline may thus combine to make the gut a major organ for aniline metabolism.

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THE PERCUTANEOUS ABSORPTION OF LEAD-203 IN HUMANS FROM COSMETIC PREPARATIONS CONTAINING LEAD ACETATE, AS ASSESSED BY WHOLE-BODY COUNTING AND OTHER TECHNIQUES

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Abstract—The percutaneous absorption of lead from two hair-darkening cosmetic preparations containing lead acetate has been measured by radioisotopic tracer techniques, using lead-203 acetate, in eight normal human male subjects. Spiked preparations were applied in fluid and dried forms to each subject's forehead (with periods of 1 month between each application) and the quantity of lead absorbed was calculated from blood counts, whole-body counts and urine radioactivity. Results were normalized for each subject by administration of an iv tracer dose of lead-203 chloride, from which absorption was calculated. It was found that absorption of lead through the skin was essentially zero, with results ranging between 0 and 0·3% of the dose applied to whole skin. Slight absorption was found when the skin was broken. The potential hazard of the use of such cosmetic preparations is therefore considered to be insignificant.

INTRODUCTION

When substances are applied to the skin, they may be absorbed and reach the dermal microcirculation by three routes—by passage through the epidermal cells, by passing between the epidermal cells and by passing through hair follicles and sweat glands. The rate-limiting step of this process is thought to lie at the level of the stratum corneum (Scheuplein & Blank, 1971). On the basis of compartmental models, Wallace & Barnett (1978) have been able to explain the penetration of the drug methotrexate through the skin at various pH values, showing that as the pH value rose a greater proportion of the drug passed through the skin by way of the hair follicles and sweat glands.

It has been suggested (Blank, Scheuplein & MacFarlane, 1967) that polar molecules will pass through the skin by way of the aqueous regions between the epidermal cell membranes, and this is the region of greatest interest with respect to the highly polar salt, lead acetate. Some problems do exist in the measurement of the rate of absorption of lead compounds, not the least of which is the great capability of lead ions to form complexes with anionic ligands such as sulphydryl groups in protein structures. It is on the basis of such bond formation that lead acetate is used as a hair dye, in which circumstance the lead combines with available organic SH groups to form dark grey or black insoluble lead sulphides and thus darken the hair. Other reactions, such as reduction of sulphur, usually present as an ingredient in the hair dyes, and subsequent reaction with the lead acetate probably contribute also to the dyeing process.

In evaluating the relative contributions of environmental sources of lead to total human lead exposure, most emphasis must be placed upon the major sources of intake as alimentary or pulmonary exposure. At any one time, considerable quantities of lead are in continuous contact with the skin from sources such as water, clothing and the air. To date it has been generally accepted that percutaneous absorption of lead is only of importance in the case of organic compounds of lead, naphthenates and alkyl-lead compounds (Rastogi & Clausen, 1976; Waldron & Stöfen, 1974). The available evidence on the cutaneous absorption of inorganic lead compounds is small and has in all cases been derived from animals. Laug & Kunze (1948) found that lead acetate could be absorbed through the skin of the rat, and they demonstrated a rise in the concentrations of lead in the kidney after such applications. They also noted that when the skin was damaged there was a three- to fourfold increase in the quantity of lead found in the kidneys, although they were unable to find an increase in urinary lead after seven daily applications of lead oleate to the skin of the back, hands and feet of four human subjects. Later experiments (Rastogi & Clausen, 1976) confirmed this. These studies, however, concentrated upon the effects of lead in the rat, in which animal the skin structure is quite different from that found in man.

The study described here examined, in a group of eight human volunteers, the rate of uptake of lead acetate through the skin from two cosmetic preparations.

EXPERIMENTAL

Test materials. Two forms of commercially-available lead acetate preparations were used in these

studies, both being designed to darken greying hairs through formation of a lead–sulphur compound within the hair. The first was a hydroalcoholic solution of lead acetate containing colloidal sulphur (alcoholic strength 6%; 6 mmol lead acetate/litre). The second was a cream containing 9 mmol lead/kg. For the experiment each of these preparations was spiked with lead-203 (^{203}Pb) acetate supplied either by the MRC Cyclotron Unit at the Hammersmith Hospital, London (Horlock, Thakur & Watson, 1975) or by New England Nuclear (Boston, MA), 0·74 mBq (20 μCi) ^{203}Pb being added to either 0·1 ml of lotion or 0·1 g of cream. The activity of this isotope (half-life 52·1 hr) was checked upon receipt by reference to a calibrated ionization counter.

Test subjects. The eight volunteer subjects used in this study were fully informed of the procedures to be carried out. These procedures had been assessed by the Research and Ethical Committee of Stobhill Hospital and by the Department of Health and Social Security Isotopes Advisory Panel. All the subjects were male. None of them handled radioactive material, had excessive environmental exposure to lead, drank excessive quantities of alcoholic beverages, took any form of drug, suffered from porphyria, had any form of skin pathology, especially in the area of the forehead to be used, had any other form of systemic pathology, or had any history of allergy. All of them were able to conform to all the required conditions of the study over the 6-month experimental period.

Basic experimental procedure. The basic procedure was as follows: lead acetate-containing lotion or cream spiked with 0·74 mBq (20 μCi) ^{203}Pb acetate as tracer was placed on the subject's forehead for a 12-hr period, after which it was washed off. Percentage absorption of the preparation was estimated by measuring the ^{203}Pb activity in blood samples taken over the 12-hr application period, by counting over the subject's calf region and also by counting 0–24- and 0–48-hr urine collections using a whole-body monitor.

Counting procedures. Gamma counting of aliquots of whole blood was carried out on a Wallac 7·5 cm (3 in.) sodium iodide crystal auto gamma counter (Wallac, Finland) and on a Selo DS7/B rectilinear scanner (Selo, Milan, Italy). This consists of two opposed 127 mm-diameter sodium iodide crystals/photomultiplier tubes. Blood for counting was placed in an 11 cm-diameter container and the two heads were brought together to enclose the pot completely. Additional 2 mm-thick lead shielding was then placed round the head to protect it from further background radiation. The counting channel chosen ranged from 60 to 420 keV to include both of the principal photopeaks, at 80 and 280 keV.

Counting of the human subjects was carried out using two pieces of equipment. The first was a single probe counter consisting of a 51 mm sodium iodide crystal/photomultiplier tube (Ekco Ltd., England) fitted with a lead collimator. This was used to count the amount of ^{203}Pb acetate solution applied to the subject's forehead. A narrow channel centred on the gamma-ray emission at 280 keV was used in this apparatus. To determine the mean ratio between the single probe counts and Selo counts a standard forehead application was made up by pippetting an aliquot of ^{203}Pb on to absorbent paper. The counts thus obtained were compared with the counts obtained from an equal aliquot used in a container filled with 150 ml water in the Selo counter. This gave a geometry conversion factor (container in Selo/single probe counter) of 94·5 $\pm$ 11·6.

Whole-body monitoring was carried out in the whole-body counter in the Southern General Hospital, Glasgow. The detection apparatus consisted of six 153 mm-diameter/102 mm-thick sodium iodide crystals, which were held stationary in a ring around the calves of the subjects during the counting period; to shield the counting heads from scattered background radiation from the forehead of the subject a lead collar was constructed and placed round the neck of each of the subjects during counting and a sheet of 6 mm-thick lead was mounted between the collimators and the subject's forehead, to diminish further any scattered radiation.

Clinical chemistry. Blood-lead levels were measured by flameless atomic absorption spectrophotometry with deuterium background correction (Perkin-Elmer 306 with HGA 72; Meredith, Moore & Goldberg, 1977). Similar techniques were used for the measurement of lead concentrations in the cosmetic preparations. Erythrocyte δ-aminolaevulinic acid (ALA) dehydratase was measured by the Commission of the European Communities' standardized method (Berlin & Schaller, 1974), while the concentrations of urinary ALA were determined by the method of Mauzerall & Granick (1956) and the concentrations of porphyrins in urine and in blood by the method of Rimington (1971).

Treatment cells. Each of the subjects was assigned to the five treatment cells in a random fashion from a table of random numbers. During the progress of the study the subjects were given careful directions on the importance of avoiding cross-contamination from the forehead to any other part of the body. The applications were as follows:

(1) The control application, in which each of the items under investigation was examined to establish baseline conditions.
(2) The wet cell, in which a corn plaster (a circular plastic foam ring of interior area 8 sq. cm) had the interior surface sealed with three coats of nail varnish and was fixed to the forehead of the subject. The test application was then placed in the centre well, and the whole of the well was sealed with two layers of impermeable tape.
(3) The dry and scratch cell, in which the forehead of the subject was cleaned and a line 2 cm long was wiped with a swab containing 70% isopropranol. This was allowed to dry and the skin was lightly scratched using a sterile hypodermic needle, avoiding the drawing of any blood. Over this scratch, 0·1 ml of the test application was placed and spread over a total circular area of 10 sq. cm, which was then dried with a hair dryer and lightly covered with a thin layer of gauze and permeable tape.
(4) The cream application cell, in which 0·1 g of cream was put on to the skin surface and again spread out in a circular area of 10 sq. cm. This area was then occluded with impervious adhesive tape.

(5) The dry cell, in which the procedure was identical to that for the scratch/dry cell without the skin being scratched. Following drying, the skin was lightly covered with a thin layer of gauze and permeable tape.

In each of the experiments the subsequent procedure was as follows: On the first day of the experiment a background whole-body count was taken, together with a 24-hr collection of urine and withdrawal of blood for measurement of blood lead concentration, porphyrins and ALA dehydratase. On the day of the application the subjects had blood removed and then the material was applied to the skin and at the same time a 24-hr urine collection was started. Blood samples were taken at 1, 2, 4, 8 and 12 hr, and a further sample was taken 24 hr after the test material had been applied. Immediately after administration of the material the forehead was counted using the single probe counter. This was repeated 12 hr later, at which time the application was removed from the head, and the forehead was carefully cleaned using Count-off (New England Nuclear), followed by two washes with soap and ten rinses with warm water. The forehead was dried and lightly covered with gauze and tape, and was then recounted on the single probe counter. The subjects were taken for whole-body counting in the whole-body monitor 12 and 24 hr after the application. Each of the blood aliquots taken over the day was counted and at the same time measurements were made of blood lead and blood ALA dehydratase activity. On the initial sample for each of the days, blood protoporphyrin was also determined. The aliquots collected during the day were then pooled in a container and counted for between 6 and 14 hr overnight in the Selo counter. The proportion of the dose present in the total blood volume was then given by:

$$\%\ \text{dose in total blood volume} = \frac{\text{sample counts} - \text{background}}{\text{counts in applied dose} \times C} \times \frac{\text{blood volume}}{\text{volume of sample}} \times 100$$

The background count used was the mean of the counts recorded on the days before and after the day when the sample was counted. The counts in the dose applied to the forehead were obtained from the single probe measurements. C is a factor to convert from single probe to Selo counts; this was obtained from an experiment in which a known aliquot of ^{203}Pb was counted on the Selo and also on the single probe counter. Lead was measured in each of the 24-hr urine collections, together with urinary ALA and urinary coproporphyrin. Each of the 24-hr urine collections was also counted in the whole-body monitor. For each cell of the study, haematological measurements were carried out, together with a total biochemical screen of serum and urine. One month was allowed to elapse between each of the cells to allow total half-life decay of residual lead on the foreheads of the subjects.

Intravenous studies. At the end of the study, the blood volume of each subject was calculated. Each subject was then injected iv with 2 ml of ^{203}Pb chloride of activity between 2·7 and 5·4 kBq (100 and 200 nCi). The subjects were counted in the whole-body monitor 15 min and 8 and 24 hr after the injection. Urine samples were also counted in the whole-body monitor.

Radiation dose to subjects. ^{203}Pb has three principal gamma emissions—81% at 280 keV, 5% at 400 keV and 0·9% at 608 keV. There are in addition 72 and 83 keV thallium X-rays. The whole-body dose from the gamma emission was calculated to be 4·94 mrad per application and the surface application dose was calculated as being between 0·58 and 0·12 rad at between 0·01 and 1 cm depth into the skin.

RESULTS

Sensitivity of methods

Using calibrated sources supplied by the Medical Research Council Cyclotron Unit, it was calculated that for a dose of 0·74 mBq (20 μCi) of ^{203}Pb, sensitivity of the whole-body counting technique would be in the region of 37 Bq (1 nCi) for the whole body, with a similar sensitivity for the urinary collections. For the Selo counter similar calculations gave a detection limit of 289 mBq (7·8 pCi) per sample. The counting of whole blood in the Wallac counter was optimized by counting 10-ml aliquots of blood for 1 hr.

ALA dehydratase can measure most efficiently when blood-lead levels are less than 2 μmol/litre (Meredith & Moore, 1979). Each of the subjects in the study fell into this range where the rate of change of ALA dehydratase with respect to blood lead was 23 units/mol blood lead. Blood-lead measurement had a limit of accuracy of 0·1 μmol/litre.

General data

The initial concentrations of lead and related indices are shown in Table 1 for each of the subjects. All were within normal limits, as defined by our laboratories. None of the cells showed any change other than normal diurnal variation in the concentrations of blood lead, blood protoporphyrin, urinary ALA or urinary coproporphyrin, or in the activity of ALA dehydratase. No change was observed between the cells in haematological or biochemical indices.

Selo and single probe monitor data

The single probe count showed that the percentage dose remaining on the head following the wash-off procedure was generally low—in the region of 1% of the applied dose—but in some cases it rose to as high as 7%. The percentages of the dose present in the blood and whole body and excreted in the urine are shown in Table 2. It is of note that for each of these measurements the highest mean figure is that for the dry and scratch cell, in which the greatest trauma to the skin, and therefore the highest rate of intake of lead into the blood, is likely to have occurred.

Intravenous data

These data established the relationship between the count-rate recorded over the calf region for each of the subjects and the activity injected (Table 3). This

Table 1. *Data on percutaneous lead absorption in volunteers*

Data	Values for subject no.							
	1	2	3	4	5	6	7	8
Age (yr)	33	27	35	35	25	23	20	23
Height (m)	1·73	1·76	1·67	1·79	1·67	1·76	1·67	1·74
Weight (kg)	82·0	69·2	67·3	60·0	55·7	87·0	62·6	72·0
Blood measurements								
Total volume (litres)	5·143	4·831	4·479	4·639	4·105	5·404	4·328	4·861
Lead concn (μM)	1·3	1·2	1·1	1·2	1·2	1·0	0·9	1·0
ALA dehydratase (μmol/min/litre)	30·6	38·7	24·6	26·9	27·8	30·1	44·8	43·2
Protoporphyrin (nM)	485	632	364	354	493	365	472	497
Haemoglobin (μg/100 ml)	14·4	17·3	16·0	14·5	15·7	13·6	15·5	14·6
Urine analyses								
ALA (μM)	4·8	18·0	9·0	8·3	7·9	12·0	9·5	20·0
Coproporphyrin (nM)	72	70	65	70	111	127	80	170

ALA = δ-Aminolaevulinic acid.

relationship was then used to establish the whole-body uptake of ^{203}Pb from the results of calf count-rate obtained for each of the cells. In addition, blood samples were collected at 4 and 24 hr. The percentage of the initial dose in 10 ml of blood was calculated and thence the percentage dose in the whole blood volume was determined.

Whole-body monitor data

The net calf count for each subject following room-background subtraction and subject-background subtraction was converted to whole-body activity in nCi using the factor obtained for each subject from the iv studies (Table 3). Percentage absorption was then

Table 2. *The percutaneous absorption of lead-203 acetate as assessed by three techniques*

Treatment		Subject no.	Radioactivity in				
			Blood	Whole body (% of dose)		Urine (% of dose)	
Cell no.	Application		(% of dose/100 ml)	12 hr	24 hr	24 hr	48 hr
2	Wet	1	0·00053	0·017	−0·006	0	0
		2	0·00086	0·100	0·245	0·020	0·025
		3	0·0043	0·092	0·252	0·004	0·008
		4	0·00082	0·189	0·40	0·032	0·036
		5	0·00040	0·103	0·285	0·003	0·007
		6	0·00013	0·012	0·020	0·001	0·001
		7	0·00015	0·006	0·040	0·010	0·011
		8	0·00006	0·037	0·179	0·008	0·013
3	Dry + scratch	1	0·00039	0·060	0·080	0·006	0·012
		2	0·00029	0·003	0·058	0·002	0·003
		3	0·00221	0·385	0·507	0·021	
		4	0·00658	0·958	1·33	0·198	
		5	0·00173	−0·03	−0·01	0·010	
		6	0·00037	0·037	0·050	0·002	
		7	0·00042	−0·01	−0·002	0·007	0·007
		8	0·00028	0·037	0·050	0·005	0·006
4	Cream	1	0·00227	0·024	0·018	0·001	0·004
		2	0·00040	0·002	0·025	0·002	0·002
		3	0·00038	0·061	0·08	0·003	0·003
		4	0·00020	0·008	0·010	0·0002	0·003
		5	0·00008	0·011	0·020	0·001	0·001
		6	0·00045	0·001	0·02	0·0005	
		7	0·00093	0·038	0·064	0·004	0·004
		8	0·00098	0·028	0·057	0·001	0·001
5	Dry	1	0·00029	0·074	0·30	0·019	0·027
		2	0·00042	−0·022	−0·014	0·00	0·004
		3	0·00004	0·069	0·044	0·004	0·011
		4	0·00005	−0·004	−0·040	0·007	0·013
		5	0·00040	0·205	0·389	0·046	0·073
		6	0·00017	0·325	0·353	0·011	0·022
		7	−0·00009	0·010	0·013	0·001	0·001
		8	0·00043	0·008	0·011	0·009	0·009

Table 3. *Levels of radioactivity excreted in the urine and retained in the body after iv injection of volunteers with lead-203 chloride*

Subject no.	Dose (nCi)	Activity in urine (nCi)			Urinary excretion of activity over 0–48 hr (% of dose)	Calf counts (nCi)/2400 sec at	
		0–24 hr	24–48 hr	0–48 hr		5 hr	24 hr
1	132	5·1	1·4	6·5	5·0	288	296
2	124	2·8	—	—	—	252	256
3	118	4·8	1·9	6·7	5·7	258	248
4	132	5·6	1·7	7·3	5·6	220	205
5	196	8·3	2·2	10·5	5·4	175	170
6	196	7·2	2·6	9·8	4·9	191	205
7	215	9·6	2·9	12·5	4·9	211	214
8	215	3·8	2·6	6·4	3·0	257	241

easily obtained as the applied activity was known. To avoid contamination of urine-sample containers, all urines were transferred to fresh containers before counting in the whole-body monitor. The counts per 2400 sec were corrected for room background and for contamination from naturally occurring potassium-40 on the assumption that each subject excreted 1·05 mequiv. potassium/kg body weight/day. The percentage of applied dose excreted was converted to the percentage absorption by multiplying by the factor 100/% iv dose excreted. This was a crude approximation since the transit time of lead from the skin to the bloodstream is not known and since there is the possibility that the mechanical trauma of the washing procedure would alter the skin sufficiently to allow the post-wash residual lead to diffuse more easily into the blood. However, when one correlates the urine activity for 0–48 hr as a function of whole-body activity in 24 hr, the correlation coefficient is 0·71 ($P < 0·01$), which leads to a regression equation of:

activity in 0–48-hr urine = 0·055 whole-body activity in 24 hr + 0·51.

This is very similar to that predicted from the iv studies, in which:

activity in 0–48-hr urine = 0·049 whole-body activity at 24 hr (Fig. 1).

The regression of absorption at 24 hr was calculated against absorption at 12 hr (Table 4). For each application this correlation was statistically significant. This suggests that contamination was not a reason for the higher absorption at 24 hr as compared to 12 hr. The most likely explanation is that ^{203}Pb on the forehead continues to be absorbed for some time after the wash-off procedures have been completed, as a function, in part, of the trauma associated with this wash-off. It is of interest to note that the greatest gradient of 2·12 occurs with the wet application. In all other cases the gradient lies near to the ideal value of 1, which would imply that on average there was no further significant absorption between 12 and 24 hr. Thus it appears that the absorption at 12 hr is at the lower limit and with the exception of the wet application the true absorption will not be more than 40% above the 12-hr value. Urine activity at 48 hr correlates significantly with the whole-body activity shown in Fig. 1. The assumptions used in translating urinary absorption are not sufficiently valid, however, to allow great reliance to be placed on these values.

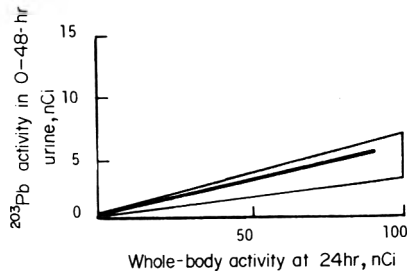

Fig. 1. The regression of activity in the 0–48 hr urine, and whole-body activity at 24 hr; showing the best fit regression line obtained from the data and the predicted behaviour envelope obtained from iv data (mean ± 2SD).

Table 4. *The regression of percentage absorption at 24 hr against the percentage absorption at 12 hr for each cell**

Treatment		m (gradient)	c (intercept)	r (regression coefficient)	Significance ($P <$)
Cell no.	Application				
2	Wet	2·12	0·02	0·95	0·05
3	Dry + scratch	1·36	0·01	0·99	0·05
4	Cream	1·11	0·01	0·89	0·05
5	Dry	1·19	0·05	0·85	0·05

*Absorption at 24 hr = m × absorption at 12 hr + c.

Table 5. *The mean percutaneous uptake of lead-203 acetate as assessed by blood, whole-body and urine counting*

	Mean uptake of ^{203}Pb activity (% of dose) in				
		Whole body at		Urine at	
Treatment	Total blood volume	12 hr	24 hr	12 hr	24 hr
Wet	0·023 ± 0·016	0·069 ± 0·063	0·177 ± 0·146	0·0097 ± 0·0110	0·0144 ± 0·0120
Dry + scratch	0·070 ± 0·099	0·180 ± 0·339	0·257 ± 0·464	0·042 ± 0·005	0·007 ± 0·004 (4)
Cream	0·035 ± 0·037	0·022 ± 0·021	0·037 ± 0·026	0·0022 ± 0·0017	0·0026 ± 0·0013 (7)
Dry	0·010 ± 0·009	0·083 ± 0·121	0·142 ± 0·173	0·012 ± 0·015	0·020 ± 0·023

Values are means ± 1SD for eight subjects except where indicated otherwise in parenthesis.

Examination of empty plastic urine bottles demonstrated that these were free of activity, with a mean count of 4 ± 171 counts/2400 sec. The mean percentage uptake of ^{203}Pb acetate in each of the cells is shown in Table 5.

DISCUSSION

These experiments have shown that one form of inorganic lead, lead acetate, may be absorbed through the skin in infinitessimal quantities. This is the most soluble lead salt in aqueous solution and will therefore represent the opposite situation to the highly lipophilic alkyllead compounds which have been shown to be absorbed relatively easily (Waldron & Stöfen, 1974). In view of the very small uptake, it is not surprising that no change was observed in any of the basic measures of lead exposure—blood lead, ALA dehydratase, blood porphyrin, urinary ALA and coproporphyrin—since the predictive accuracy of these measures is inadequate to detect such change (Meredith & Moore, 1979; Moore & Meredith, 1979). Equally, in the initial assessment of the experiments it was concluded that the most reliable figures would be those obtained from whole blood counted in the Selo counter, followed by whole-body counting and, finally, the urinary count data. The results confirm this assessment.

The counting of larger volumes of blood provided a very sensitive means of assessment of uptake into the total blood volume. The quantity of blood used varied between 140 and 200 ml, and was counted for periods of not less than 6 hr and in some cases for as long as 18 hr. This blood represented an integrated sample of early and late specimens taken throughout the day. The absorption figure thus obtained represents a midpoint measurement of lead uptake into the body over the 12-hr sampling period.

Differences were observed in the whole-body counts taken at 12 and 24 hr. These ranged from little or no change to a tenfold increase after 24 hr. These differences may be ascribed to two features: firstly overnight contamination, especially when subject compliance was not as good as expected, and secondly, the lead gradient established across the skin continued to allow lead to be absorbed over the second 12-hr period. This second probability is made more likely by the mechanical damage that took place on the epidermal surface of the skin during the cleaning procedure. It was therefore considered that the 12-hr whole-body count was the more reliable of the two taken.

Although the whole-body count and the blood counts gave equivalent figures, counts in urine did not give values in such good agreement with the others. One might ascribe this to contamination but it did not appear to come from the bottles used in counting. An alternative explanation is that excess lead absorbed following wash-off was rapidly cleared into the urine.

Some of the features of these experiments make it difficult to compare the different treatments. First, the areas of skin differed. Secondly, it was noted that a standardized scratch in the dry and scratch cell was very difficult to achieve without drawing blood and this in part accounted for the very much greater variability in this cell. Equally, in the wet application, excessive hydration of the skin over 12 hr influenced the results and in both the wet application and the cream application there was loss of material from the skin on to the cover or support, which effectively meant that the quantity of material continuing to touch the skin was less than that for the dry application.

In view of the potential hazard associated with lead absorption, it is of interest to consider the likely practical implications of the use of such lead-containing hair dyes in man. In the following calculations, the mean absorption rate was calculated from three of the cells, the dry and scratch cell being excluded. The mean absorption (± standard deviation) was calculated to be 0·023 ± 0·021% of the dose in the total blood volume, and for the whole body 0·058 ± 0·081% of the dose. Since 6 ml of cosmetic is normally applied, of which 0·18 ml will reach the scalp, 612 μg of lead will reach the scalp for each application and become available for absorption. In the whole body, the amount absorbed would represent 0·058% of this dose, which equals 0·355 μg. Similarly, for blood 0·023% will be absorbed, which is equivalent to 0·135 μg in the whole blood volume. In the initial stages of absorption, 50% of absorbed lead lies in the blood (Moore, Meredith, Campbell & Watson, 1979). Thus, extrapolating to the 12-hr absorption figure in whole blood, one arrives at a total absorption of 0·337 μg in the whole body. These figures are thus in good general agreement and any discrepancy could be accounted for on the basis of temporal differences in counting time.

It is then of consequence to consider these figures in the context of likely lead absorption from other

sources. It has been estimated that there are 170 μg lead in food eaten daily (Ministry of Agriculture, Fisheries and Food, 1975). Of this, 18% will be absorbed from the gut (Moore *et al.* 1979). Thus, around 30 μg lead/day is taken into the body in the diet. There will in addition be some absorption of lead from the air, estimated as 7·5 μg/day, following exposure to 1 $\mu g/m^3$ and assuming a total respired volume of 15 m^3 and an absorption rate of 50% (Chamberlain, Heard, Little, Newton, Wells & Wiffen, 1978). Following such exposure, metabolic balance is maintained, i.e. the output of lead in urine and faeces accounts nearly totally for the oral and respiratory input. This total quantity of absorbed lead, 38 μg/day or 266 μg/wk, must therefore be balanced against a maximal twice weekly use of cosmetics giving a total potential absorption of 0·7 μg/wk. The overall quantity of lead absorbed is thus 380-fold greater than the likely absorption from the cosmetic use of the lead acetate compound.

The challenge of these experiments was to measure nearly undetectable quantities of lead by isotopic tracer techniques. These techniques have arrived at answers which lie at the limits of sensitivity of the methods used. No conventional chemical or biochemical technique could measure this intake, which by any measure is exceedingly small, and the final conclusion must be that the overall percutaneous absorption of lead acetate except where skin damage had taken place is negligible.

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HYDROXYCITRONELLAL: A SURVEY OF CONSUMER PATCH-TEST SENSITIZATION

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Abstract—The potential of the fragrance material hydroxycitronellal to elicit human sensitization reactions or induce hypersensitivity was evaluated by analysing patch-test data for a variety of household and personal care products and fragrance blends containing hydroxycitronellal. Data from tests on hydroxycitronellal itself were also included. Data from a total of 11,638 patch tests containing hydroxycitronellal and involving approximately 9,300 subjects were evaluated. No reactions attributable to hydroxycitronellal were elicited as a result of exposure to the initial patch in any of the tests. The only reported reactions that could possibly be interpreted as elicitation of pre-existing sensitization to hydroxycitronellal occurred in a single test of one fragrance blend in which three persons reacted after multiple patching. However, follow-up tests in this one study did not consistently show reactivity to hydroxycitronellal. Induction of sensitization to hydroxycitronellal appears to be dependent on the exposure concentration. Sensitization to hydroxycitronellal appears to be induced primarily at exposure concentrations greater than 5% but one induction by 0·3% hydroxycitronellal in a fragrance blend was reported. No induced reactions occurred in 9,223 tests on consumer products. These observations provide valuable information regarding the safety of the use of hydroxycitronellal in consumer products.

INTRODUCTION

Safety assessments of consumer products or their ingredients are based on the results of various tests. When use of a product involves skin exposure there is a potential for sensitization. Patch tests involving exaggerated exposure are conducted to assess the potential for sensitization to occur as a result of direct contact with consumer products or their ingredients. Data from patch tests conducted on experimental and marketed products and their ingredients are available in many companies. Data on elicitation of reactions in previously untested subjects can indicate the extent of pre-existing sensitivity in the consumer population while reactions that occur only after prior or repeated patch test exposure can indicate the potential to cause, i.e. induce, sensitivity. Patch-test data were compiled and evaluated in this study in order to provide guidance for the safe use of hydroxycitronellal in consumer products.

Contact sensitization is frequently induced as a result of high-level chemical exposure. A 5- to 7-day period is required for the development of lymphocytes specifically sensitized to a hapten (Polak, 1977, 1978a, b). Reactions can be elicited in sensitized individuals upon sufficient subsequent exposure. Both the induction and the elicitation aspects of skin sensitization are considered in assessing the overall sensitizing potential of a fragrance ingredient.

The evaluation of published induction and elicitation data for the fragrance material hydroxycitronellal (7-hydroxy-3,7-dimethyl-octan-1-al; CAS 107-75-5; $CH_3 \cdot (HO)C(CH_3) \cdot [CH_2]_3 \cdot CH(CH_3) \cdot CH_2 \cdot CHO$) has indicated an apparent conflict: essentially there is no evidence of induced sensitization, but there are several clinical reports of elicited responses. A monograph published for the Research Institute for Fragrance Materials (RIFM), reported no induced sensitivities to 5 and 12% hydroxycitronella in petrolatum in two Kligman maximization tests (Kligman, 1966), each with 25 normal subjects (Opdyke, 1974). Jordan & King (1977) also did not induce sensitization in 25 to 30 normal panelists using the Kligman maximization test with 4% hydroxycitronellal in petrolatum. However, they sensitized one of 150 normal subjects using 4% hydroxycitronellal in petrolatum by the Draize predictive test (Marzulli & Maibach, 1976; NAS-NRC, 1977). Some dermatologists have recently reported elicitation reactions with patients using products containing fragrances with hydroxycitronellal as an ingredient. In tests on 20 subjects previously known to be sensitized to perfumes, 4% hydroxycitronellal in petrolatum elicited nine reactions (Larsen, 1977). Mitchell (1975) reported six strong and seven weak elicitation reactions to 10% solutions of hydroxycitronellal among 38 Japanese contact dermatitis patients patch tested

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to identify causative agents. van Ketel (1978) reports an elicited reaction to 10% hydroxycitronellal in petrolatum with a patient that developed dermatitis in response to an aftershave. Mathias, Cram, Ragsdale & Maibach (1978) describe a case in which a single patient showed an elicited reaction to 4% hydroxycitronellal in petrolatum. Calnan (1979) reported that a patient with allergic contact dermatitis reacted to 1% hydroxycitronellal in petrolatum as well as to other perfume ingredients. These conflicting induction and elicitation reports have caused uncertainty regarding the sensitization potential of hydroxycitronellal.

Hydroxycitronellal has been used in perfumery since the 1920s (Arctander, 1969), hence it is possible to survey epidemiologically any consequences of this extensive use. Hydroxycitronellal has also been generally recognized as safe (GRAS) for food additive applications (FEMA, 1965), and has been approved for food use by the Food and Drug Administration (21 CFR 172.515). US production has been estimated to exceed one million lbs annually; the majority of this is used by the soap and detergent industry as a minor ingredient. Hydroxycitronellal is an ingredient in many consumer and household products that have been tested to ensure safety prior to market introduction. As a result, existing data allow an assessment of the true sensitization potential of hydroxycitronellal in the general population.

EXPERIMENTAL

The survey encompassed skin patch tests on human subjects conducted in the USA by member companies of the Soap and Detergent Association or by perfume suppliers. Panel subjects were selected regardless of sex, race or occupation, but for availability reasons most subjects were female, white homemakers between the ages of 18 and 65. Subjects were interviewed regarding their previous susceptibility to allergies and this information was reviewed before selection. Volunteers were excluded if they were under treatment for skin disease at the time of testing, but were generally not excluded if they reported the previous occurrence of skin allergies.

The request for accumulated data was made in late 1977 and supplemented with additional data in early 1978. The materials tested represent a broad spectrum of commercial and non-commercial consumer products and fragrances that contain hydroxycitronellal as well as hydroxycitronellal itself. The test materials containing hydroxycitronellal were identified as belonging to one of four general categories: household products including detergents, cleaners, laundry aids, bleaches, etc.; personal care products including soaps, creams, shampoos, talc, perfumes/colognes, and other products applied directly to the skin; fragrance blends including concentrated fragrance oil mixtures which are used in consumer products; hydroxycitronellal itself. This survey thus included tests on hydroxycitronellal in a variety of mixtures. Exposure concentrations represented both typical product concentrations and exaggerated concentrations.

All of the tests used the human repeat insult patch (HRIP) or the prophetic patch procedure, both representing exaggerated exposure conditions. These techniques are used to assess the potential for materials to induce, and subsequently to elicit, the sensitization reactions previously described and have historically represented the most common and widely used methods (Marzulli & Maibach, 1976; NAS-NRC, 1977). In the HRIP procedure a nine-application induction period is generally employed in which patches are applied on Monday, Wednesday and Friday for 3 wk, Patch strips with the test materials are applied to the arm or back of subjects for a 24-hr period. Normally a number of materials (often four or more) are tested at different sites on a subject at the same time. Challenge patches are applied 2 wk after the last application of the induction phase. In the prophetic patch procedure, a single induction patch application is made for a 24- or 48-hr period. Again, the challenge patch is applied 2 wk later. In both procedures challenge patches are applied to the original site and to a new site on the subject. The patches are removed after 48 hr and the sites are graded immediately. Usually the sites are re-examined and graded at a later time, either 24 to 48 hr after the challenge patches are removed. In most of the tests from which data were collected in this survey the test materials were mixed with a vehicle before application to the skin. The vehicles used by the various companies included: water, ethanol, dimethyl phthalate, dibutyl phthalate, mineral oil and petrolatum. In some cases, a finished product was applied directly to the skin.

The method used to identify a causative agent among the numerous constituents in a consumer product was fractionation. This involves separating the product into fractions containing several components and testing each fraction separately. The fractions producing reactions are further subdivided until the reactive material is identified.

Although the details of tests in this survey were different, compilation and correlation of the data were facilitated by identification of the following common experimental information: nature of test material, percentage hydroxycitronellal in test material, percentage hydroxycitronellal in patch test application (these latter two do not include possible hydroxycitronellal contributions from the use of speciality ingredient blends of unknown composition which may contain the material), test method used, number of tests, number of sensitization reactions including elicited and induced reactions, but not irritation reactions.

RESULTS

Responses to this survey provided a broad data base with respect to several characteristics; the number of test subjects involved, the diversity of hydroxycitronellal-containing products, and the test exposure concentration ranges. A total of 11,638 human patch test results were received. An estimated 9300 different subjects were involved. The difference in the number of tests reported and subjects tested resulted from repeated testing of some of the subjects. Results were reported from tests on 127 different formulations and on the pure material. Hydroxycitronellal exposure concentrations ranged to a maximum of 10%.

Table 1. *Human sensitization survey: hydroxycitronellal in consumer products*

Product type	Percentage hydroxycitronellal in patch test mix	Percentage product in patch test vehicle	Percentage hydroxycitronellal in product	Test method	No. of tests	No. of sensitizations
Personal care	0·2	100	0·2	HRIP	61	0 to 1*
Personal care	5×10^{-8} to 0·16	Varied	$1·0 \times 10^{-5}$ to 0·8	Varied	2645	0
Household	3×10^{-8} to 1×10^{-2}	Varied	6×10^{-6} to 5×10^{-2}	Varied	6517	0

HRIP = Human repeat insult patch

*In this test, a sensitization was observed using a composition in which hydroxycitronellal was one of many components. Insufficient data were available to determine which component caused the reaction. During the induction phase the reaction grades after application 1 = 1E, 2 = 1E, 3 = 2E, 4 = 1/1E, 5 = 1E, 6 = 2, 7 = 1E/2, 8 = 2E and 9 = 2E/1. During the challenge phase the reactions grades were grades 1/1E after the first application and grades 1E/1E after the second application. The grades are defined in the footnotes to Table 3.

Data from 9223 patch tests on consumer products containing hydroxycitronellal as a fragrance component were reported (Table 1). Only one subject tested with 0·2% hydroxycitronellal exhibited sensitization, but insufficient data were available to identify the causative agent from among the hundreds of components in the product tested. The severity of the response in this subject ranged from mild erythema to moderate erythema with papules/oedema (Table 1). No other induced or pre-existing sensitivity was observed in any of these tests involving consumer products.

Data from 2298 patch tests on fragrance blends containing hydroxycitronellal were reported (Table 2). Exposure levels ranged from 0·03 to 5·0% hydroxycitronellal. Hydroxycitronellal was initially identified as the causative agent in four sensitizations out of 76 subjects in a single study with 0·3% hydroxycitronellal in a fragrance blend–mineral oil mixture. Three of the four subjects (Nos 1, 2 and 4 in Table 3), exhibited elicited reactions after the second and third patch. They also reacted later to a challenge patch containing the same level of hydroxycitronellal associated with only a portion of the fragrance blend in mineral oil, or in mineral oil alone. However, these subjects did not react to 0·3% hydroxycitronellal in a mineral oil mixture containing all the other aldehydes present in the fragrance blend. Subject no. 3 (Table 3) exhibited an induced reaction. This subject was subsequently patch tested with seven subgroups of constituents with the same ingredient concentrations as in the fragrance blend. There were no reactions to any subgroup, including the one containing hydroxycitronellal. A second challenge, which contained hydroxycitronellal alone at the same concentration (0·3%), caused a mild reaction. These four subjects showed mild to moderate erythema with papules/oedema (Table 3). Seven other subjects exhibited induced reactions from fragrance blends (Table 3), but the reactions could not be directly attributed to hydroxycitronellal since follow-up tests to identify the inducing materials were not performed. In summary, the data

Table 2. *Human sensitization survey: hydroxycitronellal in fragrance blends*

Percentage hydroxycitronellal in patch test mix	Percentage fragrance blend in patch test vehicle	Vehicle	Percentage hydroxycitronellal in fragrance blend	Test method	No. of tests	No. of sensitizations
5·0	25	Ethanol	20	HRIP	41	0 to 1*
4·5	25	Ethanol	18	HRIP	39	0
3·3	25	Ethanol	13	HRIP	44	0 to 3*
3·0	25	Dimethyl phthalate	12	HRIP	51	0
2·4	20	Petrolatum	12	HRIP	50	0
1·9	25	Ethanol	7·7	HRIP	39	0
1·3	25	Ethanol	5	HRIP	42	0 to 1*
1·2	25	Ethanol	4·7	HRIP	39	0
1·1	30	Dimethyl phthalate	3·6	HRIP	51	0
1·0	10	Mineral oil:dibutyl phthalate	10	HRIP	77	0 to 2*
0·7	2	Ethanol	37	HRIP	72	0
0·7	20	Dimethyl phthalate	3·3	HRIP	51	0
0·4	2	Ethanol	22	HRIP	39	0
0·3	3	Mineral oil	10	HRIP	76	4†
0·03 to 0·2	Varied	Varied	0·1 to 14	Varied	1587	0

HRIP = Human repeat insult patch

*In these tests, sensitizations were observed using compositions in which hydroxycitronellal was one of many components. Insufficient data were available to determine which component caused the reaction. Reaction grades for each incident are presented in Table 3.

†See also the text for the description of the puzzling nature of these reactions. Reaction grades for these reactions are presented in Table 3.

Table 3. *Reactions to fragrance blends containing hydroxycitronellal (it cannot be concluded that hydroxycitronellal was the causative agent in subjects 5 to 11)*

Percentage hydroxy-citronellal in patch test mix	Response ratio	Subject	Induction phase. Reaction grade after application at site no.									Challenge phase. Reaction grade after application no.	
			1	2	3	4	5	6	7	8	9	1	2
0·3	4/76	1*	0	0	1	2E	0/2E	A	2E	2E	2E	1E/2E	2E/2E
0·3		2*	0	1E	2E	0/2E	0	1	1	1	2	1E/0	1E/2E
0·3		3*	0	0	0	0	0	0	0	0	1E	2E/2E	2E/2E
0·3		4*	0	1E	2	0/2	1	1	2E	1/1	3	2E/1E	2E/1E
5	1/41	5†	0	0	0	0	0	0	1	4	X	4	
3·3	3/44	6†	0	1	1	0	0	0	A	X	X	6	
3·3		7†	0	0	0	0	0	2	0	2	X	4	
3·3		8†	0	0	0	0	0	2	0	0	3	4	
1·3	1/42	9†	0	0	0	0	0	0	1	4	X	4/4	
1	2/77	10*	0	0	0	0	0	0	0	1E	2E	2E/1E	2E/1E
1		11*	0	0	0	0	0	0	0	0	0	1/0	1E/1E

A = Subject absent

*The following scoring system applies to subjects 1–4, 10 and 11 in this table and to the footnote to Table 1: 0—no visible reaction, 1—mild reaction, erythema, 1E—mild erythematous reaction with papules/oedema, 2—moderate erythematous reaction, 2E—moderate erythematous reaction with papules/oedema, 3—strong reaction, erythema, 3E—strong reaction, erythema with papules/oedema, 4—severe reaction, erythema with papules/oedema, 5—bullous reaction.

†The following scoring system applies to subjects 5–9 in this table and to subjects 1–7 in Table 4: 0—no visible reaction, 1—slight erythema, 2—marked erythema, 3—erythema and papules, 4—very strong oedema/papules, 5—vesicular eruptions, 6—any reaction above grade 3 extending beyond the patch, X—test reaction precludes patch application.

A double grade during the induction phase in either of the scoring systems indicates that the patch was moved to a new adjacent site. The first number is the grade for the new site; the second is the grade for the residual reaction at the old site.

A double grade during the challenge phase indicates that the site was read at two different times after removal of a challenge patch. Patch no. 1 was applied to the old site and path no. 2 was applied to a new site.

submitted on fragrance blends containing hydroxycitronellal show that no elicited or induced reactions occurred that were directly attributable to hydroxycitronellal with the exception of the inconsistent reactions that occurred in four subjects exposed to 0·3% hydroxycitronellal.

Pure hydroxycitronellal was tested in 117 human subjects at 5·0, 7·5 and 10·0% hydroxycitronellal in ethanol (Table 4). Neither induced nor elicited reactions occurred in panelists tested with 5% hydroxycitronellal. In fact, elicited reactions attributable to hydroxycitronellal did not occur as a result of the initial patch application in any test reported in this survey. One panelist (No. 4 in Table 4), tested with 10% hydroxycitronellal, who exhibited slight erythema after the first patch application, but no reactions occurred in this subject after the second application on the same test site. This initial reaction was interpreted as mild irritation. Induced reactions occurred only at concentrations of 7·5 and 10% hydroxycitronellal. These subjects exhibited strong reactions after challenges (Table 4).

DISCUSSION

An assessment of the sensitization potential of a fragrance ingredient should consider the potential both for the elicitation of pre-existing sensitization and for the induction of new sensitization. Under

Table 4. *Human sensitization survey: pure hydroxycitronellal**

Percentage hydroxy-citronellal in patch test mix	Response ratio	Subject	Induction phase. Reaction grade after application no.†									Challenge phase. Reaction grade after application no.†	
			1	2	3	4	5	6	7	8	9	1	2
10	6/40	1	0	1	1	0	0	0	1	0	3	2	4
10		2	0	0	0	0	1	0	4	X	X	1	3
10		3	0	0	1	4	0/-	3	X	X	X	3	3
10		4	1	0	1	1	3	3/-	X	X	X	0	4
10		5	0	0	0	0	0	0	0	0	3	0	3
10		6	0	0	0	0	0	0	0	0	0	0	3
7·5	1/38	7	0	0	0	0	0	0	0	1	3	3	3
5·0	0/39												

*All tests used the human repeat insult patch procedure using ethanol as the test vehicle.

†See appropriate scoring system in the footnotes to Table 3. A double grade indicates patch moved to new adjacent site. The first number is the grade for the new site; second number is the grade for the residual reaction at the old site. A dash indicates the old site was not graded.

given exposure conditions, the frequency and severity of elicitated reactions defines the magnitude of pre-existing sensitizations in that population. Similarly, the frequency and severity of induced reactions indicates the potential for a compound to cause new sensitization reactions in the population.

Previous reports have identified elicited reactions in dermatitic patients tested with 1 to 10% hydroxycitronellal in petrolatum (Calnan, 1979; Larsen, 1977; Mathias *et al.* 1978; Mitchell, 1975; van Ketel, 1978). In the present survey, no elicited reactions attributable to hydroxycitronellal occurred after the initial exposure and before the second exposure in any of the 11,638 patch tests. Only 8 of 9300 subjects exhibited reactions as a result of exposure to the first through third patches, a time period when reactions could be interpreted as pre-existing sensitivity. The severity of these reactions never exceeded a mild to moderate rating. Four of the reactions were identified as induced. Of the remaining four reactions, which were elicited, one could not be attributed to hydroxycitronellal as follow-up tests were not performed to identify the causative agent (Table 1) and the other three elicited reactions were not consistently repeatable following hydroxycitronellal exposure (subjects 1, 2 and 4 in Table 3). The absence of elicited reactions attributable to hydroxycitronellal upon exposure to the initial patch in an estimated 9300 individuals, and the mild to moderate severity of the early reactions observed, indicates a very low, or non-existent, incidence of pre-existing sensitization to hydroxycitronellal in the survey population.

Induced hypersensitivity is the primary basis for assessing the sensitization potential of a new material. However, sensitizers may not induce if exposure concentrations are sufficiently low (Polak, 1978b). The general population has been exposed to hydroxycitronellal due to its use in perfumery and flavours for 50 yr (Arctander, 1969). The low incidence of pre-existing sensitization found in this survey suggests that there is a low probability that hydroxycitronellal will induce hypersensitivity under the actual conditions of exposure to consumer products containing the ingredient. This conclusion is substantiated by the observation that no cases of induced hypersensitivity were observed in 9223 tests on consumer products containing hydroxycitronellal.

Pure hydroxycitronellal exhibited a concentration-dependent incidence, initiation time and severity of induced reactions. No induced reactions occurred at 5% pure hydroxycitronellal. Eight induced reactions occurred in 2298 patch tests with concentrated fragrance blends. Seven of these eight induced reactions could not be attributed to hydroxycitronellal as follow-up tests were not performed to identify the causative agent. One subject exhibited an induced reaction attributed to hydroxycitronellal at a concentration of 0·3% (No. 3 in Table 3). No confirmed reactions to hydroxycitronellal were reported in 635 patch tests at 13 higher hydroxycitronellal concentrations involving various other vehicles and fragrance blend formulations. Also this one subject did not react to the hydroxycitronellal and aldehyde fraction of the fragrance blend. The predominance of data in the survey shows no clear pattern of hypersensitivity.

Challenge and induction exposure concentrations and durations were generally exaggerated in the studies reported in the present survey. The severity or intensity of induced or elicited skin reactions is dependent upon the concentration of chemical applied to the skin (Polak, 1978a, b), and the duration of exposure (Opdyke, 1977). The studies in this survey that used the highest concentrations also used the test procedure (HRIP) with the longest duration of exposure. The concentrations required to induce hypersensitivity greatly exceeded consumer usage concentrations. The exaggerated exposure conditions resulting from test methods involving occluded patches repetitively placed on the same treatment site, multiple patches applied simultaneously, regular exposure for three consecutive weeks, and the use of pure hydroxycitronellal or concentrated fragrance blends were expected to maximize the likelihood of discovering any risk associated with hydroxycitronellal in consumer products. Typical consumer use patterns for these products result in far less exposure than in the exaggerated tests since they are used at lower exposure concentrations, often intermittently and for short durations.

The apparent contradiction between published reports of elicited reactions from hydroxycitronellal at 1 to 10% concentrations in tests on contact dermatitic and perfume sensitive patients and the absence of confirmed elicited reactions to hydroxycitronellal in tests reported in this survey involving hydroxycitronellal alone or in fragrance blends at similarly high concentrations may be related to differences in subject populations. Further, the apparent contradictions between published reports of elicited reactions in such patients and the absence of either elicited or induced reactions in tests reported on consumer products, which contain lower hydroxycitronellal concentrations, may also be in part related to differences in the subject populations. In the present survey, individuals known to be under treatment for skin disease at the time of testing were excluded from tests. In contrast, elicited reactions reported in the literature occurred predominantly in subjects known to be sensitized to perfumes or under care for contact dermatitis. The reactions occurring in this latter group indicate that contact dermatitic patients and people known to be sensitized to perfumes may represent a unique segment of the population. Data to assess this hypothesis are presently unavailable.

Exposure to hydroxycitronellal in consumer products did not induce hypersensitivity or elicit reactions in the large number of tests performed (9,223). The potential to induce hypersensitivity on exposure to hydroxycitronellal alone or in concentrated fragrance blends was dependent on concentration and showed no clear pattern of hypersensitivity at concentrations below 5%. No elicited reactions to hydroxycitronellal occurred upon exposure to the initial patch in any of the 11,638 patch tests reported in this survey. The only reported elicited reactions attributed to hydroxycitronellal occurred in the one fragrance blend in which three subjects reacted after two to three exposures.

The patch test data presented here, a significantly larger data base than has been available before, indicate that hydroxycitronellal has a very low potential for human sensitization.

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CITRAL: A SURVEY OF CONSUMER PATCH-TEST SENSITIZATION

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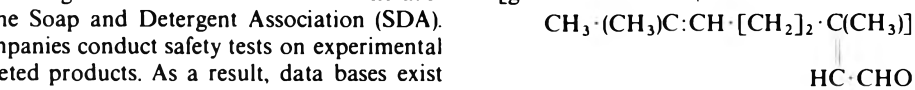
Abstract—The potential for citral, a widely used fragrance and flavour material, to elicit human sensitization reactions or induce hypersensitivity was evaluated by analysing patch-test data. The human sensitization test data presented in this report are from tests conducted on citral, fragrance blends containing citral as one of many ingredients, and a variety of consumer products containing citral. Citral could not be identified as being responsible for any elicited or induced reaction in the 12,758 patch tests with consumer products or fragrance blends containing citral. Citral was applied alone in solvent in 256 tests. At concentrations of 1·0% or greater induced hypersensitivity was observed. Induction of sensitization to citral appears to be dose related, since no sensitizations to citral occurred at citral concentrations of 0·5% or lower. No elicitation of a pre-existing sensitization attributable to citral was observed in any of the patch tests reported. These results provide valuable information regarding the safety of the use of citral in consumer products.

INTRODUCTION

A survey of sensitization data from tests on materials containing citral was conducted under the auspices of the Soap and Detergent Association (SDA). Many companies conduct safety tests on experimental and marketed products. As a result, data bases exist which contribute significant information for assessing the extent of existing sensitization in the consumer population and the potential of citral as found in consumer products to cause, or induce, sensitizations. The objective of publishing these data is to provide guidance for the safe use of citral in consumer products.

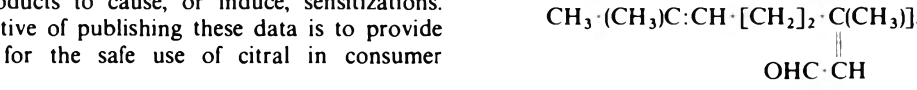
Citral (*cis*- and *trans*-3,7-dimethyl-2,6-octadien-1-al; CAS 5392-40-5) is an important component in fragrance and flavour formulations. It has a powerful lemon aroma that is useful in achieving fresh or citrus type fragrance compositions. It has been in public use in the United States since the early 1900s and the soap and detergent industry has been a major user. Citral is recognized as a safe food additive (FEMA, 1965) and is approved for food use by the Food and Drug Administration (GRAS. 21 CFR 182.60). Citral consists of two geometric isomers usually with the proportions of two thirds *trans*

[geranial or α-citral;



and one third *cis*

[neral or β-citral;



The citral of commerce is either produced synthetically (Bedoukian, 1967) or obtained from natural sources. In both cases it consists of a mixture of the *cis*- and *trans*-isomers, with a purity of not less than 96%. The natural products that are most often used as a source of citral are lemongrass (70–80% citral) and *Litsea cubeba* (70% citral). Synthetically produced and naturally derived citral are used interchangeably in the fragrance industry, and specifications do not distinguish between the two (EOA, 1975; Food Chemicals Codex, 1972).

Citral was selected for this survey since it was reported (Opdyke, 1976) that human sensitization can be induced under the conditions of the Kligman maximization test (Kligman, 1966; Kligman & Epstein, 1975) at levels of 8, 5, 4, 2, 1, 0·5 and 0·1% in petrolatum. However, lemongrass oil, of which citral is a major component, did not induce sensitization under similar test conditions (Opdyke, 1976). Further, it was observed that certain ingredients are able to quench the sensitizing potential of citral (Opdyke, 1976). Thus mixtures of four parts citral to one part of

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either *d*-limonene, α-pinene, or mixed citrus terpenes did not induce sensitization. Although these observations are not well understood and further investigations are in progress (Opdyke, 1976) it appears that there are conditions or substances that interfere with the potential for citral to induce sensitization reactions. Elicitation is not prevented by these quenchers (D. L. J. Opdyke, personal communication, 1979).

There are no cases reported in the clinical diagnostic testing literature of human sensitization to citral in the USA. Consumer experience suggests that no sensitization problems have occurred as a result of the long use of citral in consumer products. This survey presents data for assessing the extent of sensitization to citral in the general population and the potential of citral to induce sensitization.

EXPERIMENTAL

This survey was restricted to skin patch tests on human subjects conducted in the USA by member companies of the Soap and Detergent Association and by perfume suppliers. It includes tests on numerous fragrance compositions in a variety of product bases such as creams, soaps, shampoos, detergents, etc. The request for data was made in late 1977 and was supplemented with additional data in early 1978.

The data requested in this survey on citral included the same information as gathered in a previous survey on hydroxycitronellal (Steltenkamp, Booman, Dorsky, King, Rotherstein, Schwoeppe, Sedlak, Smith & Thompson, 1980). Also, the screening and selection of the test subjects were the same as described previously, as were the patch-test procedures. It was requested that the percentage citral in the test material should include both added citral (as such) whether from natural sources or produced synthetically, and that present from the use of natural material containing significant amounts of citral i.e. *Litsea cubeba*, lemongrass, and *Eucalyptus staigeriana*. The small amounts of citral that may be present due to the use of other natural products such as the citrus oils were not included.

Vehicles used by the various companies in the tests reported in this survey included: water, ethanol, dimethyl phthalate, mineral oil and petrolatum. In some cases, a finished product was applied directly to the skin. Only four formulations on which test data were reported contained citral present from the use of natural materials containing this ingredient.

RESULTS

This survey of human sensitization data includes the results of 13,014 patch tests on products or test materials containing citral. Since some subjects participated in more than one test, it is estimated that a total of 10,200 different subjects were involved in the tests. The data include results from patch tests on 127 different formulations containing citral and on citral in a vehicle.

No subject demonstrated pre-existing sensitization to any of the personal care or household products containing citral involving 10,660 patch tests (Table 1). Also, none of these products induced hypersensitivity that could be attributed to citral. One case was reported of an induced sensitization to a household product that contained a trace amount of citral (2×10^{-7}% citral in the patch test application), but it was not determined which component caused the reaction. The grades of this reaction are presented in Table 2 (subject no. 8).

There were no confirmed reactions to citral in 2098 patch tests on fragrance blends containing the material (Table 3). There were seven cases of sensitization (six inductions and one elicitation) to fragrance blends that contained a very low level of citral, but it was not possible from available data to identify the causative agent from among the many ingredients in these fragrance blends. The grades of these reactions are presented in Table 2 (subject nos 1–7).

Induced hypersensitivity to citral occurred only in tests conducted with high concentrations of pure citral under the exaggerated test conditions. Table 4 gives the results from tests conducted with four concentrations of pure citral. A total of 22 induced sensitizations occurred in 174 tests conducted at 1 to 5% pure citral in ethanol. No inductions occurred at the 0·5% citral concentration level in 82 test subjects.

DISCUSSION

No induced or elicited reactions attributed to citral were observed in 10,660 patch tests on personal care and household products containing citral. The one case of an elicited sensitization to a household product was not traced to the causative agent, but it seems unlikely that citral was responsible in view of its very low level. It can be concluded from these data that citral has a very low or virtually no potential to induce sensitization reactions at the concentrations and under the conditions of ordinary consumer ex-

Table 1. *Human sensitization survey: citral in consumer products*

Product type	Percentage citral in patch test application	Percentage citral in product	Percentage product in patch test vehicle	Test method	No. of tests	No. of sensitizations
Personal care	2×10^{-6} to 3×10^{-2}	5×10^{-4} to 3×10^{-2}	0·1 to 100	Varied*	3572	0
Household	1×10^{-8} to 3×10^{-3}	2×10^{-6} to 3×10^{-2}	0·1 to 100	Varied*	6981	0
Household	2×10^{-7}	7×10^{-5}	0·3	HRIP	107	0–1†

HRIP = Human repeat insult patch

*The test methods used were HRIP and prophetic patch procedures.

†In this test, a sensitization was observed in response to a complex product in which citral was a trace component. Insufficient data are available to determine which component caused the reaction. The reaction grades are given in Table 2 (subject no. 8).

Table 2. *Reactions to fragrance compositions or consumer products containing citral: it cannot be concluded that citral caused any of these reactions*

Percentage citral in patch test mix	Response ratio	Subject	Induction phase. Reaction grade for application no.									Challenge phase. Reaction grade after application at site no.	
			1	2	3	4	5	6	7	8	9	1	2
3×10^{-2}	2/80	1*	0	1	1	1	1	1	2	3	4/3	1/1	3E/2E
		2*	0	2	1/1	1	1	1	3	2/4	5/4	3E/3E	5/4
2×10^{-2}	1/42	3†	0	0	0	0	0	0	1	4	X	4/A	
$1{\cdot}3 \times 10^{-3}$	3/44	4†	0	1	1	0	0	0	A	X	X	6/A	
		5†	0	0	0	0	0	2	0	2	X	4/A	
		6†	0	0	0	0	0	2	0	0	3	4/A	
4×10^{-5}	1/79	7*	3EX	3EX	3EX	2X	2X	2X	2X	1X	1X	—	—
2×10^{-7}	1/107	8*	0	1	2E	0/2E	2E	1/1	1E	2X	2	2E/0	1E/0

A = Subject absent X = Test reaction precludes patch application

*The following scoring system applies to subjects 1, 2, 7 and 8 of this table: 0—no visible reaction, 1—mild reaction, erythema, 1E—mild erythematous reaction with papules/oedema, 2—moderate erythematous reaction, 2E—moderate erythematous reaction with papules/oedema, 3—strong reaction, erythema, 3E—strong reaction, erythema with papules/oedema, 4—severe reaction, erythema and papules/oedema, 5—bullous reaction.

†The following scoring system applies to subjects 3 to 6 of this table: 0—no visible reaction, 1—slight erythema, 2—marked erythema, 3—erythema and papules, 4—very strong oedema/papules, 5—vesicular eruptions, 6—any reaction above grade 3 extending beyond the patch, R—adhesive reaction.

A double grade during the induction phase in either scoring system indicates that the patch was moved to a new adjacent site. The first number is the grade for the new site; the second is the grade for the residual reaction at the old site. A dash indicates that the old site was not graded.

A double grade during the challenge phase indicates that the site was read at two different times after removal of a challenge patch. Patch no. 1 was applied to the old site and patch no. 2 was applied to a new site.

Table 3. *Human sensitization survey: citral in fragrance blends*

Percentage citral in patch test application	Percentage citral in fragrance blend	Percentage fragrance blend in patch test vehicle	Vehicle	Test method	No. of tests	No. of sensitizations
4×10^{-2} to 0·36	0·7 to 4·0	Varied	Varied	HRIP	383	0
3×10^{-2}	5·5	0·5	Ethanol–H_2O (1:1, v/v)	HRIP	80	0 to 2*
2×10^{-2}	$9{\cdot}4 \times 10^{-2}$	25	Dimethyl phthalate	HRIP	54	0
2×10^{-2}	$8{\cdot}3 \times 10^{-2}$	25	Ethanol	HRIP	42	0 to 1*
$1{\cdot}4 \times 10^{-3}$ to 1×10^{-2}	7×10^{-3} to 0·25	Varied	Varied	HRIP	628	0
$1{\cdot}3 \times 10^{-3}$	5×10^{-3}	25	Ethanol	HRIP	44	0 to 3*
5×10^{-5} to 8×10^{-4}	2×10^{-3} to 0·2	Varied	Varied	Varied†	524	0
4×10^{-5}	6×10^{-4}	6·5	Mineral oil	HRIP	79	0 to 1*
5×10^{-9} to 2×10^{-6}	1×10^{-3} to 5×10^{-3}	Varied	Varied	HRIP	264	0

HRIP = Human repeat insult patch

*In these tests, sensitizations were observed for compositions in which citral was one of many components. Insufficient data are available to determine which component caused the reaction. The reported citral quenchers were present at effective levels in the five induction cases reported (subjects 1 & 3–6 in Table 2). Thus it is unlikely that citral contributed to these induced reactions. Reaction grades are given in Table 2 (subjects 1–7).

†The HRIP and prophetic patch tests were used.

Table 4. *Human sensitization survey: pure citral*

Percentage citral in patch test mix	Response ratio	Subject	Induction phase. Reaction grade after application no.									Challenge phase. Reaction grade after application at site no.	
			1	2	3	4	5	6	7	8	9	1	2
5	16/49	1	0	0	3	0	1	0	0	0	1	3/4	4/3
		2	0	0	0	0	0	X	0	3	X	3/0	3/3
		3	0	0	0	0	0	0	1	4	X	4/4	3/3
		4	0	0	0	0	0	0	1	4	0/4	3/3	4/4
		5	0	0	3	0/-	0/-	6/6	X	X	X	X	X
		6	0	0	0	0	0	0	R	X	X	3/0	1/0
		7	0	0	0	0	1	RX	1	1	4	2/0	3/6
		8	0	0	0	0	0	0	0	3	6/6	X	X
		9	0	0	0	0	0	1	0	0R	3/6	X	X
		10	0	0	0	0	2	X/2	X/4	X/4	X/4	X	X
		11	0	0	0	0	1	0	2	X/4	X/6	1/1	4/3
		12	0	0	0	1	0	1	0	0	4	0	3
		13	0	0	1	2	1	0	0	2	3	X/6	X
		14	0	3	0	0	0	0	4	X/-	X/-	2	4
		15	1	2	1	0	2	0	0	RX	RX	4	6
		16	0	0	1	0	0	1	0	0	4	0	3
2	4/41	17	0	0	0	0	0	0	0	0	0	4/0	4/3
		18	0	0	0	0	0	0	0	0	3	3/0	4/0
		19	0	0	0	0	0	0	0	0	0	3/0	3/3
		20	0	0	0	0	0	0	A	1	4	3/0	3/3
1	2/84	21	0	0	0	0	0	0	4	0/4	1/4	4/4	2/1
		22	0	0	0	0	0	0	0	0	0	4/0	3/0
0·5	0/82												

A = Subject absent

All tests used the human repeat insult patch procedure with ethanol as the test vehicle. The following scoring system was used: 0—no visible reaction, 1—slight erythema, 2—marked erythema, 3—erythema and papules, 4—very strong oedema/papules, 5—vesicular eruptions, 6—any reaction above grade 3 extending beyond the patch, X—test reaction precludes patch application, R—adhesive reaction. A double grade during the induction phase indicates that the patch was moved to a new adjacent site. The first number is the grade for the new site; the second is the grade for the residual reaction at the old site. A dash indicates that the old site was not graded. A double grade during the challenge phase indicates that the site was read at two different itmes after removal of a challenge patch. Patch no. 1 was applied to the old site and patch no. 2 was applied to a new site.

posure to citral. In addition, the absence of elicited reactions attributable to citral in the 13,014 patch tests conducted on pure citral, on fragrance blends, and on personal care and household products containing citral indicates that sensitizations have not occurred as a result of the use of products containing citral. On the basis of the data reported in this survey, sensitization to citral as contained in consumer products is not apparent.

There were no confirmed reactions to citral in any of the 2098 patch tests on fragrance blends containing citral. Seven reactions were reported in tests on fragrance blends in which citral was a minor constituent (0·03 to 4×10^{-5}% in the patch test mixture), but it was not determined which ingredient in each case was responsible for the reactions. It is highly unlikely that citral was responsible for these reactions in view of the absence of any reactions to citral in 383 other patch tests with fragrance blend–vehicle mixtures containing higher citral concentrations, ranging from 0·04 to 0·36%. In addition, materials that have been reported to act as quenchers of citral-induced sensitization were present at many times the concentrations required for quenching (Opdyke, 1976) in each of the three fragrance blends that induced hypersensitivity.

The data reported in this survey from patch tests performed on citral confirm that, when patch tested alone, it can induce human sensitization under exaggerated test conditions. Further, the data indicate that the incidence of induced sensitizations to citral is concentration dependent (see Table 4). In a similar survey hydroxycitronellal also showed induced reactions that were concentration dependent when it was applied alone in a vehicle (Steltenkamp *et al.* 1980). Further, no elicited reactions attributable to hydroxycitronellal occurred in tests on consumer products containing the ingredient (Steltenkamp *et al.* 1980). Together, these data suggest that there is a relationship between the potential for induction and the exposure concentration of the inducing agent.

The test data reported in this survey are consistent with the phenomenon of quenching reported by Opdyke (1976 & 1979) in that induced sensitizations were reported in patch tests on pure citral in a vehicle mixture but none were reported in tests on fragrance blends or consumer products containing citral. The consumer products on which patch tests data were submitted were not intentionally formulated to include recommended quenching agents, yet no induced reactions were observed. This suggests that the conditions required to render citral innocuous are commonplace. The absence of a confirmed elicited reaction to citral within the large sample of reported

test data cannot be explained by the presence of quenching agents. Quenching agents are reported only to preclude the induction of a reaction and have not been demonstrated to prevent an elicited response in a previously sensitized subject (D. L. J. Opdyke, personal communication, 1979).

The data reported in this survey illustrate that exaggerated patch testing of an ingredient alone at high concentrations should not be the sole basis for an assessment of sensitization potential. This assessment should be based on all pertinent test results including results from sensitization tests which cover the product compositions and ingredient concentrations to which consumers have been exposed for a significant period of time.

Exposures to citral in consumer products did not induce hypersensitivity or elicit reactions in the large number of tests performed (10,660). The potential of citral to induce hypersensitivity existed only for the material alone and was concentration-dependent. No elicitation response to citral occurred in any of the 13,014 patch tests reported in this survey, nor have any been reported in the clinical literature.

The patch test data presented here, a significantly larger data base than has been available before, indicate that citral is not a sensitizer as used in consumer products.

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CINNAMIC ALCOHOL: A SURVEY OF CONSUMER PATCH-TEST SENSITIZATION

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Abstract—The potential for cinnamic alcohol, an important fragrance and flavour ingredient, to induce hypersensitivity or elicit reactions in humans was evaluated by analysing patch-test data. Data from a total of 16,530 patch tests were collected from fragrance and formulator companies on various consumer products, fragrance blends containing cinnamic alcohol, and on the material itself. This study indicates that cinnamic alcohol as present in consumer products and fragrance blends has no detectable potential to induce hypersensitivity. However, cinnamic alcohol itself can induce concentration-related hypersensitivity. The lowest cinnamic alcohol concentration which has been tested and reported as inducing hypersensitivity is 4%. Data for levels below 4%, available from the present survey ($\leq$0·4%) and from a European survey ($\leq$3·2%), reveal no induced hypersensitivity. Cinnamic alcohol at the concentrations present in consumer products and in fragrance blends has a very low potential to elicit sensitization as evidenced by only four confirmed reactions elicited by cinnamic alcohol in 16,530 patch tests. The data presented in this survey contribute valuable information regarding the safety of the use of cinnamic alcohol in consumer products and fragrances.

INTRODUCTION

A survey of skin sensitization data from human patch tests on materials containing cinnamic alcohol was conducted under the auspices of The Soap and Detergent Association (SDA). Many companies conduct safety tests on experimental and marketed products. As a result, data bases exist which can contribute valuable information for assessing the extent of existing sensitization in the consumer population and the potential of cinnamic alcohol, as found in consumer products, to induce hypersensitivity. The objective of publishing these data is to provide guidance for the safe use of cinnamic alcohol in consumer products. Surveys of hydroxycitronellal and citral were previously published (Steltenkamp, Booman, Dorsky, King, Rothenstein, Schwoeppe, Sedlak, Smith & Thompson, 1980a,b).

The data presented here on cinnamic alcohol were obtained from a survey of members of The Soap and Detergent Association and from fragrance suppliers. Participants in the survey submitted data from human patch tests on an assortment of commercial or experimental products containing cinnamic alcohol, on neat cinnamic alcohol in a vehicle, or on fragrance blends containing cinnamic alcohol and intended for use in consumer products.

Cinnamic alcohol (cinnamyl alcohol; 3-phenyl-2-propen-1-ol; CAS 104-54-1) is an important fragrance raw material used in many different fragrance types for its floral, balsam-like scent. It has been in public use since the 1930s and the soap and detergent industry has been a major user. An estimated 500,000 lbs are used annually in perfumes and flavours in the USA. Cinnamic alcohol is generally recognized as safe for food additive applications (FEMA, 1965) and is approved for food use by the Food and Drug Administration (21 CFR 172.515).

Commercial cinnamic alcohol is the trans isomer made by the hydrogenation of cinnamic aldehyde. It is more than 98% pure and contains less than 1·5% cinnamic aldehyde (EOA, 1978). Cinnamic alcohol also occurs in numerous natural products, either in the free state or as an ester. These natural products include styrax, cinnamon leaf, hyacinth, narcissus, gardenia and wisteria.

Cinnamic alcohol was selected for this survey because of recent reports in the literature of its potential to induce and elicit sensitization when applied to humans. Based upon the exaggerated predictive patch testing of the ingredient tested alone, cinnamic alcohol has a potential to induce hypersensitivity at high concentrations (Table 1). In contrast, formulations containing cinnamic alcohol as one ingredient at relatively high concentrations did not induce hypersensitivity or elicit reactions in a large number of European subjects (Table 2). Elicitation reactions have

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Table 1. *Induction by neat cinnamic alcohol in normal subjects*

Percentage cinnamic alcohol in vehicle	Vehicle	Reactions	Test method	Reference
10	Petrolatum	39/200*	KM	Opdyke, 1980
10	Hydrophilic ointment	2/25	KM	Opdyke, 1980
4	Petrolatum	0/25	KM	Greif, 1967; Opdyke, 1974
4	Petrolatum	0/25	KM	Jordan & King, 1977
4	Petrolatum	0/150	MD	Jordan & King, 1977
4	Alcohol	4/150	MD	Jordan & King, 1977
4	Alcohol	1/25	KM	Jordan & King, 1977

KM = Kligman maximization MD = Modified Draize

*Result of eight tests.

Table 2. *Induction and elicitation by formulations containing cinnamic alcohol in normal and dermatitic subjects**

Type of subjects tested	Percentage cinnamic alcohol in patch	Reactions	Experimental method	Fragrances tested
Normal	5·0	0/30	Prophetic patch	1
Approx. 40% with known sensitivity to chemicals	0·87†	0/130	Repeat insult patch	13
Approx. 30% with known sensitivity to chemicals	2·5‡	0/5620	Single closed patch	64

*These data are taken from F. Grundschober, IFRA, personal communication, 1979 (summarized in Opdyke, 1980).
†This is the mean value; all the values were less than or equal to 3·2%.
‡This is the mean value; all the values were less than or equal to 13·5%.

resulted from exposure of dermatitic patients to cinnamic alcohol alone (Table 3). In many cases, patients involved in these tests were simultaneously exposed to multiple ingredients and/or serial concentrations of the same ingredient. Many of the patients also reacted to at least one other fragrance ingredient in addition to cinnamic alcohol. In the literature, the incidence of elicited reactions in dermatitic patients contrasts sharply with the absence of elicited reactions both in subjects exposed to formulations containing cinnamic alcohol and in normal individuals exposed to cinnamic alcohol alone.

This report presents a survey of data on a wide variety of products that should help in assessing the potential for cinnamic alcohol to induce delayed hypersensitivity or elicit sensitization reactions in the

Table 3. *Elicitation by neat cinnamic alcohol in dermatitic patients**

Type of subjects tested	Percentage cinnamic alcohol in vehicle	Vehicle	Reactions	Reference
Known sensitive to Peru Balsam, reacted to more than one ingredient	5	Petrolatum	26/144	Hjorth, 1961
Known sensitive to chemicals	5	Alcohol	52–116/ 900–2000	Rudner, 1977
Known sensitive to an aftershave, reacted to three ingredients	5	Petrolatum	1/1	Van Ketel, 1978
Known sensitive to perfume, reacted to more than one ingredient	4–5	Petrolatum	15/20	Larsen, 1977
Known sensitive to cosmetics, multiple simultaneous exposure and reactions	2	Petrolatum	2/102	Ishihara, 1978†
Known sensitive to cosmetics, multiple simultaneous exposure and reactions	2	Petrolatum	4/52	Ishihara, 1978†
Known sensitive to cosmetics, multiple simultaneous exposure and reactions	1	Lanolin	5/183	Nakayama, 1974†
70% with known dermatitis, eczema and itching	10–50	Alcohol and olive oil	13/97	Gutman & Somov, 1968
Known sensitive to perfumes, reacted to more than one ingredient	0·25	Oil of Helianthin	3/10	Novak, 1974

*In all cases the test method used was the 48-hr diagnostic patch test.
†The data from Ishihara (1978) are summarized in Opdyke (1980) and those from Nakayama (1974) are summarized in Mitchell (1975).

Table 4. *Human sensitization survey: cinnamic alcohol in consumer products*

Product type	Percentage cinnamic alcohol in patch test mixture	Percentage cinnamic alcohol in product	Percentage product in patch test vehicle	Test method	No. of tests	No. of elicited sensitizations*
Personal care	8×10^{-2} to 1×10^{-1}	1×10^{-1}	Varied	HRIP	176	0
	5×10^{-2}	5×10^{-2}	100	HRIP	139	1†
	4×10^{-2}	5×10^{-2}	80	HRIP	129	1†
	2×10^{-7} to 3×10^{-2}	8×10^{-6} to 2×10^{-1}	6×10^{-2} to 100	Varied‡	5390	0
Household	7×10^{-2}	7×10^{-2}	100	HRIP	104	0
	3×10^{-7} to 4×10^{-2}	5×10^{-6} to 6×10^{-2}	0·1 to 100	Varied‡	6312	0

HRIP = Human repeat insult patch

*There were no induced reactions.
†See Table 5 for reaction grades.
‡Both the HRIP and the prophetic patch test procedures were used.

general population. Very few data have been published from sensitization tests with normal subjects conducted on the kinds of products and formulations reported in this paper.

EXPERIMENTAL

This survey was initiated in May, 1979, to collect data from human patch tests conducted in the USA by member companies of The Soap and Detergent Association and fragrance suppliers. Participants were requested to submit all available patch test data both on commercial products and experimental, or non-commercial, products containing cinnamic alcohol, on concentrated fragrance blends containing cinnamic alcohol, and on cinnamic alcohol alone. Consumers are not exposed to concentrated fragrance blends or cinnamic alcohol alone.

The data requested in this survey included the same type of information as gathered in a previous survey on hydroxycitronellal (Stelkenkamp *et al.* 1980a). Also, the screening and selection of the test subjects were the same as previously described, as were the patch test procedures.

Reported cinnamic alcohol concentrations were from the direct use of the material itself and did not include any amounts that might be present due to the use of natural products or specialities in which it is a constituent.

RESULTS

This survey of human sensitization data includes the results of 16,530 patch tests on products or test materials containing a wide range of cinnamic alcohol concentrations. Since some subjects participated in more than one test, it is estimated that a total of approximately 13,000 different subjects were involved. Results were reported from tests on 119 formulations and on cinnamic alcohol itself. Vehicles used in the tests included water, ethanol, petrolatum, dimethyl phthalate, and isopropyl palmitate. In some cases a finished product was applied directly to the skin.

The results were reported from a total of 12,250 patch tests on personal care and household products where the applied concentrations of cinnamic alcohol ranged from 2×10^{-7} to 1×10^{-1}% (Table 4). No induced hypersensitivity or elicited sensitization reactions were observed in the 6416 patch tests conducted on household products. However, with personal care products, two cases of elicited sensitization (subjects 4 & 6, Table 5) to unmarketed experimental products were observed among 5834 patch tests. A mild sensitization reaction was elicited on subject 4 after the first application during the induction phase. On challenge, subject 4 developed moderate to strong reactions while subject 6 reacted mildly. These two cases of elicitation were attributable to cinnamic alcohol contained in the fragrance portion of the personal care products.

In addition to the two elicited reactions attributed to cinnamic alcohol, there was one additional elicited reaction reported to the fragrance contained in a personal care product for which the causative agent was not identified. The one personal care product, tested at a cinnamic alcohol concentration of 4×10^{-2}%, elicited mild to moderate reactions during induction and mild reactions upon challenge (subject 8 in Table 5). The fragrance in this product was not fractionated and consequently a definite link between the elicited reaction and cinnamic alcohol was not established. Another component of the product could have been reponsible for this reaction. The data on this possible case are reported for completeness.

Results from 4226 patch tests were reported on fragrance blends tested at cinnamic alcohol concentrations of 9×10^{-8} to 4×10^{-1}% (Table 6). As with personal care and household products, sensitization was not induced in any subject exposed to fragrance blends. However, two elicited sensitizations attributed to cinnamic alcohol were observed. One of 69 panelists (subject 3 in Table 5) developed mild to severe elicited reactions to a fragrance blend tested at 8×10^{-2}% cinnamic alcohol. After each of the first six applications in the induction phase, the elicited reactions were mild to moderate. After the seventh, eighth, and ninth patches, severe reactions were observed. During the challenge phase the reactions were only mild to moderate in this subject. Cinnamic alcohol tested at 4×10^{-2}% as a component of another fragrance blend elicited one sensitization in 72 subjects (no. 5 in Table 5). The elicited sensitization was mild to moderate during the induction and challenge phases. There were four further elicited sensitization reactions among the 4226 subjects exposed to fragrance blends containing cinnamic alcohol in which the causative agent was not identified (subjects 7, 9, 10, and 11 in Table 5). No definite

Table 5. *Human sensitization survey: sensitization reactions attributable to cinnamic alcohol and reactions to materials containing cinnamic alcohol where the causative agent was not identified*

Percentage cinnamic alcohol in patch test mix	Product type	Response ratios	Subject	Induction phase. Reaction grade after application no.									Challenge phase. Reaction grade after application at site no.	
				1	2	3	4	5	6	7	8	9	1	2
Reactions attributable to cinnamic alcohol														
6·0	Neat	2/54	1*	0	1	1	0	1	1	0	0	0	1/1	1/1
			2*	0	0	0	0	0	0	0	0	1	1/1	0/0
8×10^{-2}	Fragrance blend	1/69	3†	1	2	1/1	1	2	2/1	5/1	4X	4X	1E/2E	1E/2E
5×10^{-2}	Personal care	1/19	4†	1E	2	1/1	1	2	2EX	1EX	2X	1	2E/3E	2E/3E
4×10^{-2}	Fragrance blend	1/72	5†	0	1E	2E	1/1E	2E	2E/2X	2EX	1X	1	2E/2	1/1
4×10^{-2}	Personal care	1/129	6†	0	1E	1E	2E	1E/1	2E	1/2E	2EX	3E	1E/1	1E/1E
Elicited reactions to materials containing cinnamic alcohol														
2×10^{-1}	Fragrance blend	1/79	7†	3EX	3EX	3EX	2X	2X	2X	2X	1X	1X	X/X	X/X
4×10^{-2}	Personal care	1/41	8†	1	2E	2/2	1/1	2	2/X	1X	1X	1X	1/1	1/1
4×10^{-2}	Fragrance blend	2/72	9†	0	0	2E	0/1E	1E	2E	1/2EX	2E/2X	2	1/1	1/1
			10†	0	1	0	1	1E	1	1E	1E	1	2E/1	1E/0
9×10^{-8}	Fragrance blend	1/80	11†	0	0	1E	1E	2E	0/1E	1	1E	1	2E/1E	2E/2E

*The following scoring system applies to subjects 1 and 2: 0—no visible reaction, 1—redness, slight puffiness, scaliness and occasional, small, pinpoint-sized papules.

†The following scoring system applies to subjects 3 to 11: 0—no visible reaction, 1—mild reaction, erythema, 1E—mild erythematous reaction with papules/oedema, 2—moderate erythematous reaction, 2E—moderate erythematous reaction with papules/oedema, 3—strong reaction, erythema, 3E—strong reaction, erythema with papules/oedema, 4—severe reaction, erythema with papules/oedema, 5—bullous reaction, X—next patch not applied and next grade is residual reaction.

A double grade during the induction phase indicates that the patch was moved to a new adjacent site. The first number is the grade for the new site; the second is the grade for the residual reaction at the old site.

A double grade during the challenge phase indicates that the site was read at two different times after removal of a challenge patch. Patch no. 1 was applied to the old site and patch no. 2 was applied to a new site.

Table 6. *Human sensitization survey: cinnamic alcohol in fragrance blends*

Percentage cinnamic alcohol in patch test mixture	Percentage cinnamic alcohol in fragrance blends	Percentage fragrance blend in patch test vehicle	Test methods	No. of tests	No. of elicited sensitizations*
1×10^{-1} to 4×10^{-1}	0·5 to 18·0	2 to 25	HRIP	854	0
8×10^{-2}	4	2	HRIP	69	1†
5×10^{-2} to 7×10^{-2}	0·5 to 3·6	2 to 10	HRIP	416	0
4×10^{-2}	7×10^{-1}	5	HRIP	72	1†
9×10^{-8} to 4×10^{-2}	5×10^{-6} to 3	1 to 100	Varied‡	2815	0

HRIP = Human repeat insult patch

*There were no induced reactions.

†See Table 5 for reaction grades.

‡Both the HRIP and the prophetic patch test procedures were used.

link between these reactions and cinnamic alcohol could be made because the fragrances were not fractionated.

Six percent neat cinnamic alcohol in dimethyl phthalate applied by the repeat insult patch technique induced mild sensitization in 2 of 54 test subjects. Both subjects (nos 1 and 2 in Table 5) developed mild reactions during the induction phase. Reactions during the challenge phase were also mild.

DISCUSSION

Assessment of the sensitization potential of a fragrance ingredient such as cinnamic alcohol, which has been used for many years in consumer products, is based on its potential to elicit pre-existing sensitization and to induce new cases of hypersensitivity in the consumer population. The present study was concerned with both aspects of sensitization, namely elicitation and induction.

Consumer product and fragrance blend formulations tested at cinnamic alcohol concentrations of 0·4% and lower in this survey did not induce hypersensitivity in 16,476 patch tests. Similarly, no induced reactions were reported in a European survey involving 160 patch tests on formulations tested at cinnamic alcohol concentrations up to 5% (F. Grundschober, IFRA, personal communication, 1979). The lowest cinnamic alcohol concentration which has been tested and reported as inducing hypersensitivity is 4% cinnamic alcohol tested alone in vehicle (Table 1). Cinnamic alcohol tested alone in this survey induced two mild sensitization reactions in 54 panelists exposed to 6%. The results of this survey support the concentration-dependent incidence of induced reactions observed in the available literature.

There were only four confirmed elicited reactions to cinnamic alcohol in this survey of 16,476 patch tests on consumer products and fragrance blends tested at 4×10^{-1} to 9×10^{-8}% cinnamic alcohol. No confirmed elicited reactions occurred when exposure was less than 4×10^{-2}%. Two of the elicited reactions involved personal care products tested at 4×10^{-2} and 5×10^{-2}% cinnamic alcohol and two involved fragrance blends tested at 4×10^{-2} and 8×10^{-2}% cinnamic alcohol. The extent to which these results reflect inter-laboratory variation cannot presently be assessed, but the absence of elicitations in 1084 patch tests with cinnamic alcohol concentrations equal to or greater than the highest concentrations producing an elicitation reaction must be considered in making an overall safety assessment of cinnamic alcohol. In another survey in Europe (see Table 2), formulations containing cinnamic alcohol produced no elicited reactions in 5780 patch tests at concentrations ranging up to 13·5% and with about 30% of the subjects known to be sensitive to chemicals.

In contrast to the four elicited reactions in normal subjects observed in tests reported in this survey on formulations containing cinnamic alcohol and on cinnamic alcohol alone in vehicle, dermatitic patients treated with cinnamic alcohol alone in vehicle reportedly exhibited variably high incidences of elicitation (Table 3). However, these patients were often exposed simultaneously to multiple fragrance ingredients and/or concentrations of the same ingredient. Every patient that exhibited reactions elicited by cinnamic alcohol and for whom data were reported also exhibited reactions elicited by other fragrance ingredients. The expected difference between dermatitic patients and normal subjects is borne out by the high incidence of elicited reactions to cinnamic alcohol in the former and the low incidence reported for the latter in this broad survey. This supports the findings of the hydroxycitronellal survey (Steltenkamp *et al.* 1980a), that patch-test data obtained from dermatitic patients do not predict the sensitization potential of a material for the normal population.

Exposures to cinnamic alcohol in consumer products did not induce hypersensitivity in the large number of patch tests performed (12,250). Cinnamic alcohol has a very low potential to elicit sensitization reactions at the concentrations used in consumer products as evidenced by two elicited reactions in 12,250 patch tests on consumer product formulations and two elicited reactions in 4226 patch tests on fragrance blends containing cinnamic alcohol. Induced hypersensitivity is concentration related. The lowest concentration of cinnamic alcohol which has been tested and reported as inducing hypersensitivity is 4%. Below 4%, data available from the present survey ($\leq$0·4%) and the European survey ($\leq$3·2%) reveals no induced hypersensitivity.

The patch test data presented here, a significantly larger data base than has been available before, indicate that the potential for sensitization through consumer product use of cinnamic alcohol is very low.

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SHORT PAPER

OESTROGENIC ACTIVITY OF SOYA-BEAN PRODUCTS

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Abstract—Normal rat cake containing soya meal was found to be oestrogenic. Sixteen samples of soya meal were examined in the mouse uterine weight bioassay and all were found to have oestrogenic activity. Ethyl-acetate extracts of the meals also had oestrogenic activity. Genistein and daidzein were present in the extracts.

Introduction

It has previously been reported from this laboratory (Drane, Patterson, Roberts & Saba, 1975) that rat cake used as a control feed in routine mouse bioassays for oestrogens had developed significant uterotrophic activity over a period of a few months. We have recently encountered another oestrogenic control feed, higher basal uterine weights than expected being found in mice fed this ration. Investigation of the components of the feed showed that the oestrogenic activity was due to soya meal, which made up 10% of the rat cubes.

Little attention seems to have been paid to soya meal as a possible source of oestrogenicity although daidzein and genistein were isolated from soya beans nearly 50 yr ago (Walz, 1931). The oestrogenic activity of these and other isoflavones is well documented (Bickoff, Livingston, Hendrickson & Booth, 1962; Carter, Smart & Matrone, 1953; Cheng, Story, Yoder, Hale & Burroughs, 1953). A new isoflavone, glycitein, has been isolated from soya beans (Naim, Gestetner, Kirson, Burk & Bondi, 1973) and recently coumestrol was also shown to be present at levels ranging from 0·05–30 μg/g (Lockhart, Jones & Finney, 1978). The present report provides bioassay data showing that oestrogenic activity was present in all sixteen samples of soya meal examined.

Experimental

Materials. Sample 1 was the extracted soya-bean meal that had been used in the manufacture of the control feed (Porton Rat Diet) associated with the original problem. Samples 2–14 were soya-bean meals of various origins destined for the manufacture of farm-animal feeds. Sample 11 was a pelleted form of feed. Samples 15 and 16 were soya-bean products intended for human consumption. Semi-synthetic (SS) feed supplied by RHM Labsure Ltd. was used as a soya-free control.

Three extracts were prepared. For the first, 90 g soya-bean meal was exhaustively extracted with ethyl acetate in a Soxhlet apparatus. The solvent was evaporated to dryness and the residue was re-dissolved in a convenient volume of ethanol–ethyl acetate (1:1, v/v). A second, 70%-ethanol extract was prepared as described for the extraction of oestrogens from white clover (Saba, Drane, Hebert & Holdsworth, 1974). A third extract in aqueous acetonitrile was also prepared (Drane *et al.* 1975).

Oestrogen bioassay. Eighteen-day-old MF1 weanling female mice weighing 7–9 g were supplied by OLAC 1976 Ltd., Bicester, Oxon. They were housed in groups of six to a cage and each group was given 40 g of feed over a period of 3·5 days. On the following day the mice were killed and the uteri were dissected out, blotted on filter paper and weighed. Each assay included a control group given only the SS feed and three or four groups given SS feed containing known amounts of diethylstilboestrol (DES). The test soya meal samples were fed alone, or mixed with SS diet, or as an extract mixed into SS diet and air dried.

Mycology and mycotoxin screening. Samples of soya-bean meal were screened for possible mycotoxin contamination by the method described by Roberts & Patterson (1975) as modified by Patterson & Roberts (1979). The mycological examination of six samples was carried out by the methods described by Shreeve, Patterson & Roberts (1975).

Thin-layer chromatography (TLC). Biologically-active ethyl-acetate extracts were examined for phyto-oestrogens by TLC using Polygram Sil G/UV_{254} sheets and methanol–chloroform (7:93, v/v) as the developing solvent. Genistein, daidzein and formononetin (minimum detectable levels 10 μg/g) and coumestrol (minimum detectable level 1 μg/g) were run as reference compounds. The developed chromatogram was examined under long- (360 nm) and shortwave (250 nm) ultra-violet light for fluorescing and absorbing spots both before and after exposure to ammonia vapour. These active extracts were also analysed for zearalenone using the mycotoxin method cited above (analytical limit 20 μg/kg).

Results and Discussion

No mould growth was evident in any of the six soya meals sampled and Fusarium species were not

isolated in mycological cultures of the meals. Neither zearalenone nor any other mycotoxin was detected.

The mouse uterine weight bioassay data are summarized in Table 1. Samples 2 and 3 were oestrogenic when fed as whole meal but their extracts were not tested. Nine other samples were active when fed as whole meal and also when fed in the form of ethyl-acetate extracts, while five further samples were active only when fed as ethyl-acetate extracts. Thus all sixteen samples showed biological activity. No oestrogenic activity was found in extracts in 70% aqueous ethanol, which is routinely used to extract substances with oestrogenic activity from red and white clover (Bickoff, Loper, Hanson, Graham, Witt & Spencer, 1967; Saba *et al.* 1974). Neither was it found in extracts in acetonitrile, which has previously been used to isolate an active fraction 6bII from oestrogenic rat cake (Drane *et al.* 1975). However, all of the ethyl-acetate extracts were oestrogenically active, although when appropriate comparisons were made, it was found that the recovery of the source of the activity present in the original samples of soya meal was poor. Hydrolysed ethyl-acetate extracts examined by TLC were found to contain genistein and daidzein and preliminary experiments suggested that the former isoflavone contributed most of the oestrogenic activity. No other reference oestrogen was detected.

Various reproductive disturbances in animals have been traced to the ingestion of oestrogenic feeds. Cattle became infertile whilst grazing lucerne containing high concentrations of coumestrol (Adler & Trainin, 1967), hyperoestrogenism was reported in pigs fed diets containing 0·1–6·8 μg zearalenone/g (Mirocha, Pathre & Christensen, 1977), the conception rate was lowered in sheep fed 8–16 μg DES/day and conception was prevented altogether in sheep given 32 μg DES/day (Morley, Bennett & Axelsen, 1963). The present results suggest that comparable levels of oestrogenic activity might be provided by diets containing soya products; in those whole soya meals in which quantifiable amounts of oestrogenic activity were present, levels equivalent to 8–37 ng DES/g soya were detected. On the basis of our own estimate that the potency of the mycotoxin zearalenone in the mouse bioassay is $8{\cdot}5 \times 10^{-4}$ that of DES, the observed oestrogenic activity of these soya meals was equivalent to 9·4–43·3 μg zearalenone/g soya.

There is little published information on the oestro-

Table 1. *Oestrogenic activity of whole soya meal and of ethyl-acetate extracts of whole soya meal in the mouse uterine weight assay*

	Results for mice fed whole soya meal				Results for mice fed ethyl-acetate extracts of soya meal			
Sample no.	Maximum total dose† (g whole soya meal/mouse)	Uterine wt (geometric mean; mg)	DES equivalent (ng/g soya)	Calculated zearalenone equivalent‡ (μg/g soya)	Maximum total dose† (g soya meal extracted/ mouse)	Uterine wt (geometric mean; mg)	DES equivalent (ng/g)	Calculated zearalenone equivalent‡ (μg/g soya)
1	6	10·4*	<10§		NT			
	6	16·5**	8	9·4	15	19·4***	5	5·9
2	6	11·7	<10		NT			
	6	13·9*	17	19·9	NT			
3	6	16·0***	10–20		NT			
	6	17·4***	17	19·9	NT			
	6	19·0***	10	11·7	NT			
4	6	11·3***	<20		NT			
	6	13·6***	10	11·7	20	18·6***	3·5	4·1
5	3	12·2**	<10		15	16·6***	4	4·7
6	3	8·5	0·0		15	19·0***	5	5·9
7	3	11·0*	<10		14	33·5***	8	9·4
8	2·5	6·3	0·0		12	29·1***	7	8·2
	5	10·5	<10		NT			
9	2·5	7·7	0·0		14	21·3***	4	4·7
	5	9·0	<10		NT			
10	2·5	10·1**	<10		12	24·0***	5	5·9
11	3	7·8	0·0		12	15·5***	3	3·5
12	2·5	9·9	<10		12	22·8***	5	5·9
13	3	31·5***	37	43·3	15	56·5***	12	14·0
14	3	13·6***	12	14·0	15	32·7***	7	8·2
15	5	34·0***	24	28·1	15	36·3***	8·7	10·2
16	4	15·5***	16	18·7	15	25·3***	7	8·2

NT = Not tested

†The mice ate poorly, and therefore the dose is only approximate.

‡By the mouse uterine weight assay the zearalenone equivalent per unit wt of DES = 1170 (850–1600).

§Oestrogenic activity present at levels equivalent to < 10 ng DES/g soya could not be quantified.

The values marked with asterisks differ significantly from those of the controls that were given soya-free semi-synthetic feed (*$P < 0{\cdot}05$; **$P < 0{\cdot}01$; ***$P < 0{\cdot}001$). Sample 1 was a soya-bean meal used in the manufacture of rat feed. Samples 2–14 were soya-bean meals used for the manufacture of farm-animal feeds. Samples 15 and 16 were soya-bean products intended for human consumption.

genic activity of foodstuffs for human consumption (Schoental, 1977) and this report helps to remedy the situation. Since soya meal is an important source of protein for animal feeds and is now increasingly used in human food, we feel that this apparently constant source of oestrogenic activity should not be overlooked, even though it is at a low level. However, species differ greatly in their susceptibility to the effects of oestrogens and caution must therefore be exercised when attempting to extrapolate data from species to species or from the biological effects of one oestrogenic substance to another.

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Review Section

REVIEWS OF RECENT PUBLICATIONS

Nasopharyngeal Carcinoma: Etiology and Control. Edited by G. de-Thé and Y. Ito. IARC Scientific Publications No. 20. International Agency for Research on Cancer, Lyon, 1978. pp. xvii + 610. Sw.fr. 100.00.

Tumours with a high incidence in specific localities are frequently attributed to some environmental factor and it has often been claimed that an intensive study of their biology may provide a valuable insight into the aetiology of human tumours. The attention currently being paid to nasopharyngeal carcinoma is very much in line with such thinking. The incidence of this tumour is high in some Far Eastern countries, notably China, while in others, including Western Europe, it is very low indeed. Furthermore there are indications that certain ethnic groups are more prone than others to its development.

The salient features of the research on this disfiguring and fatal form of cancer are presented in these proceedings of an international symposium held in Kyoto, Japan, 2 years ago. The venue for the symposium was particularly appropriate, since a major part of the research effort discussed at the symposium had apparently been carried out in Japan. The communications fell broadly into four main groups, dealing respectively with the histopathological appearances of nasopharyngeal carcinomas, the clinical course of the disease, epidemiological studies and investigations into the aetiology of the tumour.

Most of the papers on histopathology were concerned with the definition of the controversial undifferentiated tumours called by some carcinoma and by others lymphoepithelioma. No fresh evidence seems to have been presented for settling this issue, although some serological typing techniques appear to hold promise for the future. The papers on the clinical aspects of the disease emphasized the difficulties of making an early diagnosis: the nasopharynx is surrounded by cranial nerves and by organs of the special senses and the first symptoms are usually referable to one of these rather than to the nasopharynx itself. An excellent account of the clinical course of this disease and its response to radiotherapy revealed that the rate of recurrence after treatment is disturbingly high and bears no clear relation to the histological type of the tumour.

Epidemiological studies are being pursued with the aims of defining more clearly the geographical areas where the disease shows the greatest prevalence and of identifying the ethnic groups that seem to be most susceptible to it. The reports presented at the symposium indicated that, in areas of low incidence, epidemiologists were paying greater attention to the identification of certain groups showing an incidence of the tumour that was higher than expected in that particular community as a whole. There are indications that certain occupations offer a greater risk of tumours of the nasal cavity than do other walks of life. Unfortunately the papers presented made no specific mention of any such occupations, although in view of previous claims that woodworkers appear to be at greater risk of developing this type of tumour than other workers, one might have expected greater attention to be given to the occupations that apparently carry such risk.

Considerable attention was devoted to the possibility that a viral agent might be responsible for the development of nasopharyngeal carcinomas. In view of the prevalence of lymphoma-like tumours in the nasopharynx, the Epstein-Barr virus (EBV) has been suspected, presumably because of its connection with malignant lymphoma in some parts of Africa. Detailed accounts of the quest for this virus were presented in many of the communications. Several techniques, including ultrastructural and serological studies and nucleic acid hybridization, have been used in the search. Unfortunately the fruits of these extensive—and expensive—efforts have been disappointing. Yet some workers still believe that a viral aetiology is the likely explanation, and the serological investigations have been widened to include a search for viruses other than EBV.

It was disappointing to find that the symposium participants had paid so little attention to the possibility of chemical induction of nasopharyngeal carcinoma. Many chemicals can induce this tumour in rodents, not only when inhaled but also when administered by the oral or parenteral routes, and a discussion of relevant studies would have added considerably to the value of this book. In fact, only two out of some forty communications were devoted to chemical induction of the tumour and the fields selected were so narrow and the experimental designs so poor that they make little impact.

On the whole the book is aimed at those seeking to understand the nature and epidemiology of the human disease. There is little in it for those involved in occupational medicine or for the scientist working in the field of chemical carcinogenesis.

Residue Reviews: Residues of Pesticides and Other Contaminants in the Total Environment. Vol. 69. Edited by F. A. Gunther. Springer-Verlag, New York, 1978. pp. viii + 146. $22.00.

Residues Reviews: Residues of Pesticides and Other Contaminants in the Total Environment. Vol. 70. Edited by F. A. Gunther. Springer-Verlag, New York, 1979. pp. viii + 144. $21.80.

Volume 69 of Residue Reviews opens with a survey of the physico-chemical interactions of parathion with soils. A picture is built up of the fate of this organo-

phosphorus pesticide, which is used to control both plant- and soil-borne insects, and of the speed (or relative slowness) of its degradation. The non-toxic metabolites formed by hydrolysis of the phosphate ester bond tend to be retained by the soil colloids.

For many years, toxaphene has been the most heavily used pesticide in the USA, but its importance has not been reflected in the frequency of its appearances in the literature. In recent years, however, research on toxaphene has intensified, and now two US academics, seeking to remedy the literary neglect, have reviewed the available data. Surveying its acute, subacute and chronic toxicity in various species, including man, they conclude that while toxaphene shows, in most animals, an intermediate level of toxicity when compared with other organochlorine pesticides, it is extremely toxic to fish. Data on its biodegradability and on the persistence of its residues in crops, animal tissues and milk present a confusing picture, largely because of the analytical problems presented by a substance said to be a mixture of at least 177 separate components. For the same reason, metabolic studies are still at a very early stage. All in all, the impression remains that here is a fruitful field for study, the success of which will depend on the development of more discriminating and sensitive analytical methods and on the careful recording of all known details about any test samples used, particularly the method of preparation and the toxaphene detection limits of the analytical methods.

These two pesticide contributions are separated by a review of the occurrence of polycyclic aromatic hydrocarbons (PAH) in food and their possible significance in terms of human health. This wide-ranging survey considers the types of food in which PAH have been found and identifies 11 that have been shown to be carcinogenic in experimental animals out of the total of 22 detected in foods. Lengthy tables detail the carcinogenicity findings on these 11 compounds and provide other evidence in support of the text. Consideration is also given to the many metabolites of the PAH, including the K-region epoxides. Ideas that the high incidence of stomach cancer in some areas may be related to a high consumption of smoked fish or meat are aired in this review, but the authors conclude that, with this possible exception, there is no epidemiological evidence to link the PAH in foods with any specific human disease.

The next issue in this series carries the bright red cover indicating the cumulative indexes for the latest ten volumes. Accompanying these indexes are a survey of chemical, physical and biological methods for the disposal and detoxication of pesticides and a step-by-step outline of the field studies necessary to establish safe re-entry intervals after the application of organophosphates to crops. The volume also contains a discussion on the effects of herbicides on the structure and function of plant-cell membranes and, more specifically, on their interaction with the lipid components of the cell membrane.

Much has been written recently on the 're-entry problem'. The approach offered in this volume is an essentially practical one—re-entry intervals are viewed as occupational health standards and particular attention is paid to study design, project organization, requirements for laboratory work and statistical analyses. A brief section at the end of this chapter refers to the establishment of re-entry intervals for applications of carbamates. This topic has previously received little attention but presents special problems, particularly because the reversibility of carbamate effects on cholinesterase activity necessitates great speed in carrying out laboratory procedures.

Pharmacological Methods in Toxicology. Edited by G. Zbinden and F. Gross. Pergamon Press Ltd., Oxford, 1979. pp. xi + 612. £57.50.

The difficulties encountered in recognizing functional disturbances in conventional toxicological studies call for a careful assessment of the possible application of both established and new experimental techniques adopted by pharmacologists for the study and measurement of organ function. This book is a major response to this need. Derived initially from the proceedings of a 1977 International Workshop on the Use of Pharmacological Methods in Safety Evaluation of New Drugs, it appears to be the first in which a group of distinguished pharmacologists has attempted to provide some guidelines on the application of current pharmacological techniques to the assessment of toxic effects, particularly those reflected in disturbances of organ function. Emphasis is placed on methods that have proved useful in the detection of side effects or adverse reactions. In addition to a large number of individual contributions, the volume contains the reports of the working parties of pharmacologists, toxicologists and clinicians, whose discussions on the possible use of pharmacological methods in the preclinical safety evaluation of new drugs preceded the Workshop.

While the introductory chapter, entitled "General concepts" may perhaps be considered somewhat lacking in detail, in comparison with the depth of coverage given to some of the more specific topics, it nevertheless provides an interesting view of the similarities and differences in the approaches of pharmacologists and toxicologists, and outlines the problems involved in applying the methods of the one discipline to the other. At the same time, it pinpoints the justifications for introducing pharmacological methods into some aspects of toxicity testing, and indicates areas where this is likely to be of particular benefit.

The rest of the book follows a pattern that has become traditional in the study of pharmacology. Beginning with autonomic pharmacology, it analyses, section by section, the pharmacology of the major organs and systems. Outstanding are the sections on the cardiovascular system, on blood coagulation and platelet function and on behavioural pharmacology. The section on bronchopulmonary function is surprisingly brief in view of the great importance of inhalation as a route of entry and elimination of toxic compounds. The only contribution on this topic is the report of the main working party; no individual contributions are presented, as is the case in all the other sections. Other notable deficiences are the omission of skin pharmacology and immunology from the topics considered at the Workshop. However, it is a common disadvantage of volumes based on the proceedings of meetings, that they lack the comprehensive

coverage that could be provided by a standard textbook. Overall this publication contains much that will be of interest to those concerned with the continuing development of toxicity testing.

Hepatotoxicity. The Adverse Effects of Drugs and other Chemicals on the Liver. By H. J. Zimmerman. Prentice-Hall International, London, 1978. pp. x + 597. £35.40.

The author of this volume has attempted to cover the vast field of chemically induced liver injury, and to summarize the work of biochemists, pathologists and clinicians. To accomplish this task he has divided his book into several sections. The first deals, in seven chapters, with basic biochemical mechanisms, the structural and biochemical manifestations of injury, a classification of hepatotoxins and the mechanisms underlying their effect and, finally, chemical carcinogenesis in the liver. In the second section, on specific aspects of experimental hepatotoxicity, the author deals separately with direct and with indirect hepatotoxins, dividing the latter into those that have cytotoxic effects and those that cause cholestasis. Sections three and four deal respectively with environmental and iatrogenic hepatic injury. In general the chapters in the latter sections are based on groups of compounds, and in many of the reports of drug effects, a brief introduction is followed by a consideration of clinical features, incidence, facts affecting susceptibility, prognosis, mechanisms and histopathology.

Each section can be read in isolation, without reference to the other three. The fact that the chapters form self-contained essays is at once the book's greatest strength and greatest weakness. As each one can be read independently, the reader can pick his area of interest and gain useful information rapidly, but this approach means much repetition. One example of this is the discussion of the role of diet, sex and age on the mixed-function oxidase system, together with induction and inhibition of enzyme activity. This topic is discussed in chapter 2 and to a large extent is repeated in chapter 3. Similarly carbon tetrachloride toxicity is covered in detail in chapter 9, but many of the points are made also in earlier chapters, in some cases on more than one occasion. A few other minor criticisms could be made of this otherwise excellent book. In the second chapter, which deals in an introductory manner with drug metabolism, the pivotal position of cytochrome *P*-450 is described and the distinction is made between phase 1 and phase 2 reactions, but a few pages later type 1 and type 2 spectral interactions are mentioned and it may not be clear to the uninitiated that the terms are used in somewhat different contexts. Some further clarification might have been helpful here. Secondly, the chapter on chemical carcinogenicity seems sparse in comparison with other chapters, particularly with regard to carcinogen metabolism, site of activity and the precise meaning and significance of the initiated state. There are few statements of fact with which one would actively disagree (interpretation, as always, is another matter). However the suggestion (p. 77) that most of the cholangiocarcinomas attributable to experimental hepatocarcinogens are variants of hepatocellular carcinoma is probably an overstatement and the position of butter yellow (dimethylaminobenzene) and its derivatives should have been discussed.

Apart from these few small points, the author has made a fine job of reviewing this complex subject in 597 pages. The data are presented in a lucid and consistent manner, and are supported by useful summary tables and diagrams, which will no doubt be of great value to experienced and inexperienced workers alike. At £35.40 the volume is a sound investment for those interested in this subject.

Chemical Diagnosis of Disease. Edited by S. S. Brown, F. L. Mitchell and D. S. Young. Elsevier/North-Holland Biomedical Press, Amsterdam, 1979. pp. xviii + 1383. Dfl. 153.00.

This book is essentially a collection of monographs on the interpretation of clinical chemistry in the context of medical practice. As such, it is comprehensive, authoritative, up-to-date and lucid. However, the deliberate avoidance of methodological detail in favour of in-depth discussion of interpretation, while laudable, entails the risk of ignoring those circumstances in which the significance of an observation must be judged against the background of its method of acquisition.

An introductory contribution of "Biological variability" by Professor D. S. Young sets the tone of circumspection for the whole volume and is followed by chapters on specific topics of analytical practice, including the chemical analysis of body fluids other than blood, and hydrogen ions, carbon dioxide and oxygen as components of blood, as well as glucose metabolism, lipids, proteins, enzymes, and calcium and magnesium. Several chapters concentrate on the clinical-chemical consequences of pathological conditions in the kidney, liver, gastro-intestinal tract, pancreas and thyroid, while others discuss topics of current interest such as the measurement of steroid hormones and their metabolites, haemoglobin variants and drug monitoring. The volume ends with two chapters on cancer.

The editors are to be congratulated on a systematically organized and extremely readable book. There can be no doubt of its appeal to clinical-chemical pathologists; one hopes that its merits will also command attention from the clinicians who "tick the boxes"!

BOOKS RECEIVED FOR REVIEW

Trace Metals: Exposure and Health Effects. Proceedings of the Research Seminar held at the University of Surrey, Guildford, United Kingdom, 10–13 July 1978. Edited by E. Di Ferrante. Pergamon Press Ltd., Oxford, 1979. pp. viii + 262. £15.00.

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Information Section

ARTICLES OF GENERAL INTEREST

A WHIFF OF PETROL

The complex network of ecological problems associated with the broad range of petroleum products is demonstrated in a 50-page 'ecotoxicological review' of the industry by Korte & Boedefeld (*Ecotoxic. envir. Safety* 1978, **2**, 55). The enormous tonnages involved, the possible bioaccumulation of some additives and other constituents, and the high volatility of many of the bulk compounds are some of the factors influencing the 'global impact' of the various branches of the petroleum industry. In particular, the volatility of many of these products has clear repercussions in the field of industrial health.

Pulmonary damage resulting from inhalation of petrol vapour has been studied by a group at the University of New South Wales, Sydney. In the first of a series of papers on this work, Lykke & Stewart (*Experientia* 1978, **34**, 498) reported the changes induced in the lungs of male rats exposed to air containing 100 ppm petrol vapour for 8 hr/day on 5 days/wk for up to 12 wk. The petrol used throughout the study was a commercial grade, octane rating 98%, and contained 0·45 g tetraethyllead/litre. In 22 of 28 rats exposed for 6–12 wk, there were unequivocal changes in lung histology, ranging from the appearance of minor foci of interstitial fibrosis to widespread sclerosis. Most of the remainder had been exposed only for 6 or 7 wk, and none for more than 9 wk. The pulmonary parenchyma involved in the advanced lesions showed aggregation of fibroblasts with occasional macrophages, and irregular deposition of collagen which extended into the surrounding parenchyma in the plane of the basement membrane. From wk 6, prolongation of the exposure period was accompanied by an increasing incidence of irregular alveolar collapse and the development of sclerotic foci and by variable overdistension of other alveoli. Shorter periods of exposure were associated with sporadic hypertrophy and hyperplasia of type 2 pneumocytes; later on, these pneumocytes decreased in number and developed vacuolated surfactant lamellae. No animal exposed for less than 6 wk showed clear evidence of any alterations in lung structure. Tolerance diminished in rats exposed to petrol vapour for longer than 8 wk and prostration and tachypnoea developed, although recovery was usually rapid following removal to a normal atmosphere. Because of these findings, and particularly the rapidity with which the lesions developed, the rat was considered to be a suitable model for ultrastructural and biochemical studies of the development of pulmonary fibrosis.

The observations outlined above were largely confirmed in female rats similarly exposed (Lykke *et al. Pathology* 1979, **11**, 71), although the histological changes were less marked. However, most of the rats showed pathological changes at the ultrastructural level after exposure to 100 ppm petrol vapour for 6 wk or more. There were wide individual variations in the severity and extent of these ultrastructural changes and in their onset in relation to exposure time, but an overall pattern involving three major steps emerged.

The three stages were marked by predominantly degenerative changes, by mainly hypertrophic and/or hyperplastic changes and finally by the development of interstitial sclerosis and irregular alveolar collapse. The irregularly distributed degenerative changes, which were apparent in a few rats exposed for 5 wk but were more generally apparent after wk 6, consisted of hydropic degeneration of the cytoplasm of interstitial fibroblasts, leading to interstitial thickening, irregular distension of the endoplasmic reticulum and an irregular distribution of electron-lucent vacuoles in the cell sap. Focal areas of grossly hydropic endothelial degeneration were also observed. Nevertheless, complete endothelial rupture was rare and inter-endothelial junctions were usually closed. Generally between wk 6 and 10, type 2 (surfactant-producing) pneumocytes increased in size and their cytoplasmic density was increased by large numbers of intensely-staining surfactant lamellar bodies. The enlarged cells often had two distinct nuclei, although no mitotic figures were detected at any stage, and many of the cells appeared to be embedded in or to have invaginated the interstitium. Their tendency to adhere to opposite surfaces of the alveolar walls was apparently one cause of an observed distortion of the microcirculation. An increasing occurrence of irregular foci of fibrosis during wk 9–12 was associated with alveolar distortion and collapse, and some distension of the associated alveolar sacs. In these areas, the type 2 pneumocytes showed vacuolar cytoplasmic degeneration, with loss of the lamellar phospholipid structure of the surfactant granules. Moderate deposition of collagen fibrils and interstitial matrix occurred in the disordered interstitial planes.

The observations that petroleum vapour caused first hypertrophy and/or hyperplasia and then degeneration of the type 2 surfactant-producing pneumocytes while their type 1 counterparts were unaffected and, furthermore, that in the type 2 cells the degenerative changes involved only the intracellular organelles concerned with surfactant secretion led Le Mesurier *et al.* (*ibid* 1979, **11**, 81) to study the effects of petrol inhalation on the amount and composition of pulmonary surfactant in the rat. Pulmonary surfactant is secreted into the alveolar spaces and is a complex substance rich in saturated phosphatidyl cholines (lecithins) with lesser amounts of lipid, other phospholipids and protein. Its main functions are to

decrease surface tension and to stabilize surface forces between large and small alveoli during the inflation/deflation cycle (Morgan, *New Eng. J. Med.* 1971, **284**, 1185). Using female rats and the exposure procedure described above (except that exposure was terminated after 45 days), Le Mesurier *et al.* (*loc. cit.*) found that by day 5 there was a marked reduction in the yield of surfactant obtained by endobronchial lavage. Thereafter the yield continued to decline, reaching a minimum by day 15. Subsequently there was some fluctuating recovery, and between days 35 and 45 a relatively constant yield, approximately 50% of the control mean, was maintained. Chromatography of the surfactant showed no qualitative change in its phospholipid composition during the course of the exposure.

While the mechanisms underlying these effects cannot be said to be established, these workers deduced several hypotheses from their findings. One possible rationale for the series of events observed is that surfactant secretion may be inhibited by the direct effect of the petrol vapour on the surfactant-producing organelles of the type 2 pneumocytes and that the resulting deficiency of surfactant precipitates alveolar collapse. Collapse of the alveoli may, in turn, cause respiratory distress and morphological changes, the latter ('pseudofibrosis') resulting directly from the apposition of collapsed alveolar walls. True fibrosis, however, is a later event, and certain early changes are not accounted for by this hypothesis either. Thus both the vascular endothelium and interstitial fibroblasts showed early degeneration but apparently recovered, the fibroblasts being stimulated later to an excessive level of collagen production. The tentative suggestion is made, therefore, that different factors in the petrol vapour may be acting independently on these different cells of the pulmonary parenchyma.

Further studies on this topic will be of interest, not merely in connection with assessing the toxic hazard of petrol inhalation but as a possible contribution to a greater understanding of the syndrome of fibrosing alveolitis in man. Such studies may perhaps provide confirmation of the evidence presented here, which seems to point to the type 2 pneumocyte as the primary target for some constituent of petrol vapour.

[P. Cooper—BIBRA]

STYRENE AND THE CHROMOSOME

The primary metabolite of styrene, its epoxide, has been found to be a potent mutagen in a number of test systems, with and without metabolic activation (*Cited in F.C.T.* 1978, **16**, 300; *ibid* 1978, **16**, 397; *ibid* 1979, **17**, 298). Styrene itself has been found to increase the chromosomal aberration rate of the lymphocytes of occupationally-exposed workers (*ibid* 1979, **17**, 299), but the results of experimental studies have been equivocal, although they have established that the presence of a metabolic activation system is an undoubted prerequisite of *in vitro* demonstrations of styrene mutagenicity.

Loprieno *et al.* (*Scand. J. Work Envir. Hlth* 1978, **4**, Suppl. 2, 169) studied the incidence of chromosomal aberrations in the bone-marrow cells of mice given a single dose of styrene or styrene oxide by gavage and killed 24 hr after treatment. Styrene oxide caused a significant increase in the number of aberrations at doses of 50, 500 and 1000 mg/kg. A dose-dependent response was found, although the highest dose was toxic. The aberration rate was unaffected by styrene. By contrast, Fabry *et al.* (*Mutation Res.* 1978, **51**, 377) gave adult male mice a single ip injection of 250 mg styrene oxide/kg and found no increase in the number of chromosomal aberrations in bone-marrow cells after 1, 2, 6 or 13 days [but no results were shown either for positive or for negative controls]. Similarly, largely negative results were obtained by Norppa *et al.* (*Chemico-Biol. Interactions* 1979, **26**, 305) using the bone-marrow of Chinese hamsters. The animals were exposed to 25, 50, 75 or 100 ppm styrene oxide for 9 hr (6 hr on day 1 and 3 hr on day 2) or 21 hr (6 hr on days 1, 2 and 3 and 3 hr on day 4). At the lowest dose the effects of exposure for 3 wk (6 hr/day, 5 days/wk) were also studied, and at the highest dose only the 9 hr duration was used because of serious signs of poisoning in the animals. The animals were killed immediately after the last exposure. None of the animals treated by inhalation showed any significant increase in the frequency of cells with chromosomal aberrations. However in animals given a single ip injection of 500 mg styrene oxide/kg there was a significant increase in the number of chromosomal aberrations in the cells of animals that died 18–22 hr after treatment but not in those that were killed after 24 hr.

Chromosomal aberrations were also observed in human lymphocytes taken from a single donor and exposed to 0·1 or 0·5 mM-styrene oxide for 48 hr (Fabry *et al. loc. cit.*). Most of the anomalies were chromatid gaps and breaks. Linnainmaa *et al.* (*Mutation Res.* 1978, **58**, 277) studied human lymphocytes exposed to 2·6 mM-styrene during the entire culture period or to 0·7 mM-styrene oxide during the last 8 hr of culture (both toxic levels). The styrene treatment induced chromosome breaks, and styrene oxide caused severe chromosome destruction resulting in pulverized chromosomes. In many metaphases that did not show chromosomal fragmentation the chromosomes acquired a non-specific banded staining, and with both compounds there was a significant increase in the number of interphase cells with micronuclei or nuclear bridges. Onion (*Allium cepa*) root-tip cells were also used by Linnainmaa *et al.* (*loc. cit.*) to study the production of chromosomal aberrations *in vivo*. The results with this sytem were, for both styrene and styrene oxide, very similar to those obtained with human lymphocytes *in vitro*.

These results and those of previous studies in rats and in man (*Cited in F.C.T.* 1979, **17**, 299) show that both styrene and its oxide undoubtedly produce chromosomal aberrations under certain conditions. It seems likely that the assortment of results obtained

arises from the widely different routes, levels and durations of exposure used.

Styrene did not increase the mutation rate of *Salmonella typhimurium* strain TA1535 at concentrations ranging from 1×10^{-3} to 10 μmol/plate either with or without S-9 mix (Loprieno *et al. loc. cit.*). Survival ranged from 0·8 to 100% under these conditions. Styrene oxide was mutagenic both with and without the S-9 mix at levels of 1–100 μmol/plate and the response was dose-related except at the highest dose-level which was extremely toxic. These results support those of Stoltz *et al.* (*Bull. envir. Contam. Toxicol.* 1977, **17,** 739; *Cited in F.C.T.* 1978, **16,** 397) but contradict those of De Meester *et al.* (*Mutation Res.* 1977, **56,** 147; *Cited in F.C.T.* 1979, **17,** 298) who found that styrene itself was mutagenic towards this strain when a metabolic activating system was used.

In cultured Chinese hamster bone-marrow cells there was no increase in the mean number of sister-chromatid exchanges in any of the styrene oxide inhalation exposure groups used by Norppa *et al.* (*loc. cit.*). However the group given 500 mg styrene oxide/kg by ip injection showed a marginally significant ($P < 0·05$) increase in the number of sister-chromatid exchanges, but the increase was only seen in one out of six animals. Styrene oxide was previously shown to be a potent inducer of sister-chromatid exchanges *in vitro* in Chinese hamster ovary cells (*Cited in F.C.T.* 1979, **17,** 298).

Fabry *et al.* (*loc. cit.*) found no evidence of mutagenicity when polychromatic erythrocytes from 8-wk-old mice were examined 30 hr after a single ip injection of 250 mg styrene oxide/kg. They also obtained negative results of the meiotic chromosomes of treated male mice and in dominant lethal tests performed 1–3 wk after the injection. DNA repair in cultured human (EUE) cells was stimulated by styrene oxide at a level of 4·4 mM, but styrene did not increase DNA repair even in the presence of a metabolic activation system (Loprieno *et al. loc. cit.*). These authors also found that styrene oxide, but not styrene, produced point mutations in the Chinese hamster cell line V-79. They consider that the lack of mutagenic activity of styrene itself may be due to rapid conversion of its active metabolite, styrene oxide, to phenylethylene glycol. The latter conversion, which is activated by microsomal epoxide hydratase, occurs at a faster rate than that of styrene to styrene oxide. Moreover, it has been shown (*Cited in F.C.T.* 1978, **16,** 300) that epoxide-hydratase activity is markedly increased by the treatment of rats with styrene but is largely unaffected by similar treatment with styrene oxide.

[P. Cooper—BIBRA]

THE VARYING FATE OF VINYLIDENE CHLORIDE

Vinylidene chloride (1,1-dichloroethylene; VDC) is used as a monomer in plastics manufacture, particularly for the production of wrapping films. Acute inhalation of VDC has been shown to decrease glutathione stores in the rat liver (Jaeger *et al. Expl mol. Path.* 1974, **20,** 187) and inhalation of 25 ppm VDC for 52 wk has been associated with the development of malignant tumours of the kidney in male mice, although 200 ppm had no such effect in rats (Maltoni *et al. Medna Lav.* 1977, **68,** 241). Several recent studies of VDC have highlighted the ways in which its metabolism differs in mice and rats as well as the effects of inadequate food intake and of various routes of administration on its fate in rats. In other work, the species differences have been reflected in the results of short-term mutagenecity tests on VDC.

In the study by Jaeger *et al.* (*loc. cit.*), depletion of hepatic glutathione stores by VDC and the accompanying hepatotoxicity were found to be exacerbated by a period of fasting prior to VDC exposure. Later McKenna *et al.* (*Toxic. appl. Pharmac.* 1978. **45,** 599 & 821) studied the pharmacokinetics of VDC administered by inhalation or orally to both fed and fasted rats. In the inhalation study (p. 599), groups of male rats fed normally or fasted for 18 hr before treatment were exposed to 10 or 200 ppm [^{14}C]VDC vapour for 6 hr, and the elimination of ^{14}C activity was studied for the following 72 hr.

After exposure to 10 ppm, about 98% of the absorbed VDC was converted to non-volatile metabolites both in normal and fasted rats. However, after exposure to 200 ppm, the proportion of VDC metabolized was significantly lower and showed a significant difference between the fed and fasted rats, at 96 and 92% respectively of the total amount absorbed. There was also a significant difference between the total amounts absorbed by the fed and by the fasted rats, the total body burdens in the fasted rats being lower at both exposure levels. Despite the markedly higher pulmonary elimination of unchanged [^{14}C]VDC by fasted rats exposed to 200 ppm, compared with rats exposed to 10 ppm, the former also showed a higher level of retention of ^{14}C activity. Retention in the fed rats exposed to 200 ppm, however, differed little from that in the 10-ppm groups. The main sites of VDC retention in all the groups were the liver, kidneys and lungs. Liver and kidney lesions developed after 200-ppm exposure of the fasted but not the fed rats. No such effect resulted in any rats treated with 10 ppm VDC. The affected livers showed centrilobular necrosis and the kidneys marked degeneration of the proximal tubular epithelium. The liver lesions were associated with a higher level of covalently bound ^{14}C activity than was seen in fed rats and the levels in the latter were in turn considerably higher than those in normally fed rats exposed to 10 ppm VDC.

Similar results were obtained in rats given an oral dose of 1 or 50 mg [^{14}C]VDC/kg (McKenna *et al. loc. cit.* p. 821). In normally fed rats the proportions of the lower and higher dose exhaled unchanged were 1–3 and 19%, respectively, and the metabolism of the larger dose was further reduced (29% being exhaled unchanged) in rats fasted for 18 hr before treatment.

Elimination of non-volatile metabolites was somewhat greater in the fed than in the fasting rats given the higher dose and, again as was found in the rats inhaling VDC vapour, fasting increased the concentration of covalently-bound ^{14}C activity in the liver after ingestion of 50 mg [^{14}C]VDC/kg.

In both studies, two of the major metabolites of VDC in the urine were identified as *N*-acetyl-*S*-(2-hydroxyethyl)cysteine and thiodiglycollic acid. Conjugation with liver glutathione therefore seems to be a major pathway for the detoxication of VDC in the rat, but the evidence points also to some metabolism of VDC to a more reactive compound which binds to subcellular components. It seems possible that when a high dose of VDC saturates the detoxifying capacity of liver glutathione stores depleted by fasting, the covalent binding of VDC metabolite(s) to tissue constituents is enhanced, with the resulting development of overt toxicity.

A comparison of the patterns of VDC elimination in rats following administration of similar doses by different routes was made by Jones & Hathway (*Chemico-Biol. Interactions* 1978, **20**, 27). Male rats were given [^{14}C]VDC in single doses of 0·5 or 350 mg/kg by the intragastric or ip route and of 0·5 mg/kg iv, and ^{14}C activity was measured in the urine, faeces and exhaled air over 72 hr. The change in excretion patterns with dose was clearly demonstrated by the use of these widely differing doses. At the higher level, a high proportion of the administered ^{14}C (68 and 92% after intragastric and ip dosing, respectively) was eliminated via the lungs, mainly as unchanged VDC (67 and 91% of the dose), with urinary excretion in these groups accounting for 30 and 8%, respectively. In contrast, the lower dose was excreted mainly in the urine, which accounted for 80% of the intragastric and 69% of the ip dose. At this level, about 5% of the intragastric dose was exhaled as CO_2 and less than 1% as unchanged VDC, with about 8% in the faeces, while after ip dosing, some 4% was exhaled as CO_2, 12% was unmetabolized and about 16% was in the faeces.

The authors relate these findings not only to the possible saturation of the metabolizing capacity but also to an efficient arterial–alveolar transfer, which rapidly reduces the concentration of unchanged VDC in systemic blood, thus leaving little available for transformation in successive passes through the liver. Support for this contention comes from their finding that more than 60% of the small iv dose was exhaled unchanged within 5 min of injection and 80% within 60 min. The change in pulmonary excretion of unchanged VDC over a range of intragastric doses between 0·5 and 650 mg/kg suggested a rapid release of VDC which had not been absorbed by the tissues or which exceeded the saturation of the body pool and a subsequent slower release from the tissues (probably adipose tissue and liver). Metabolites appeared in the urine over 3 days and this relatively slow rate of excretion was consistent with the view that at least part of the urinary radioactivity had been transported in the bile. Like McKenna *et al.* (*loc. cit.* p. 599), Jones & Hathway (*loc. cit.*) identified thiodiglycollic acid as the ultimate detoxication product of VDC. Another major product was an *N*-acetyl-*S*-cysteinyl acetyl derivative. and they also demonstrated substantial amounts of chloroacetic acid, dithioglycollic acid and thioglycollic acid. It is probable that chloroacetic acid, which may be derived from VDC by migration of one chlorine atom and loss of the other, is on the main metabolic pathway for VDC, since the two compounds have several metabolites in common. The VDC-derived CO_2 may be the product of a minor oxidative pathway for chloroacetic acid or possibly of the action of epoxide hydratase on 1,1-dichloroethylene oxide. The reaction of 1,1-dichloroethylene oxide with glutathione *S*-epoxide transferase probably accounts for the *N*-acetyl-*S*-cysteinyl acetyl derivative.

These authors also demonstrated some differences in the metabolism of VDC in rats and mice (Jones & Hathway, *Br. J. Cancer* 1978, **37**, 411). After an oral dose of 50 mg [^{14}C]VDC/kg, mice and rats, respectively, excreted 6 and 28% of the dose as unchanged VDC in exhaled air, and 3 and 22% as thiodiglycollic acid, 23 and 5% as dithioglycollic acid and 50 and 28% as the *N*-actyl-*S*-cysteinyl acetyl derivative in the urine. Mice excreted no chloroacetic acid and rats no *N*-acetyl-*S*-(2-carboxymethyl)cysteine. At this dose level, mice metabolized over 20% more of the VDC dose than did rats. These findings are in accordance with known differences in certain enzyme activities, in particular the higher levels of *P*-450 activity that have been demonstrated in mice, as well as with the difference in the toxic effects of VDC in the two species. Not only is VDC more readily metabolized in mice but it also shows a much higher degree of acute toxicity in that species and, as previously mentioned, has induced kidney tumours in mice but not in rats. The authors of this paper suggest that the murine carcinogenicity of VDC may reflect the greater availability of the reactive metabolite 1,1-dichloroethylene oxide and its rearrangement product chloroacetyl chloride, resulting from the relatively high level of cytochrome *P*-450 activity, as well as a greater likelihood that these products will react with DNA than is the case in the rat.

Some support for this contention was obtained by Jones & Hathway (*Cancer Lett.* 1978, **5**, 1) from a short-term mutagenicity study. Exposure of *Salmonella typhimurium* strain TA1535 to VDC vapour indicated that VDC was weakly mutagenic in the presence of a post-mitochondrial supernatant (S-9 mix) from normal mouse kidney or liver and was strongly mutagenic in the presence of S-9 mix from Aroclor 1254-induced murine organs. With S-9 mixes derived from rats, however, only the liver preparation from Aroclor-induced animals was associated with a positive bacterial response to VDC, and this was relatively weak. VDC showed no significant mutagenic effect in the presence of kidney or liver preparations from rats that had not been pretreated with Aroclor. Similar results were obtained with *S. typhimurium* strain TA100. Mention is made in this paper of some very limited data from Ames tests in which S-9 mixes derived from human or marmoset livers were used. These suggested that primates may respond more like rats than mice as far as the generation of alkylating metabolites of VDC and their reaction with bacterial DNA are concerned, but clearly a lot more work will be required before any conclusion on that aspect can be drawn.

[P. Cooper—BIBRA]

PATTERNS OF CARCINOGENICITY

Over the last few years a range of short-term tests has been developed with the aim of accurately predicting carcinogenic activity and so reducing the need for long-term animal studies. Another approach to predicting carcinogenicity is the study of structure–activity relationships (SARs). Knowledge of the molecular basis of carcinogenic activity is still far from complete, but SAR methods are being used to find correlations between structure and carcinogenic activity in animal studies. An extensive analysis of the available SAR data for different groups of carcinogens has been published (Arcos *et al. Chemical Induction of Cancer I*, Academic Press, New York, 1968; Arcos & Argus, *Chemical Induction of Cancer IIA and IIB*, Academic Press, New York, 1974) and the broad SARs for several series of carcinogens have been reviewed by Ashby (*Br. J. Cancer* 1978, **37**, 904).

There have been numerous searches for physicochemical indices that correlate with carcinogenic activity. For example, using methods closely following those developed for the study of SARs in drug systems, Wishnok & Archer (*ibid* 1976, **33**, 307) studied a set of 47 *N*-nitrosamines on the basis of their carcinogenicity in the BD rat. They found statistically significant correlations between carcinogenic activity, toxicity and the number of carbon atoms per molecule. In further studies, Wishnok *et al.* (*Chemico-Biol. Interactions* 1978, **20**, 43) established that carcinogenic activity was correlated with water–hexane partition coefficients and electronic factors for *N*-nitroso compounds. Smith *et al.* (*Cancer Res.* 1978, **38**, 2968) used theoretical reactivity indices to examine the metabolic reactions of 25 representative polycyclic aromatic hydrocarbons and found strong correlations between several of the indices and carcinogenic activity. Using a pattern-recognition technique, Dunn & Wold (*J. mednl Chem.* 1978, **21**, 1001) carried out an SAR study on 4-nitro- and 4-hydroxyamino-quinoline 1-oxides. Each of the ten non-carcinogens and 18 carcinogenic compounds was described by physico-chemical variables and a model was derived for the carcinogens. This model was 82% successful in predicting the carcinogenic potential of the compounds.

Recently, computer-assisted pattern recognition SAR techniques have been applied to a large number of compounds representing a wide range of chemical groups, and the results of two such studies indicate that these methods have considerable potential in the accurate prediction of carcinogenicity. Jurs *et al.* (*ibid* 1979, **22**, 476) worked on a set of 209 heterogeneous compounds of known carcinogenic activity. These compounds were drawn from more than 12 structural classes and included aromatic amines, alkyl halides, *N*-nitroso compounds, polycyclic aromatics, azo dyes and naturally occurring compounds such as simple sugars, amino acids and fungal toxins. The set was made up of 130 carcinogens and 79 non-carcinogens.

The structures of the chemicals were entered into the computer by sketching them on the screen of a graphics display terminal. The classification of the compounds as carcinogenic or non-carcinogenic (as given by McCann *et al. Proc. natn. Acad. Sci. U.S.A.* 1975, **72**, 5135; McCann & Ames, *ibid* 1976, **73**, 950) was also entered. The next step was to use the computer to generate molecular structure descriptors. The following types of descriptors were used: fragment descriptors (number of atoms of each type, number of bonds of each type, molecular weight, number of basis rings, number of ring atoms), substructure descriptors (number of occurrences of a particular substructure of interest), environment descriptors (giving information about the interconnection of fragments and substructures), molecular-connectivity descriptors (a measure of the branching of the structure) and geometric descriptors (representing the shape of the molecule). A large number of descriptors was developed and tested. Pattern recognition techniques were then used to discriminate between carcinogens and non-carcinogens. [The descriptors developed are used to represent each molecule as a point in multidimensional space. The value of each co-ordinate is the numerical value of one of the descriptors. The aim is to find a set of descriptors that will cause the carcinogenic compounds to cluster in one limited region of the space and the non-carcinogenic compounds to cluster elsewhere. Discrimination functions (discriminants) are then developed that will define the boundaries between the clusters.] No set of descriptors was found that would support a discriminant that could separate all the carcinogens from all the non-carcinogens. The best sets of descriptors produced recognition percentages of 90–95%. It was found that 17 compounds were preventing linear separation of the carcinogens and non-carcinogens. When these had been eliminated a set of 26 descriptors was identified that was sufficient to allow complete separation of the remaining 192 carcinogens and non-carcinogens.

The predictive ability of these 26 descriptors was assessed. A 'prediction set' of ten compounds (six carcinogens and four non-carcinogens, a ratio reflecting that in the total population under consideration) was randomly selected from the 192 compounds remaining. A linear discriminant was identified that would separate the remaining 'training set' of 182 compounds into carcinogens and non-carcinogens and then without further change this discriminant was used to predict the carcinogenic activity of each of the ten test compounds. From a total of 300 individual predictions an overall predictive ability of 85·3% was obtained. The predictive success for carcinogenicity (90%) was much higher than for non-carcinogenicity (78%).

Chou & Jurs (*J. mednl Chem.* 1979, **22**, 792) went on to apply the same techniques to 153 *N*-nitroso compounds. A set of 15 descriptors was identified that would allow linear discrimination between 116 carcinogens and 28 non-carcinogens. (Two pairs of diastereoisomers and five misclassified compounds were eliminated.) The set was made up of six fragment descriptors, five molecular-connectivity descriptors, one geometric descriptor, one environmental descriptor and two σ-charge descriptors (coding the electronic properties of molecules). Prediction sets of ten carcinogens and three non-carcinogens were used and the overall success rate in a total of 650 predictions was 91%, with success rates of 93% for carcinogens and 85% for non-carcinogens.

These studies demonstrated that chemical carcino-

gens and non-carcinogenic compounds can be represented by groups of calculated molecular descriptors that will allow the separation of large numbers of carcinogens from non-carcinogens. Furthermore, the discriminants derived were shown to be able to predict with considerable accuracy the carcinogenic activity of 'unknown' compounds.

The data on carcinogenicity used in the computer-assisted SAR study of the heterogeneous group of 209 compounds (Jurs *et al. loc. cit.*) was taken from published complications of the carcinogenic activity of about 300 compounds (McCann *et al. loc. cit.*; McCann & Ames, *loc. cit.*) which were assessed for mutagenic activity in the Ames test. It was found that 90% (157/175) of the carcinogens were mutagenic and that 87% (94/108) of the non-carcinogens were non-mutagenic. Therefore, the same level (10%) of false-negative results was obtained in the Ames test as in the computer-assisted SAR study. However, the percentage of false positives was considerably higher in the SAR study (22%) than in the Ames test (13%). The figures for the Ames test depend very much on the choice of chemicals for the test, just as those from the computer-assisted SAR study are influenced by the lack of true 'unknown' compounds. Both sets of figures must therefore be viewed cautiously.

It would be interesting to know whether Jurs *et al.* (*loc. cit.*) and Chou & Jurs (*loc. cit.*) found any particular individual or groups of compounds that consistently gave false results in their predictive trials. The 17 compounds that were eliminated from the heterogeneous compound data set because they prevented linear separation included two (safrole and 1'-hydroxysafrole) that were classified as false negatives in the screening programme using the Ames test (McCann *et al. loc. cit.*; McCann & Ames *loc. cit.*), one that gave a weak false-positive result (5-nitro-2-furamidoxime) and two that gave stronger false-positive responses (1-[(5-nitrofurfurylidene)amino]hydantoin and dibenz[*a.h*]anthracene 5,6-oxide). The 15 descriptors used in the computer-assisted SAR study of *N*-nitroso compounds correlated with indices that had previously been suggested to be related to the carcinogenic activity of this group of compounds. Correlations have been reported between the partition coefficients of nitrosamines and their carcinogenic activity (Wishnok *et al. loc. cit.*; Singer *et al. Chemico-Biol. Interactions* 1977, **19**, 133). Molecular connectivity is known to correlate with various physico-chemical properties, including the partition coefficient. Kier *et al.* (*J. pharm. Sci.* 1978, **67**, 725) related molecular-connectivity indices to the mutagenicity of nitrosamines.

Would computer-assisted SAR studies identify non-mutagenic carcinogens? Could they be used to fill the gaps left by mutagenicity screening tests, such as the Ames test? How well would the computer-based SAR methods do when tested with genuinely 'unknown' compounds? These are some of the questions that will need to be answered before the full value of these apparently promising new methods of carcinogenicity screening can be determined. Clearly they have considerable value in furthering our understanding of the structural features of molecules that can lead to carcinogenic potential.

[M. E. Morris—BIBRA]

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ABSTRACTS AND COMMENTS

FOOD ADDITIVES AND CONTAMINANTS

Amaranth and the teratogenicity of cadmium

Gale, T. F. (1979). Toxic effects of cadmium and amaranth on the developing hamster embryo. *Bull. envir. Contam. Toxicol.* **22**, 175.

Several studies have shown that cadmium interferes with prenatal mammalian development and that it causes foetal malformations if administered at a critical period during pregnancy (Webster, *Archs envir. Hlth* 1978, **33**, 36). There has been controversy, however, about the teratogenic potential of the food colouring, amaranth. Whilst the bulk of the evidence suggests that amaranth does not produce teratogenic effects (Drake, *Fd Cosmet. Toxicol.* 1977, **15**, 153; *Cited in F.C.T.* 1977, **15**, 487), there have been some reports of effects on foetal development, most recently in the cat (*ibid* 1977, **15**, 487). The present investigation was therefore carried out to determine the effects of cadmium, amaranth, and cadmium in combination with amaranth, on the developing hamster embryo.

On day 8 of gestation, ten pregnant golden hamsters were injected iv with 100 mg amaranth/kg, ten others were administered 2 mg cadmium sulphate/kg, and another ten were administered 100 mg amaranth/kg and 2 mg cadmium sulphate/kg in two separate injections. On day 15 of gestation, all the hamsters were killed and the uteri and foetuses were examined. Half of the foetuses were subsequently dissected for identification of internal malformations and the rest were studied for defects in skeletal development.

The results indicated that under these conditions, amaranth alone was neither embryolethal nor teratogenic, whereas cadmium alone caused a significant increase in both resorptions and malformations. Furthermore, it was found that the administration of amaranth with cadmium protected the foetuses from cadmium-induced damage of the brain and of the bones of the skull and hindlimbs, but not from the other cadmium effects. These studies thus provide further evidence for the non-teratogenicity of amaranth, and for the teratogenic potential of cadmium. They also serve as yet another illustration of how the teratogenic effect of one substance can be modified by the simultaneous administration of another.

Local sarcomas from olefine oxides

Dunkelberg, H. (1979). On the oncogenic activity of ethylene oxide and propylene oxide in mice. *Br. J. Cancer* **39**, 588.

Propylene oxide and ethylene oxide have been used for food sterilization as well as for the sterilization of surgical materials. Propylene oxide is known to form chloropropanols in the presence of chlorides, and these derivatives have been found to be mutagenic for the TA1530, TA1535 and TA100 strains of *Salmonella typhimurium* (*Cited in F.C.T.* 1977, **15**, 252; Pfeiffer & Dunkelberg, *Fd Cosmet. Toxicol.* 1980, **18**, 115). Moreover, propylene oxide has been shown to alkylate DNA (*Cited in F.C.T.* 1973, **11**, 1143). 2-Chloroethanol, which has been detected in cereal products, spices, flour and other foods, following sterilization with ethylene oxide, has also shown some mutagenic activity in *S. typhimurium* (*ibid* 1972, **10**, 592; Pfeiffer & Dunkelberg, *loc. cit.*) The paper cited above, however, is concerned with the direct actions of the parent compounds on mammalian tissues.

Female mice were given a weekly sc injection of ethylene oxide in a dose of 0·1, 0·3 or 1·0 mg/animal or of propylene oxide in a dose of 0·1, 0·3, 1·0 or 2·5 mg/animal, dissolved in each case in tricaprylin. At the time of this preliminary report, dosing had continued for 91 wk, at which time 25–45% of the mice in the various test groups were still surviving.

Sarcomas developed at the injection site in groups treated with ethylene oxide or propylene oxide, but not in the tricaprylin-treated controls. In the group given the highest dose level of ethylene oxide, 77 were alive at wk 50, when the first tumour was detected; 75 of these were dead by wk 91, twelve with injection-site tumours. The corresponding numbers for the top-dose propylene oxide group were 81 alive at wk 39, 68 dead at wk 91 and 15 with injection-site tumours. The treatments showed a clear dose-response relationship, apart from the 0·1 and 0·3 mg doses of propylene oxide, which produced a similar incidence of local tumours (3·8 and 2·3%, respectively). Ethylene oxide at these dose levels produced a somewhat higher incidence (7·1 and 8·7%, respectively), although after 91 doses of 1 mg of either compound the findings were somewhat closer, 15·6% of ethylene oxide-treated mice and 12·4% of the propylene oxide group being affected. The tumours that occurred at sites remote from the injection area were mostly lymphomas and showed a similar incidence in the treated groups and in the tricaprylin-treated and untreated controls. Histological findings are to be reported in detail at the end of the study.

[So far, this study has produced no evidence to suggest that either ethylene oxide or propylene oxide has any systemic carcinogenic effect. The formation of sarcomas at the site of repeated injection of a test substance is frequently a direct response to tissue damage, which is in turn a reflection of the physicochemical properties of the injected material (Grasso & Golberg, *Fd Cosmet. Toxicol.* 1966, **4**, 297; Grasso *et al. ibid* 1971, **9**, 463). The occurrence of such tumours cannot be taken, therefore, as a pointer to the ability of a chemical to induce tumours when administered by the oral route.]

Lead contamination from wine-bottle caps

Wai, C. M., Knowles, C. R. & Keely, J. F. (1979). Lead caps on wine bottles and their potential problems. *Bull. envir. Contam. Toxicol.* **21**, 4.

The storage of wine and fruit juices in lead-glazed earthenware utensils has given rise to lead poisoning (Klein *et al. New Engl. J. Med.* 1970, **283**, 669). Another source of lead for the wine drinker is now revealed.

Metal caps used to seal wine corks were found in a US study to contain 96–98% lead with a small proportion of tin. A white powdery substance frequently found on the cork and around the mouth of the bottle, especially in the case of old wines that were tending to seep through the cork, contained lead carbonate hydroxide, $2PbCO_3.Pb(OH)_2$, together with a hydrated form, $3PbCO_3.2Pb(OH)_2.2H_2O$. The relative amounts of each compound varied from sample to sample. Another, amorphous, corrosion product possessed in infra-red spectrum characteristic of carbonate absorption.

Samples were removed from the bottles by pipetting and also by normal decantation without prior removal of the corrosion deposits, and their lead content was compared. Analysis of one red and one white wine from the bottles with marked seal corrosion showed that the pipetted samples contained 0·31 and 0·23 ppm lead, compared with 1·6 and 1·7 ppm, respectively, in the decanted samples. In another wine, from a bottle with an only slightly corroded seal, the lead levels were 0·15 and 0·4 ppm for the pipetted and decanted samples respectively. The pH of the wines was about 3·5, making them capable of attacking metallic foil, particularly during storage in the traditional horizontal position. The UK limit for lead in wine is currently 1 ppm (SI 1979 No. 1254) and Canada and Germany are reported to have proposed maximum levels of 0·5 and 0·3 ppm, respectively. Regulations (and taste) apart, wine drinkers who observe corrosion of a foil seal should obviously take the precaution of removing any incrustation before extracting the cork.

Fate of nitrosopyrrolidine and dimethylnitrosamine in mice

Johansson-Brittebo, E. & Tjälve, H. (1979). Studies on the distribution and metabolism of *N*-[^{14}C]nitrosopyrrolidine in mice. *Chemico-Biol. Interactions* **25**, 243.

Johansson-Brittebo, E. & Tjälve, H. (1979). Studies on the distribution and metabolism of ^{14}C-dimethylnitrosamine in foetal and young mice. *Acta pharmac. tox.* **45**, 73.

N-Nitroso compounds have been implicated as carcinogens in the human environment; the carcinogenic risk associated with *N*-nitrosopyrrolidine (NPYR) and *N*-nitrosodimethylamine (NDMA) has been evaluated by the IARC (*Cited in F.C.T.* 1979, **17**, 167). The clarification of the distribution and metabolism of NPYR in adult mice and NDMA in foetal and young mice was achieved using low-temperature autoradiography and autoradiography with dry tape sections, thus facilitating distinction between the volatile non-metabolized compound and its non-volatile metabolites.

The distribution of both a single iv injection and an orally administered dose of [^{14}C]NPYR was found to be uniform in adult mice treated 2 hr previously with pyrazole, ethanol, nialamide or dithiocarbamate (known inhibitors of NDMA metabolism). In non-pretreated animals killed 1 and 5 min after iv administration of [^{14}C]NPYR, the distribution of labelled non-volatile material indicated local metabolite formation in the liver, the tracheo–bronchial and nasal mucosa and the Harderian gland. In those killed at later intervals (0·5–24 hr after dosing), high levels of metabolites were found, in addition, in tissues with a rapid cell turnover and a high rate of protein synthesis, and in brown fat. This may represent incorporation of the unidentified products of NPYR metabolism into normal biosynthetic pathways.

The high levels of radioactivity found in the kidney, bladder and bile indicate routes of excretion. In non-pretreated adult mice, about 60% of the radiolabelled carbon was rapidly exhaled as $^{14}CO_2$. However this ability was strongly depressed in the pretreated animals and the incorporation of radioactivity into the acid-insoluble fraction of the liver was also reduced.

The distribution of radioactivity in pregnant mice killed 5 min after iv administration of ^{14}C-labelled NDMA was found to be relatively uniform, apart from higher levels in the maternal liver and kidney and a lower, though uniform, concentration throughout the foetuses, placentae and amniotic fluid. Only the maternal liver and kidney retained any radioactivity (non-volatile metabolites) after drying. Similar treatment of pregnant mice with an ip dose identified the liver as the main site of metabolism.

In very young mice (1–10 days of age) there was also an accumulation of non-volatile radioactive material in the skeleton but this was not observed in 60-day-old mice. This probably reflected some disparity in the metabolism of formaldehyde (liberated during NDMA metabolism) between young and adult mice.

The 21- and 60-day-old mice exhaled a greater percentage (50–60%) of the label than did the 10- and 14-day-old mice (40%). The older mice incorporated between 1·3 times and twice as much radioactivity into the acid-insoluble fraction of the liver, which suggests that these functions do not develop in parallel and therefore cannot be used concomitantly as indices of the degradation of NDMA.

Although enzymatic competence was absent from foetal livers incubated on the last day of gestation with ^{14}C-labelled NDMA, *in vitro* activity was demonstrable on the first day of life, and by day 5 both $^{14}CO_2$ production and the incorporation of radiocarbon into the acid-insoluble fraction of the liver were at approximately adult levels.

These studies identify the tissues responsible for the metabolism of NPYR in the adult mouse and suggest that the enzyme system responsible for the metabolism of NDMA may be closely related to those involved in NPYR metabolism. The end products of metabolism of both these compounds are mainly

excreted via the lungs, but the fate of NDMA *in vivo* is not the same in young as in old mice, although the necessary enzyme systems, not present in the foetus, develop very rapidly after birth. It is suggested that the absence of NDMA metabolism in the foetal liver *in vivo* and *in vitro* is responsible for the weak transplacental carcinogenicity of this compound.

AGRICULTURAL CHEMICALS

Slow-release dichlorvos for pigs

Stanton, H. C., Albert, J. R. & Mersmann, H. J. (1979). Studies on the pharmacology and safety of dichlorvos in pigs and pregnant sows. *Am. J. vet. Res.* **40**, 315.

Dichlorvos is widely used as an insecticide, particularly in the form of slow-release resin strips. Its ability to alkylate mammalian DNA has been reported (*Cited in F.C.T.* 1974, **12**, 769) but no such reaction was detected in a study of rats exposed to a concentration of the order of those likely to be encountered under practical conditions of use (*ibid* 1978, **16**, 628). The cholinesterase-inhibiting properties of dichlorvos have been demonstrated in man and other mammals following its ingestion, and the marketing of anthelmintic preparations for dogs, cats, horses and swine, consisting of controlled-release dichlorvos in a polyvinyl chloride resin base (PVC–DDVP), has prompted further investigation of the possible toxic effects of this insecticide when ingested. In addition, the oral toxicity of dichlorvos itself has been compared with that of the PVC formulation.

The acute oral LD_{50} of dichlorvos in swine aged 40–60 days was 157 mg/kg. Doses of this order induced signs of acute cholinesterase inhibition but no delayed effects. PVC-DDVP given orally in doses yielding up to 1000 mg dichlorvos/kg was not lethal. Several animals given 320 or 1000 mg dichlorvos/kg in this form showed signs of organophosphate toxicosis, but those given 180 mg/kg did not. Vomiting, which limited the dose of PVC–DDVP that could be given, was common. Young pigs given 1–16 mg dichlorvos/kg as PVC–DDVP daily from 30 to 60 days of age showed depression of cholinesterase activity in plasma and red blood cells. No effects on weight gain, food consumption, electrolyte balance, carbohydrate metabolism, lipid mobilization or liver mitochondrial enzyme activities were demonstrated.

In sows fed 5 or 25 mg dichlorvos/kg/day as PVC–DDVP during the last 30 days of gestation, cholinesterase activity in plasma and red cells was depressed. At the higher dose level, acetylcholinesterase activity in the uterine muscle was depressed, but placental and rhombencephalic (hind-brain) cholinesterase showed no change. In foetuses from these sows, rhombencephalic cholinesterase showed a significant increase, but plasma and red-cell cholinesterase activity remained unaltered. There was no change in choline acetyltransferase activity in the brain or uterus of the sows, in the sow placenta or in the foetal brain. Thus, transplacental transfer of dichlorvos released from PVC–DDVP appears to be negligible. The noted increase in foetal rhombencephalic cholinesterase has not been explained.

The relative lack of effect of PVC–DDVP compared with free dichlorvos can be accounted for by the balance between the slow release of dichlorvos from the PVC formulation in the gut and its rapid metabolism either in the intestinal lumen or after its absorption. Conversion in the latter case probably occurs mainly in the intestinal wall and hepatic–portal system.

Immunosuppression by pesticides

Lee, T. P., Moscati, R. & Park, B. H. (1979). Effects of pesticides on human leukocyte functions. *Res. Commun. chem. Path. Pharmac.* **23**, 597.

Immunosuppression, involving alteration of cellular and humoral immune responses and atrophy of the thymus, spleen and other lymphoid tissue, has been found to follow exposure of experimental animals to certain pesticides, which at the same time affect liver function and the activity of several endocrine glands. The latter changes can lead to abnormal metabolism of biogenic amines, steroids, hormones and vitamins, and since many of these compounds are also known to have an effect on immune function, it is questionable whether the immunosuppressive effects of pesticides result from direct action or are an indirect effect brought about by changes in other organ systems. The authors cited above have sought to throw some light on this question by studying the direct effects of some pesticides and auxiliary compounds on leucocyte function *in vitro*.

A series of pesticides, including a carbamate (propham), organophosphates (trithion, methylparathion and ruelene) and organochlorines (BHC, endrin and DDT), as well as a synergist (butoxide), a repellant (benzyl benzoate), an attractant (eugenol), benzyl thiocyanate and Aroclor 1254, were studied *in vitro* for their effects on the mitogenic response of human lymphocytes to phytohaemagglutinin stimulation, on E-rosette formation between human lymphocytes and sheep red blood cells, and on neutrophil chemotaxis. In a diluted whole-blood culture, 10 μM-butoxide caused 25% inhibition of the lymphocyte response to phytohaemagglutinin, while inhibition by the other compounds in the same concentration varied from 5 to 20%. Pretreatment of lymphocytes with these compounds in the absence of serum led to higher degrees of inhibition by three of the test compounds, namely DDT (71%), trithion (49%) and benzyl thiocyanate

(30%). Inhibition of E-rosette formation was less marked than that of the response to phytohaemagglutinin for all compounds except DDT, which at 100 μM inhibited E-rosette formation by 50%. The only compounds that inhibited neutrophil chemotaxis to a significant degree at 10 μM were methylparathion and benzyl benzoate.

The results obtained in this work show that pesticidal compounds of widely differing structure can exert direct effects upon leucocyte function, suggesting that the reported immunosuppressive effect of pesticides could be a direct action. The modifying effects of serum on these interactions was confirmed in further tests using foetal calf serum, and studies on the biochemical basis of these inhibitory effects are in progress.

OCCUPATIONAL HEALTH

Talc deposits (mineral and ovarian) unearthed again

Gamble, J. F., Fellner, W. & Dimeo, M. J. (1979). An epidemiologic study of a group of talc workers. *Am. Rev. resp. Dis.* **119**, 741.

Longo, D. L. & Young, R. C. (1979). Cosmetic talc and ovarian cancer. *Lancet* **II**, 349.

The heterogeneous group of hydrated magnesium silicates known collectively as talc commonly occur in mineral deposits containing asbestos-forming minerals, notably the amphiboles and serpentines. Studies of occupational lung disease associated with talc exposure must take into account, therefore, the possible presence of asbestiform fibres in the sample.

The first paper cited above describes a study of 121 men who had been engaged for an average of 10·2 yr in the mining or milling of talc. Airborne fibres collected in the mine were 38% anthophyllite, 19% tremolite and 3% chrysotile, the corresponding figures for the mill atmosphere being 45, 12 and 2%. Studies of personal air samples indicated time-weighted average exposures to respirable particles ranging from 0·23 to 1·20 mg/m^3 in the mine and from 0·25 to 2·96 mg/m^3 in the mill. Median fibre diameter and length for anthophyllite were 0·13 and 1·50 μm. and for tremolite 0·19 and 1·60 μm, respectively. The overall concentration of fibres longer than 5 μm exceeded 0·5/ml. Exposure to free silica was in most cases below 0·02 mg/m^3, the maximum recorded being 0·04 mg/m^3. Respiratory findings in these workers were compared with those for groups of potash workers comparable in respect of age, height, smoking habits and duration of employment.

Symptoms such as cough, phlegm, dyspnoea and haemoptysis were more frequent in smokers than in non-smokers and were not a lot more common in the talc workers than in the potash miners, but pulmonary function showed a greater degree of impairment in the talc workers. Talc workers with 15 or more years of employment showed a significantly higher incidence of pleural calcification and pneumoconiosis than did potash miners, as well as a 31% incidence of pleural thickening. Occurrence of the latter was associated with a further decrease in pulmonary function. Exposure to respirable particles and asbestiform fibres was associated with a decreased forced expiratory volume at 1 sec, and a reduced forced vital capacity.

Outside the talc mining and milling industry, exposure to talc may be associated with the handling of dusting powders and deodorants, with the production of medicinal tablets, crayons and textiles and with food processing. The second paper cited above draws attention again to the fact that cosmetic talc powder may be deposited in the vagina and suggests that an occasional particle may reach the surface of the ovary where it could alter the normal hormone-controlled proliferation of ovarian epithelium and give rise to local cancer. The authors consider that talc particles should be sought in ovarian cancer specimens obtained by surgery, that studies of the risks of talc application to the genital region should be undertaken, that further epidemiological studies on female talc workers should be carried out and that clinicians should collect data on the cosmetic use of talc by patients who present with ovarian cancer. However, others hold the view that the cosmetic use of talc complying with the asbestos-free specification agreed by the major manufacturers is unlikely to present a health hazard (*Lancet* 1977, **I**, 1348). In a detailed comment on the Longo & Young review cited here, Roe (*ibid* 1979, **II**, 744) stresses the reported finding of talc and other particles in normal as well as tumorous tissues of the female genital tract, the lack of any evidence of carcinogenic potential in animal studies on talc and the complexities of the epidemiology of ovarian cancer. One epidemiological approach to the talc question, involving a study of female workers in a pharmaceutical factory using only non-fibrous talc, did not point to any involvement of talc in the aetiology of ovarian cancer (Newhouse, *ibid* 1979, **II**, 528).

[As we reported last year (*Cited in F.C.T.* 1979, **17**, 559), the levels of talc found in normal and cystic ovaries and in ovarian adenocarcinomas showed no correlation to support a direct association between the talc and ovarian cancer. The source of the talc found in ovarian tissue remains unknown. Earlier work in rabbits (Phillips *et al. Fd Cosmet. Toxicol.* 1978, **16**, 161) yielded no evidence of any transfer of intravaginally deposited doses of tritium-labelled talc to the ovaries. The degree of priority that should be given to research on the safety of cosmetic talc seems likely to remain a controversial issue for some time.]

Is acrylonitrile a carcinogen?

Parent, R. A. & Casto, B. C. (1979). Effect of acrylonitrile on primary Syrian golden hamster embryo cells

in culture: Transformation and DNA fragmentation. *J. natn. Cancer Inst.* **62**, 1025.

Acrylonitrile (ACN) is produced industrially on a large scale and is often used for the manufacture of rubber and synthetic fibres. Although the acute toxicological effects of ACN have been fairly thoroughly investigated, its mutagenic, teratogenic, and carcinogenic effects have only recently been studied. The mutagenic properties of ACN in various strains of *Salmonella typhimurium* have been reported (*Cited in F.C.T.* 1979, **17**, 179) and preliminary investigations have indicated that ACN may be carcinogenic in rats (*Food Chemical News* 1977, **18** (45), 20) and industrial workers (*ibid* 1977, **19** (11), 26).

Further work has now been carried out by the authors cited above to determine the effect of ACN on primary Syrian golden hamster embryo cells (HEC) in culture. Three studies were carried out. In one investigation, the ability of ACN to transform HEC was determined. The authors observed that foci of morphologically transformed cells were formed when HEC were treated for 6 days with ACN at a concentration of 50 μg ACN/ml.

In another experiment, the effect of ACN on the frequency of virus transformation of HEC was studied. It was found that when cells were pretreated with simian adenovirus (SA7) and subsequently treated with 200 μg ACN/ml, an 8·9-fold increase in the frequency of virus-transformed foci was produced over the frequency noted for cultures treated only with SA7. A concentration of 100 μg ACN/ml produced an 8·4-fold increase in frequency.

Thirdly, a study was carried out to determine whether ACN caused any overt damage to DNA. It was found that treatment of [^{3}H]thymidine-labelled cells with ACN (at concentrations two to four times higher than those used in the chemical transformation and enhancement experiments) caused a shift in the sedimentation pattern of the labelled cellular DNA in alkaline sucrose gradients. This shift in the sedimentation pattern of the treated DNA was similar to that observed previously with known chemical carcinogens (but not with non-carcinogens). Furthermore, DNA breakage was apparent when ACN concentrations of 400 and 200 μg/ml were used.

The authors point out that the enhancement of virus transformation has recently been reported to have a 94% correlation with known carcinogenic activity, and chemical transformation *in vitro* a 92% correlation. On this basis, the results obtained in all three investigations were consistent with those expected of a known carcinogen and provide further evidence for the possible carcinogenicity of ACN. Since, however, the hamster embryo system contains a fairly active mixed-function oxidase, which may be capable of epoxidizing ACN, the observed results may have been due to an ACN metabolite rather than to ACN itself.

[The assay systems described in this paper are not very well characterized and too much emphasis should not be placed on the results obtained until the methods have been substantiated. Nevertheless, these results, taken in conjunction with the positive mutagenic data already obtained, appear to provide further support for the view that ACN is a carcinogen.]

Another metabolite of styrene oxide?

Battistini, C., Bellucci, G. & Mastrorilli, E. (1979). The formation of phenylethane-1,2-diol 2-acetate in the metabolism of styrene oxide by rabbit liver microsomes *in vitro*. *Xenobiotica* **9**, 57.

Styrene, a widely used industrial chemical, can be metabolized in the body to form an epoxide, styrene oxide. The hydration of epoxides by microsomal enzymes is an important stage in the detoxication of these mutagenic and potentially carcinogenic compounds, and one needs, therefore, to be able to estimate the amount of epoxide hydration that takes place. One product of the enzymic hydration of styrene oxide has been identified as racemic phenylethane-1,2-diol (Jerina *et al. J. Am. chem. Soc.* 1970, **92**, 1056), and several attempts have been made to isolate this diol. In one investigation (Oesch *et al. Biochim. biophys. Acta* 1971, **227**, 685), [7-^{3}H]styrene was used as the substrate for a radiometric assay of the epoxide-hydratase activity of microsomal preparations, the unmetabolized substrate being removed by extraction with petroleum ether and the diol being extracted into ethyl acetate for assay by scintillation spectrometry. In another method (Belvedere *et al. J. Chromat.* 1976, **118**, 387), a gas–liquid chromatographic (GLC) assay was performed, the diol being assayed after esterification with 1-butaneboronic acid. In addition to phenylethane-1,2-diol, several other metabolites of styrene oxide have also been identified (Leibman, *Envir. Hlth Perspect.* 1975, **11**, 115).

In the present investigation, the styrene oxide hydratase activity of rabbit-liver microsomes was studied by carrying out a direct GLC determination of phenylethane-1,2-diol. The authors observed during the course of their experiments the formation of a second product in the incubation mixtures. This minor product was isolated by preparative thin-layer chromatography and was shown to consist principally of phenylethane-1,2-diol 2-acetate. The ratio of this diol acetate to phenylethane-1,2-diol in the incubation mixtures varied with different microsomal preparations and with the length of incubation. After complete hydrolysis of styrene oxide, however, the ratio varied from 1:7 to 1:19 with different microsomal preparations. Having excluded the possibility of transacetylation of the diol by the ethyl acetate used for extracting the incubation mixtures, the authors conclude that the diol acetate was formed by the microsomal acetylation of phenylethane-1,2-diol, which in turn was formed from styrene oxide.

Apparently, no acetate of phenylethane-1,2-diol has previously been reported as a metabolite of styrene oxide. These authors point out, therefore, that the formation of this metabolite may have been a source of error in previous determinations of styrene epoxide-hydratase activity, since in the radiochemical method (Oesch *et al. loc. cit.*) the ester would be extracted together with the unreacted epoxide into the petroleum ether, and in the GLC method (Belvedere *et al. loc. cit.*) the ester would not react with 1-butaneboronic acid. Thus previous estimates of styrene epoxide-hydratase activity may have been too low.

[This study emphasizes the value of using a variety of methods for determining the activity of a specific enzyme system.]

Solvents on the skin

Kronevi, T., Wahlberg, J. & Holmberg, B. (1979). Histopathology of skin, liver, and kidney after epicutaneous administration of five industrial solvents to guinea pigs. *Envir. Res.* **19**, 56.

Five industrial solvents, *n*-hexane, toluene, carbon tetrachloride, 2-chloroethanol (ethylene chlorohydrin) and *n*-butyl acetate, were applied in glass cells containing 1 ml of solvent to 3·1-cm^2 areas of the clipped dorsal skin of guinea-pigs for 15 min, or for 1, 4 or 16 hr. Whole-skin samples from the treated areas were then taken for examination. The first four of these solvents caused progressive nuclear pyknosis and separation of the basement membrane from the basal cells. The former effect was apparent within 15 min of application; the latter generally developed somewhat later although it was found in all the samples taken after *n*-hexane treatment. Pseudoeosinophilic infiltration appeared in the upper dermis 4 hr after the start of exposure to the first three solvents. Spongiosis (intercellular oedema in the epidermis) was observed 15 min after application of toluene or carbon tetrachloride, but not after other compounds. The fifth solvent (*n*-butyl acetate) failed to induce any lesion.

Carbon tetrachloride caused marked hydropic changes in the hepatocytes of the central areas of the liver lobules and an accompanying loss of glycogen 16 hr after the start of exposure. With 2-chloroethanol, there were centrilobular hydropic changes after 15 min or 1 hr of treatment, again with reductions in glycogen stores; at 16 hr these centrilobular hydropic changes were less pronounced than those seen with carbon tetrachloride, but necrosis was greater. Hexane, toluene and *n*-butyl acetate induced no changes in liver morphology. None of the five solvents altered kidney morphology.

Thus among these five solvents, the least water-soluble and more lipophilic compounds, *n*-hexane and toluene, produced the most severe skin lesions, while systemic effects in the form of liver lesions were a bigger problem with the water-soluble carbon tetrachloride and 2-chloroethanol. The exception, *n*-butyl acetate, with a solubility in water lying between those of carbon tetrachloride and 2-chloroethanol, showed neither dermal nor systemic toxicity under these experimental conditions.

Carcinogenicity of epoxy resins

Holland, J. M., Gosslee, D. G. & Williams, N. J. (1979). Epidermal carcinogenicity of bis(2,3-epoxycyclopentyl)ether, 2,2-bis(*p*-glycidyloxyphenyl)propane and *m*-phenylenediamine in male and female C3H and C57BL/6 mice. *Cancer Res.* **39**, 1718.

Epoxy resins are used extensively for protective coatings, in paints and in adhesives. During 40 yr of production they have been found to be allergens (*Cited in F.C.T.* 1978, **16**, 503) but there has been no definitive evidence of carcinogenicity. The uncured resins are bifunctional alkylating epoxides, and some aromatic epoxy resin monomers are known to be mutagenic in strains of *Salmonella typhimurium* (*ibid* 1979, **17**, 420). Such properties suggest that the uncured resins should be investigated as potential mutagens or carcinogens in higher organisms. Previous skin-painting and injection studies using mice have been inconclusive, but in the paper cited above Holland *et al.* claim to have demonstrated weak carcinogenic activity in two uncured epoxy resin monomers.

The monomers, bis-(2,3-epoxycyclopentyl) ether (I) and 2,2-bis-(*p*-glycidyloxyphenyl)propane (II), and a polymerizing agent, *m*-phenylenediamine, were tested for carcinogenic potential on mouse skin. Preliminary investigations, which indicated hepatorenal toxicity by I and *m*-phenylenediamine, were used to determine suitable dose levels for the carcinogenicity study, the two dose levels given in each case being the maximum tolerated dose and 20% of that level. Each compound was applied 3 times/wk for 2 yr to the shaved dorsal skin of groups of 40 C3Hf/Bd mice of each sex and 20 C57BL/6Bd mice of each sex.

Compound I increased epidermal-tumour incidence at the high dose levels in both strains (75 mg/wk in C3Hf/Bd and 37·5 mg/wk in C57BL/6Bd mice) but had no such effect at the lower doses. Compound II produced skin tumours only in C57BL/6Bd mice, with a total of eight tumours, including seven carcinomas in the 40 mice given 75 mg/wk but only a single papilloma amongst 40 animals tested with the lower dose. Control mice of this strain did not develop any skin tumours. An equal-parts mixture of I and II exhibited a dramatic synergistic effect on the incidence of skin tumours in both strains. In the more sensitive C57BL/6Bd strain, for example, the 40 mice treated with the mixture of 75 mg/wk included two with papilloma, 24 with localized carcinoma and six with metastatic carcinoma. Individually the high doses of I and II produced four and seven mice, respectively, with localized carcinoma, but none with metastatic carcinoma, and in addition there was one mouse with a papilloma in each of these groups. The potencies of I, II and the mixture were shown statistically to be low compared with the results obtained with the established skin carcinogen benzo[*a*]pyrene (BP). Further, gross inspection and autopsy demonstrated that systemic absorption of I and II had not been sufficient to increase tumorigenesis significantly in other body tissues, except in the lungs of C3Hf/Bd mice exposed to the high dose of I.

Whilst *m*-phenylenediamine was extremely toxic to both strains, it did not produce any skin tumours or substantially increase the incidence of other tumours when tested at the maximum tolerated dose (3 mg/wk). This result will be of interest in connection with the debate on the alleged carcinogenicity of hair dyes.

The study brought out other interesting observations. The two strains of mice showed a constant ratio of sensitivity to the induction of epidermal tumours. It was suggested that with knowledge of this ratio, the two strains might be used interchangeably in assessing the relative potency of epidermal carcinogens. The synergistic carcinogenesis with the I–II mixture lead to the speculation that I might be a weak initiator and II a tumour promoter. Alternatively, the effect might have been due to enhanced metabolic activation of one or both compounds by the other within

the target cell, or to an increase in penetration of either or both monomers of the mixture as a result of the differences in their viscosity and volatility.

Extrapulmonary effects of trimellitic anhydride

Ahmad, D., Morgan, W. K. C., Patterson, R., Williams, T. & Zeiss, C. R. (1979). Pulmonary haemorrhage and haemolytic anaemia due to trimellitic anhydride. *Lancet* **II**, 328.

Trimellitic anhydride (TMA), the anhydride of 1,2,4-benzenetricarboxylic acid, is used in the manufacture of plasticizers, as a constituent of alkyd resins, and as a curing agent for epoxy resins. It has been found to induce asthma, rhinitis and a late-onset respiratory syndrome with systemic symptoms, as well as an irritative bronchitis. The data now reported suggest the possible involvement of an immunological mechanism in some responses to occupational exposure to this compound.

Two youths, both aged 17 yr, were admitted to hospital because of the repeated occurrence of haemoptysis, over a period of 1 wk in one case and 6 wk in the other. Both complained of increasing shortness of breath over a more prolonged period and one also of wheezing, and both had haemolytic anaemia. The symptoms were most severe when the patients were at work. Both men worked in a room where cattle pens were first cleaned in a caustic liquid, then sprayed with a powdered mixture of epoxy resin and TMA and finally heated, to enable the resin to liquefy. The powder, which was applied by an air jet and was precipitated on the pens electrostatically, contained 5·5% TMA, 60–70% epoxy resin and about 30% titanium dioxide and other pigments. The procedures were carried out only in 'semiclosed' chambers and the standard of ventilation at the time of the mens' employment appears to have been in some doubt, although at a somewhat later date, after changes had been made, it was described as excellent. A requirement for masks to be worn by those involved with the spraying was not rigidly enforced.

In view of their anaemia, both men were treated with iron. They received no other treatment, but they ceased to work at the factory and within a few weeks both the anaemia and other symptoms disappeared. Subsequent examination demonstrated in both men the presence of antibodies against trimellityl human serum albumin and trimellityl human erythrocytes.

Consideration of these findings and of the details of several similar cases reported at recent meetings indicates that TMA inhalation may have severe systemic effects, including intra-alveolar haemorrhage and haemolytic anaemia, and suggests that an immunological mechanism may be involved.

The neurotoxic effect of triphenyl phosphate

Wills, J. H., Barron, K., Groblewski, G. E., Benitz, K. F. & Johnson, M. K. (1979). Does triphenyl phosphate produce delayed neurotoxic effects? *Toxicology Lett.* **4**, 21.

Whilst it is generally accepted that tri-*o*-cresyl phosphate is a neurotoxic agent, comparatively little work has been carried out on the neurotoxicity of triphenyl phosphate. A more detailed investigation of this compound's effect would be desirable, since triphenyl phosphate is a potentially useful component of hydraulic fluids and plasticizers. Whilst it has been shown that triphenyl phosphate (of questionable purity) may be neurotoxic to hens and cats, the pure compound has not been shown to produce this effect in hens and, furthermore, does not appear to inhibit the hen-brain enzyme known as neurotoxic esterase (Johnson, *Arch. Tox.* 1975, **34**, 259).

Further work has now confirmed the view that pure triphenyl phosphate does not cause delayed neurotoxic effects. In a limited study using 99·99% pure synthetic triphenyl phosphate, five cats were given an sc dose of 0·4, 0·7 or 1·0 g/kg. The two cats given 0·4 g triphenyl phosphate/kg appeared relatively unaffected by the treatment and were not subjected to autopsy at the end of the experiment. The two higher doses, however, caused prostration a few days after dosing, although histological examination did not reveal any evidence of axon degeneration or demyelination in the spinal cord. These doses of 0·7 and 1·0 g/kg were, in fact, higher than those that had previously been reported to produce such effects in the cat and that apparently also produced neurotoxic effects (Smith *et al. Natn. Inst. Hlth Bull.* 1932, **160**, 1).

The authors conclude that pure triphenyl phosphate is not neurotoxic and that previous results associating the compound with neurotoxicity were probably due to the presence of impurities in the sample used. There may well be a case, therefore, for using triphenyl phosphate prepared from synthetic phenol instead of the relatively impure triphenyl phosphate which is generally used and which may be obtained from coal-tar sources.

[The results indicated much variation between animals, and cannot be considered totally conclusive in view of the small number of animals tested. A repetition of the experiment on a larger scale may well be desirable in order to substantiate the interesting results obtained.]

NATURAL PRODUCTS

Cassava, diabetes and cyanide

Davidson, J. C. (1979). Cyanide, cassava, and diabetes. *Lancet* **II**, 635.

Maduagwu, E. N. (1979). Cyanide content of gari. *Toxicology Lett.* **3**, 21.

A possible relationship between endemic pancreatic

disease, including diabetes mellitus, and the chronic consumption of large quantities of cassava has previously been reported in the malnourished populations of Southern India and Nigeria (*Cited in F.C.T.* 1975, **13**, 157). A similar relationship has also been noted by Davidson (cited above) in Zambia. In regions where cassava is the major dietary component and undernourishment is common, there was a prevalence rate of 1·1% new cases of diabetes. This contrasted markedly with the remainder of the country where maize is the staple food and diabetes is uncommon.

The recognized clinical syndromes of chronic cassava toxicity, including degenerative neurological disease and endemic goitre (*ibid* 1967, **5**, 125), appear to be attributable to cyanogenetic glycosides present in the crop; these are hydrolyzed to hydrocyanic acid by the endogenous enzyme linamarase when the plant tissue is damaged. Among the wide range of methods traditionally used for the detoxication of cassava is one in which the roots are grated, fermented and roasted into flour. The product is known as gari.

The second paper reports that the assay of 500 random samples of gari being sold in Nigerian markets showed that only a very small proportion (0·4%) contained no cyanide. There was no significant difference between the mean concentrations of total (glycoside-bound plus non-glycosidic) cyanide and of free (non-glycosidic) cyanide (8·0 ± 6·9 and 7·2 ± 5·8 mg HCN/kg, respectively), which indicated that virtually all the residual cyanide content of commercial gari could be in the free form. Of the samples analysed, 82% contained less than 10 mg HCN/kg.

Testing bergamot oil for phototoxicity

Girard, J., Unkovic, J., Delahayes, J. et Lafille, C. (1979). Etude expérimentale de la phototoxicité de l'essence de Bergamote. Corrélations entre l'homme et le cobaye. *Dermatologica* **158**, 229.

The phototoxic effect of bergamot oil, which is frequently used in perfumes, has been ascribed to the furocoumarin bergapten (5-methyl-psoralen; 5-MOP), a common though not invariable component of the oil (*Cited in F.C.T.* 1978, **16**, 507).

Close correlation has now been reported between the phototoxic effects elicited in human volunteers and in guinea-pigs by a sample of bergamot oil containing 3200 ppm 5-MOP. Guinea-pigs were treated with various dilutions of the oil, in ethanol, applied in doses of 4 $\mu l/cm^2$ to depilated skin. The applications were followed 1 hr later by irradiation with a minimal erythema-inducing dose either of solar-simulating light (SSL; wavelength 290 nm and longer) or of light in the long ultraviolet range (LUVL; 320 nm and longer) and augmentation of erythema was assessed 24 hr after the irradiation. The effects were compared with the augmented pigmentation of forearm skin in adult volunteers similarly treated with the bergamot oil and exposed to the same types of irradiation, the test sites being assessed for augmented pigmentation 8 days after irradiation with the minimal dose causing immediate pigment darkening.

With both types of irradiation, there was a clear parallel between the degrees of reaction in the guinea-pig and in man to different concentrations of 5-MOP, although the volunteers seemed to be slightly more sensitive than the guinea-pigs. The lowest doses effective in the guinea-pig were 2·5% bergamot oil (80 ppm 5-MOP) with SSL irradiation and 0·312% bergamot oil (10 ppm 5-MOP) with LUVL. In man, the minimal doses augmenting pigmentation were 1·25% with SSL and 0·15% with LUVL. Very similar results were also obtained from readings of the human skin at 24 hr, but at each dose level the effect was slightly more marked at 8 days. Thus in both guinea-pig and man, phototoxicity was some eight times greater when LUVL was used than with SSL and seemed to depend essentially on long ultraviolet radiation. For a given degree of phototoxic reaction there was apparently, in the guinea-pig, an inverse relation between the concentration of bergamot oil and the quantity of irradiation energy attributable to LUVL. In man the situation was less clear and a greater involvement of ultraviolet rays in the 290–320 nm range seems likely. Nevertheless this work suggests that the guinea-pig is a particularly appropriate experimental model for studying the phototoxic potential of psoralen-containing products to which man may be exposed.

Primrose and rosewood sensitizers

Schulz, K. H., Garbe, I., Hausen, B. M. & Simatupang, M. H. (1979). The sensitizing capacity of naturally occurring quinones. Experimental studies in guinea pigs. II. Benzoquinones. *Archs Derm. Res.* **264**, 275.

An eczematous dermatitis has been reported (*Cited in F.C.T.* 1968, **6**, 678) in people handling *Machaerium scleroxylon*, a substitute for Rio rosewood containing R-3,4-dimethoxydalbergione (R-5,6-dimethoxy-2-(1′-phenylallyl)-1,4-benzoquinone). In view of the apparent increase in the incidence of sensitization to ornamental woods, further details of the sensitizing capacity of dalbergiones and related benzoquinones and naphthoquinones are of interest.

Five of seven benzoquinone derivatives tested in guinea-pigs for sensitizing capacity gave positive results. These were primin, mansonone A, 4-methoxydalbergione racemate, R-3,4-dimethoxydalbergione and S-4,4′-dimethoxydalbergione. Primin (2-methoxy-6-pentyl-1,4-benzoquinone from the primrose, *Primula obconica*) was the most powerful, eliciting allergic reactions in concentrations of 0·5–0·2 mM in three of ten animals. Next came R-3,4-dimethoxydalbergione from *M. scleroxylon* and mansonone A (1,2-dioxy-3,8-dimethyl-5-isopropyl-5,6,7,8-tetrahydronaphthalene from *Mansonia altissima*). Two compounds from *Dalbergia nigra*, 4-methoxydalbergione and S-4,4′-dimethoxydalbergione, were weaker sensitizers. No sensitization was elicited by 2,6-dimethoxy-1,4-benzoquinone or rapanone (2,5-dihydroxy-6-tridecyl-1,4-benzoquinone) in these experiments. Allergic cross-sensitization was demonstrated in guinea-pigs sensitized with mansonone A and challenged with five related naphthoquinones, all derived from *Mansonia altissima*.

These results support the hypothesis that the sensitizing capacity of naturally occurring quinones depends upon their quinoid structure and the length, position and configuration of their side-chain. It is clearly advisable for persons who show sensitivity to some exotic wood or plant containing quinones to avoid exposure to other species containing related compounds.

ENVIRONMENTAL CONTAMINANTS

Chlorinated guaiacols from the pulp mill

Chu, I., Ritter, L., Marino, I. A., Yagminas, A. P. & Villeneuve, D. C. (1979). Toxicity studies on chlorinated guaiacols in the rat. *Bull. envir. Contam. Toxicol.* **22,** 293.

Chu, I., Villeneuve, D. C., Yagminas, A. P. & Valli, V. E. (1979). Tri- and tetrachloroguaiacol: Results of a three and six-month feeding study in rats. *Archs envir. Contam. Toxicol.* **8,** 589.

Trichloroguaiacol (3CG) and tetrachloroguaiacol (4CG) are formed during pulp and paper manufacture by reaction of chlorinating agents with phenolic lignins. Consequently they tend to be found as contaminants of river water receiving pulp mill effluents, and they have been shown to be toxic for fish.

The first paper cited above describes acute and subacute toxicity studies in male rats. The oral LD_{50} of 3CG was 3 g/kg and that of 4CG was 1·69 g/kg, indicating mild toxicity. Rats fed 50 ppm 3CG in the diet for 28 days showed a decrease in body-weight gain, but paradoxically this effect did not appear with higher levels of 500 or 5000 ppm. None of the dietary levels affected food consumption. 4CG tested at the same dietary levels had no effect on either weight gain or food consumption. In rats fed 50 ppm 3CG, there appeared to be a slight increase in liver weight expressed as a percentage of the [somewhat low] body weight, but at other feed levels and with 4CG no such effect was demonstrated. Aniline hydroxylase activity was increased by 3CG at the 50 and 5000 ppm levels, and by 4CG at all dietary levels, but there was no correlation between the extent of the increase in enzyme activity and the dose. Neither compound altered sorbitol dehydrogenase activity and there were no haematological or histological changes in any of the test groups. Tissue levels of 3CG were not significant, indicating the compound's rapid metabolism and/or excretion, but with 4CG low dose-related concentrations were found in the liver and kidneys of rats fed the 500 or 5000 ppm diets.

In the longer rat feeding studies described in the second paper, 50, 500 or 5000 ppm 3CG or 4CG fed in the diet for 96 or 182 days had no effect on body-weight gain or food intake. Slight increases in relative liver weights and kidney weights were neither consistent over the two periods nor dose-related. Neither compound affected aspartate aminotransferase, but all doses of 4CG lowered lactic dehydrogenase activity at 96 days, and 5000 ppm 4CG increased alkaline phosphatase activity at that time. No haematological abnormalities were detected in these studies. Both compounds produced mild kidney lesions at 96 days but not at 182 days, a possible indication of an adaptive response. The 3CG-induced lesions were large hyaline inclusions in the proximal tubular epithelium, while 4CG induced focal or diffuse hypertrophy of the distal tubular epithelium. Mild liver lesions were produced by both compounds, periportal lipidosis with 3CG at 182 days, and a lobular mosaic pattern with lymphoid aggregates or periportal perinuclear clear zones with 4CG. One rat, which received 5000 ppm 4CG, developed a malignant liver lymphoma, but this isolated occurrence was not attributed to the treatment. The results therefore supported the findings in the shorter studies indicating that both 3CG and 4CG are only moderately toxic to rats.

Persistent problems in waste-water renovation

Saxena, J. & Schwartz, D. J. (1979). Mutagens in wastewaters renovated by advanced wastewater treatment. *Bull. envir. Contam. Toxicol.* **22,** 319.

There is an increasing drive towards the re-use of waste waters, but advanced waste-water technology is alleged to leave unchanged many potentially hazardous compounds derived from the environment and even to introduce new and potentially toxic chemicals. The need to detect possible hazards from unidentified contaminants of waters destined for re-use in irrigation, in the establishment of recreational lakes, in industry and, especially, in domestic supplies, demands the development and use of appropriate screening tests for toxicity, mutagenicity and carcinogenicity. One screening test that offers some promise in this context involves the possible induction of arylhydrocarbon hydroxylase in cultured rat-hepatoma cells as an index of toxicity. The *Salmonella typhimurium* plate assay or 'Ames test' is now well known as a short-term test for mutagens and carcinogens and its use in the waste-water context is demonstrated in the paper cited above. This reports a study of municipal waste waters from three treatment plants, using samples taken at different stages of the treatments and from different levels of a sand-filtration system. *S. typhimurium* strains TA100 and TA1535 were used to detect base-pair substitution mutagens and strain TA98 for frame-shift mutagens and 24-hr composite samples were taken from each treatment plant.

Effluents from two plants were significantly mutagenic in strains TA100 and TA1535, although the responsible mutagens were largely detoxicated by inclusion of mammalian liver enzymes in the assay. Histidine, which could have given a false indication of a mutagenic response, could not be detected in any of the samples. Samples from these first two plants had

no mutagenic effect on strain TA98 but a high degree of toxicity was indicated by a marked reduction in the spontaneous reversion rate. Effluent samples from the third plant, a village sewage-treatment plant not exposed to industrial effluents and using a process based on filtration through natural delta sand beds, showed no mutagenesis in any strain but inhibited the spontaneous reversion rate of strain TA98 in the same way as did the other two samples. Whether the negative results obtained on the samples from this plant indicated that microbial synthesis of mutagens does not occur during the sand-filtration process or merely that suitable precursors were not available remains questionable. Sampling at intermediate stages in the waste-water treatments suggested that lime addition or recarbonation could promote the synthesis of mutagens, some of them with a capacity for direct action and others requiring metabolic activation. Partial removal of these mutagens was accomplished during subsequent chlorination and/or adsorption on activated carbon but, overall, the mutagen concentration in the final effluents was no less than that in the influent waste waters.

These findings suggest that physico-chemical and biological methods currently used for treating waste waters are not adequate for the removal of the wide range of potentially hazardous chemicals that may result from industrial processes, particularly if such renovated waste-waters are to be considered for potable use.

COSMETICS, TOILETRIES AND PHARMACEUTICALS

Mechanism of butylparaben absorption

Komatsu, H. & Suzuki, M. (1979). Percutaneous absorption of butylparaben through guinea pig skin *in vitro*. *J. pharm. Sci.* **68**, 596.

Esters of *p*-hydroxybenzoic acid (parabens) have been widely used as antimicrobial preservatives in pharmaceutical and cosmetic preparations. Although they have been assumed to be generally inert, they have shown some biological activity, stimulating the growth of organ cultures (*Cited in F.C.T.* 1968, **6**, 410) and causing dermatitis and sensitization in some individuals (*ibid* 1973, **11**, 147).

In an examination of the absorption of butylparaben through isolated guinea-pig skin, a sample labelled with carbon-14 was dissolved at levels of 0·1 or 0·02% in different aqueous solvent systems containing 1 or 2% polysorbate 80, 10 or 20% propylene glycol or 10 or 20% polyethylene glycol (PEG) 400, or mixtures of some of these, and the solution was placed in contact with excised guinea-pig skin in a diffusion chamber. A 0·015% solution in water was also tested.

Absorption of the butylparaben through the disc of skin into the receptor saline, calculated when the system had achieved a steady state, ranged from 23·7% for the plain aqueous solution to 1·1% for 0·1% butylparaben in a 2% polysorbate 80–10% PEG 400 vehicle. The second and third highest penetrations (20·22 and 9·54%) occurred with 0·02% butylparaben in 10 and 20% propylene glycol, respectively. Polysorbate 80 was most effective in depressing penetration and PEG 400 had an intermediate effect. These results showed a clear correlation with the partition coefficient, indicating that the system–skin partition coefficient determined the penetration.

The antimicrobial effect of these various butylparaben systems was also studied in an attempt to relate it to the effect on percutaneous absorption. The preservative effects of butylparaben in water only and of the four systems containing either propylene glycol or PEG 400 were similar, but the systems with polysorbate 80 showed a reduction in preservative activity. Since the preservative activity depends on the effective concentration in the outer (aqueous) phase, these results indicate that the polysorbate surfactant increased the amount of butylparaben trapped in micelles and reduced its concentration in the outer phase.

Chlorhexidine through neonatal skin

Cowen, J., Ellis, S. H. & McAinsh, J. (1979). Absorption of chlorhexidine from the intact skin of newborn infants. *Archs Dis. Childh.* **54**, 379.

In the past decade, chlorhexidine has largely taken the place of hexachlorophene as an antimicrobial constituent of toiletries and creams for the control of neonatal cross-infection, following the accumulation of evidence that hexachlorophene is absorbed through the skin and may cause lesions of the central nervous system (*Cited in F.C.T.* 1976, **14**, 642; *ibid* 1977, **15**, 353). Chlorhexidine rarely causes sensitization (*ibid* 1974, **12**, 799) and its percutaneous absorption has been shown to be low in rats (*ibid* 1979, **17**, 100) and in human adults (Case *et al.* In *Chemotherapy, Vol. 3*, edited by J. D. Williams and A. M. Geddes; p. 367; Plenum Press, New York, 1976). Studies of percutaneous absorption in human infants have now been published.

Thirty-four newborn infants were given daily Hibiscrub (4% chlorhexidine gluconate) baths by application of the cream by hand to the head, excluding the face, and then to the rest of the body, excluding areas of broken skin. The head and subsequently the rest of the body were rinsed with plain water immediately after the application, in the latter case by immersion up to the neck. Chlorhexidine was estimated in capillary blood samples taken by heel prick 1 and 4 hr after the first bath, or in venous blood taken from the hand 4 hr after the first bath or, at approximately weekly intervals, 12 hr after other daily baths.

Chlorhexidine was detected in all ten babies from whom capillary samples were taken, but subsequently, when the strength of its adsorption on the skin sur-

face was recognized, this was attributed to adsorbed material that had not been removed by alcohol swabbing. Chlorhexidine was also detected in five of 24 babies whose venous blood was sampled. Capillary blood contained 53–607 ng/ml at 1 hr; of the eight samples taken after 4 hr, six covered the range 67–1021 ng/ml while two did not differ significantly from zero (i.e. the 95% confidence limits included zero). Six of the seven venous blood samples taken on day 1 were positive for chlorhexidine, but only three of these concentrations differed significantly from zero, the range for these three being 101–460 ng/ml. Only one significant value was recorded on each of days 11 (14 samples) and 18 (eight samples), the levels being 214 and 91 ng/ml, respectively.

These results indicated some percutaneous chlorhexidine absorption but the blood concentrations differed widely, showing considerable variation not only with the method and time of sampling but also within similarly sampled groups. It was therefore impossible to draw conclusions about the degree of absorption. No correlation was apparent between the gestational or postnatal age of the infant and the concentration of chlorhexidine in its blood.

TEST PROCEDURES

Implications of the slimmer rat

Tucker, M. J. (1979). The effect of long-term food restriction on tumours in rodents. *Int. J. Cancer* **23**, 803.

Hayashi, Y., Kato, M. & Otsuka, H. (1979). Inhibitory effects of diet-reduction on monocrotaline intoxication in rats. *Toxicology Lett.* **3**, 151.

Almost 40 years ago Tannenbaum reported that dietary restriction improved survival and reduced the spontaneous development of neoplasms in otherwise untreated rats or mice (*Archs Path.* 1940, **30**, 509; *Cancer Res.* 1942, **2**, 460; *ibid* 1945, **3**, 616), observations that have been confirmed in more recent studies (Conybeare, *Fd Cosmet. Toxicol.* 1980, **18**, 65; Roe & Tucker, *Proc. Eur. Soc. Study of Drug Toxicity* 1973, **15**, 171). These results have considerable implications for both the planning and the interpretation of the results of animal studies in carcinogenicity testing. We describe below two more studies of the effects of dietary restriction. In one, the effects of a relatively small decrease in food intake on tumorigenesis in otherwise untreated animals was studied, while the second deals with the modification, by rather more drastic dietary restriction, of the response of rats to monocrotaline, a toxic pyrrolizidine alkaloid that is known to produce hypertensive pulmonary vascular lesions and cor pulmonale in laboratory animals (*Cited in F.C.T.* 1979, **17**, 422).

In the first study cited above, groups of about 50 male and 50 female specific-pathogen-free albino Wistar-derived rats or Swiss albino mice were fed either *ad lib.* or at levels about 20% below those of the corresponding *ad lib.* groups. The male and female rats fed *ad lib.* consumed about 20 and 17 g feed/rat/day, respectively, while the *ad lib.*-fed mice ate about 5 g feed/mouse/day. Although the batches of feed used in the study were not analysed for nitrosamines or mycotoxins it is reported that analysis of batches used in other experiments did not reveal the presence of these contaminants.

In the feed-restricted groups, 45 out of 51 male and 44 out of 49 female rats survived to the end of the experiment (24 months) compared with 36 out of 50 males and 34 out of 50 females fed *ad lib.* However these differences between the groups on the two dietary regimes were not statistically significant. The mice were studied over their entire life-span (up to 36 months) and mortality was significantly decreased in the groups with restricted food intake. In both species there was a significant reduction in tumour incidence in the animals given a restricted diet, and the increased longevity in mice indicated a retardation of the onset of tumour development. Although the numbers of most tumours in both species were too small for statistical evaluation, in rats fed restrictively the incidence of fibrous tumours of the skin in males and of pituitary and mammary adenomas in females was significantly decreased, while brain tumours were apparently unaffected by either dietary regime. In mice, restriction of food intake significantly decreased the numbers of hepatomas in males and of pituitary adenomas in females, but had no apparent effect on tumours of the Harderian gland, testes or thymus.

The author suggests that it is necessary first to establish whether the species and strain of animal used in a carcinogenicity test is susceptible to dietary control of tumour incidence and then to formulate a feed that will maintain the animals in good health and reduce the incidence of spontaneous tumours to the lowest possible level. It will be necessary to investigate the effects such a diet may have on other factors, including metabolic activation.

In the second study cited above, 24 male Sprague–Dawley rats, 5 wk old, were injected sc with 60 mg monocrotaline/kg and 24 control rats were injected with physiological saline. Half of the rats in each group were given feed and water *ad lib.* while the remaining animals were given free access to water but only 8 g feed/rat/day. The rats were killed 4 wk after injection and examined for pathological changes in the heart and lung. No cardio-pulmonary alterations were observed in control rats on either dietary regime. Rats given monocrotaline and fed *ad lib.* showed severe pulmonary alterations such as haemorrhage, swelling or focal necrosis of the alveolar walls, capillary thrombi and arterial thickening. Right ventricular hypertrophy was also observed in these rats. In contrast there was no right ventricular hypertrophy in the monocrotaline-treated rats fed restrictively. The gross appearance of the lungs of these animals was normal and only focal swelling of the alveolar walls was noted in a histological examination.

In a similar experiment designed to assess the effects of diet restriction on the survival of monocrotaline-treated rats, 36 rats were injected sc with 60 mg monocrotaline/kg. Twelve of the rats were given food and water *ad lib.* and consumed 17·8 ± 1·7 g feed/rat/day. A second group was given only 8 g feed/rat/day and the remaining 12 rats were given 8 g feed/rat/day for 30 days after injection and were then given food *ad lib.* All surviving rats were killed 90 days after injection. The group fed *ad lib.* showed laboured breathing from about 20 days after injection, and 11 of these rats died between days 22 and 40. The diet-reduced group survived the 90 days with no sign of respiratory distress; throughout the experiment their growth was almost completely suppressed. The growth of the animals in the third group was also suppressed for the 30 days when food intake was limited and during this time there was no respiratory dysfunction. However once *ad lib.* feeding was resumed these animals began to show laboured breathing and seven of them died between days 45 and 84.

It therefore seems that restriction of food intake can inhibit the toxic effects of monocrotaline in rats, although whether these inhibitory effects are related to the reduced food intake or to the suppression of their growth remains to be determined.

PHARMACOLOGY

A cardiovascular response to injected propylene glycol

Gross, D. R., Kitzman, J. V. & Adams, H. R. (1979). Cardiovascular effects of intravenous administration of propylene glycol and of oxytetracycline in propylene glycol in calves. *Am. J. vet. Res.* **40**, 783.

Propylene glycol (PG) has been found to be capable of inducing primary irritation and dermatitis (*Cited in F.C.T.* 1975, **13**, 403; *ibid* 1977, **15**, 84). The pharmacological effects of PG are generally slight, and despite mild irritancy and its possible effects on some liver microsomal-enzyme systems (*ibid* 1975, **13**, 582), it has been widely used as a solvent for drugs to be injected. Its implication in the aetiology of a cardiovascular-collapse syndrome associated with injections of normally-well-tolerated doses of tetracycline and related antibiotics has now been reported. This reaction has been encountered in a variety of experimental situations and in cattle undergoing tetracycline therapy, although signs of overt cardiovascular depression following the clinical use of tetracycline antibiotics are relatively rare.

Intravenous injections of aqueous solutions of oxytetracycline into calves failed to induce any alteration in heart rate, or in pulmonary and renal arterial resistance, measured by means of indwelling catheters and electromagnetic flowmeter transducers, although the doses used (11·2–56 mg/kg) were the same as those that had been shown to induce a transient (1–4 min) reaction in the same calves when given in 79·2% PG. This cardiovascular reaction was characterized by periods of asystole, lasting for 5–10 sec and followed by atrioventricular nodal blockade, together with an increase in pressure and decrease in flow in both pulmonary and renal arteries. Haemolysis and haemoglobinuria occurred in all the calves after the oxytetracycline in PG injection and persisted for up to 36 hr. The same reactions occurred in all the calves after injection of an equivalent volume of 79·2% PG alone, the degree of response in comparison with the baseline data being very similar following the two treatments. Subsequently, on different days, the same dose of oxytetracycline in PG or PG alone was injected following administration of one of three autonomic blocking agents (phentolamine, propanolol and atropine) or combinations of the first two or of all three of these. All of the blocking agents altered the responses to PG alone and to oxytetracycline in PG and this aspect of the study has provided some basis for speculation on the possible mechanisms involved in this reaction to PG.

PATHOLOGY

Trimethyltin levels and brain lesions

Brown, A. W., Aldridge, W. N., Street, B. W. & Verschoyle, R. D. (1979). The behavioral and neuropathologic sequelae of intoxication by trimethyltin compounds in the rat. *Am. J. Path.* **97**, 59.

Trimethyltin chloride (TMC) affects the central nervous system. In man, it is alleged to produce memory defects, hyperactivity, insomnia and disorientation, followed by mental confusion and convulsions. In the rat, tremors and convulsions have been reported.

A detailed study of the neurotoxicity of TMC in rats has shown that after a single dose of 15·8, 25 or 40 mg TMC kg, given in arachis oil by gavage, tremors and prostration developed within 48 hr and deaths were recorded between 48 and 120 hr after treatment. The earlier deaths occurred in the rats given the larger doses. Rats receiving a single dose of 10 mg/kg became extremely aggressive and at 72 hr had to be caged individually. No deaths occurred after this dose, and 7 days after treatment the signs of aggression had abated.

The pathological lesion in the brain was studied in

animals given a single dose of 10 mg TMC/kg and then killed sequentially on days 1–4, 10, 21 and 70 after treatment. No histological changes in the brain were observed after 1 day. After 2 days, damage was largely confined to the hippocampus and fascia dentata and consisted of loss of Nissl granules, cytoplasmic eosinophilia and degenerative changes in the nuclei consisting of condensation and fragmentation of nuclear material. However, some loss of Nissl granules and cytoplasmic eosinophilia, a mild and reversible form of cell damage, were also seen in the pyriform cortex and amygdaloid nucleus. Nuclear damage was observed in a few neuronal cells in these two areas 3 and 4 days after treatment. The number of cells showing nuclear changes was much greater at day 10, indicating that the lesion was progressive, and the damage showed some increase in severity at days 21 and 70. Other areas of the brain were unaffected, with the exception of the Purkinje cell layer, some cells of which were damaged in one animal. No oedema of the white matter was observed.

Similar behavioural and pathological changes occurred when TMC was administered in smaller doses repeated weekly. Dosing of rats with 4 mg/kg weekly by oral intubation induced body tremors after the second dose, and after the third dose the rats became irritable and hyperexcitable. Histological examination of brains (fixed by a perfusion technique) from some of the animals killed after each injection revealed no changes after the first dose. After the second dose, there was a loss of Nissl granules in many neurones in the hippocampus, amygdaloid nucleus and pyriform cortex. After the third dose, some degenerate neurones were also found in these areas. Both of these changes occurred first in the hippocampus.

A fourth dose of 4 mg TMC/kg was followed by aggressive behaviour, convulsions and weight loss and some animals died. Brain damage in the vulnerable areas was observed in the animals killed 1, 8, 11 and 14 days after this last dose. When the fourth injection was only 2 mg/kg no weight loss or deaths occurred, but aggressive behaviour and convulsions were observed and the histopathological changes were similar to those found after the larger fourth dose and were also observed 49 days after the last intubation.

Several techniques, including one using the radioactive tin isotope ^{113}Sn, were used to determine the TMC content of the brain and blood of the treated animals. Levels in the blood were found to be approximately 40 times as high as those in the brain, which were estimated as 1·80, 2·75, 3·08 and 4·82 μg TMC/g wet weight 1 day after one, two, three and four 4-mg/kg doses, respectively. Since just under 50% of each of these amounts was calculated to be accounted for by TMC present in contaminating erythrocytes, the minimal brain-tissue level of TMC found to be associated with the appearance of neuronal damage was approximately 1·4 μg/g wet weight.

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LETTER TO THE EDITOR

SACCHARIN: PARA FORMS OF SOME IMPURITIES ARE NOT MUTAGENIC IN *SALMONELLA TYPHIMURIUM*

Sir,—Saccharin is synthesized by two major processes which lead to the formation of different impurities. When prepared by the Remsen–Fahlberg procedure (Remsen & Fahlberg, *Am. Chem. J.* 1879, **1,** 426) the artificial sweetener is contaminated with variable amounts of impurities: *o*- and *p*-toluenesulphonamide, *o*- and *p*-sulphamoylbenzoic acid and *o*- and *p*-sulphobenzoic acid. A much smaller amount of impurities, mainly *o*-derivatives, contaminates the saccharin prepared by the Maumee procedure (Subcommittee on Non-nutritive Sweeteners, *Safety of Saccharin and Sodium Saccharin in the Human Diet.* Publn no. PB-238-137. Committee of Food Protection, NAS-NRC, 1974, Washington).

The results of mutagenicity studies carried out on commercial saccharin suggest that the positive data obtained could be attributed to the presence of some of those impurities (Kramers, *Mutation Res.* 1975, **32,** 81; *idem ibid* 1977, **56,** 163). Crude extracts from such commercial sweetener preparations have been analysed and have been shown to contain unidentified compounds that were able to induce mutations in *Salmonella typhimurium* strains TA98 and TA100 (Stolz *et al. J. envir. Path. Toxicol.* 1977, **1,** 139). Mutagenic activity was also detected in the urine of mice fed the impure saccharin as well as in a host-mediated assay. Highly purified saccharin was not directly mutagenic *in vitro* but the urine of mice fed this product exhibited mutagenic activity towards TA100 (Batzinger *et al. Science, N.Y.* 1977, **198,** 944). Recently, the problem of saccharin, its impurities and their possible mutagenicity has been reviewed (Report of the National Academy of Sciences, Washington, DC, Nov. 1978). In a previous report (Poncelet *et al. Fd Cosmet. Toxicol.* 1979, **17,** 229) we presented results indicating the absence of mutagenic activity of o-derivatives found in saccharin. The present study was undertaken to investigate the possible mutagenicity of three *p*-derivatives that are common contaminants of commercial saccharin.

p-Sulphobenzoic acid monopotassium salt was obtained from Eastman Kodak, Rochester, NY, *p*-sulphamoylbenzoic acid from Ega-Chemie, Steinheim, Federal Republic of Germany and *p*-toluenesulphonamide from Merck, Darmstadt, Federal Republic of Germany. All the 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 a RAL diet. The animals were injected ip with 500 mg Aroclor 1254/kg body weight diluted in corn-oil (200 mg/ml). They were killed 5 days later and liver fractions were prepared (Ames *et al. Mutation Res.* 1975, **31,** 347). *S. typhimurium* strains TA1530, TA1535, TA1538, TA98 and TA100 were kindly provided by Professor B. N. Ames. The post-mitochondrial (S9) fractions were obtained from three pooled rat livers, by centrifuging the homogenate (3 ml of 0·15 M-KCl/g wet liver (Ames *et al. loc. cit.*)). 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 triplicate by mixing dilutions of test materials (0·1 ml/plate), $1–8 \times 10^7$ bacterial cells from an overnight culture in nutrient broth (Difco)/plate, 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.

The results showed that no direct mutagenic effect was obtained towards any of the tested strains of *S. typhimurium* with any of the three substrates. Moreover, in the presence of fortified liver post-mitochondrial fractions from rats pretreated with Aroclor 1254, the reversion rates observed were never significantly higher than the spontaneous reversion rates at doses of test substrate ranging from 4×10^{-7} mol up to 4×10^{-2} mol/plate. In these experimental conditions, *p*-sulphamoylbenzoic acid and *p*-toluene sulphonamide exerted a cytotoxic effect on strains TA1538 and TA98.

The para isomers of the most frequent impurities contaminating lots of commercial saccharin were ineffective towards the common strains of *S. typhimurium* both in the absence and in the presence of fortified rat-liver fractions.

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MEETING ANNOUNCEMENT

STABLE ISOTOPES

The Fourth International Conference on Stable Isotopes will be held in Jülich, Federal Republic of Germany from 23 to 26 March 1981. The programme will include papers on radioactive labelling techniques and analysis and on the use of stable isotopic tracers in pharmacology, toxicology, clinical diagnosis, biochemistry, life sciences and agriculture. Other topics will include the cause and interpretation of isotope effects, the fractionation of isotopes in biological systems and the role of stable isotopes in geochemical and environmental research. The language of the conference will be English. Further information may be obtained from Dr. H. Förstel, Kernforschungsanlage Radioagronomie (im ICH), Postfach 1913, 5170 Jülich, Federal Republic of Germany.

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FORTHCOMING PAPERS

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

N-Nitrosodimethylamine in dried dairy products. By L. M. Libbey, R. A. Scanlan and J. F. Barbour.

Synergistic effect of chlorogenic acid and thiocyanate on *in vitro* formation of *N*-methyl-*N*-nitrosoaniline under physiological conditions. By D. Lathia and U. Frentzen.

Mutagenicity of peanut oils and effect of repeated cooking. By L. Y. Y. Fong, C. C. T. Ton, P. Koonanuwatchaidet and D. P. Huang.

An investigation of the genetic toxicology of irradiated foodstuffs using short-term test systems. II. Sister chromatid exchange and mutation assays in cultured Chinese hamster ovary cells. By B. J. Phillips, E. Kranz and P. S. Elias.

Influence of solvents and adsorbents on dermal and intestinal absorption of TCDD. By H. Poiger and Ch. Schlatter.

A toxin from the palmyra palm, *Borassus flabellifer*: Partial purification and effects in rats. By J. B. Greig, S. J. E. Kay and R. J. Bennetts.

Inhibitory action of citrinin on cultured hepatoma cells. By G. Lorkowski, E. E. Creppy, G. Beck, G. Dirheimer and R. Röschenthaler.

Evidence for *in vitro* and *in vivo* interaction between ochratoxin A and three acid drugs. By P. Galtier, R. Camguilhem and G. Bodin.

Chromosome aberrations in mammalian cells exposed to vitamin C and multiple vitamin pills. By H. F. Stich, L. Wei and R. F. Whiting.

Comparative metabolism of phenobarbitone in the rat (CFE) and mouse (CF1). By J. V. Crayford and D. H. Hutson.

Lysis of rabbit polymorphonuclear leucocyte granules by surfactants of differing structure and irritancy. By W. T. Gibson.

Effects of housing conditions on food intake, body weight and spontaneous lesions in mice. A review of the literature and results of an 18-month study. By M. Chvédoff, M. R. Clarke, E. Irisarri, J. M. Faccini and A. M. Monro.

Mutagenicity of *p*-nitrosophenol. By P. Gilbert, J. Rondelet, F. Poncelet and M. Mercier (Short paper)

Repeatability and reproducibility of measurements of vinyl chloride concentrations in materials and articles made of polyvinyl chloride. By L. Rossi, J. Waibel and C. G. vom Bruck (Review paper)

[*Contents continued*]

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).

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

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