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INFORMATION SECTION

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Research Section

EFFECT OF DIETARY BUTYLATED HYDROXYANISOLE ON METHYLAZOXYMETHANOL ACETATE-INDUCED TOXICITY IN MICE

B. S. REDDY, K. FURUYA, D. HANSON, J. DIBELLO and B. BERKE

Naylor Dana Institute for Disease Prevention, American Health Foundation, Valhalla, NY 10595, USA

(*Received 1 March 1982*)

Abstract—Administration of butylated hydroxyanisole (BHA), a widely used food additive, has been found to inhibit the carcinogenic and toxic effects of various chemicals in animal models. To study the relationship of dietary BHA to the acute toxicity of methylazoxymethanol (MAM) acetate, a colon-specific carcinogenic compound, groups of female CF_1 mice were fed NIN-07 diet containing 0, 300, 1000, 3000 or 6000 ppm BHA or a semipurified diet containing 0 or 6000 ppm BHA for 4 wk, and were injected ip with MAM acetate (20 mg/kg body weight) at the end of the first 2 wk and again 4 days later. At levels of 300–6000 ppm, BHA was found to protect against death caused by MAM acetate. The mortality rates in MAM-treated mice were 80 and 92% in those fed the diets with no BHA and 0 and 1% in those fed 6000 ppm BHA, and were inversely related to the amount of BHA in the diet. The protection was associated with increased levels of hepatic cytochrome *P*-450 and b_5 and with a reduction in necrotic changes in the liver.

INTRODUCTION

The phenolic antioxidants butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are extensively used as food additives. Evidence is accumulating that suggests that these phenolic antioxidants may inhibit the carcinogenic as well as the toxic effects of a variety of chemicals in animal models (Cumming & Walton, 1973; Miranda, Reed, Cheeke & Buhler, 1981; Pamukcu, Yalçiner & Bryan, 1977; Slaga & Bracken, 1977; Ulland, Weisburger, Yamamoto & Weisburger, 1973; Wattenberg, 1979; Weisburger, Evarts & Wenk, 1977). At present, BHA is probably the least toxic of these two inhibitors of chemical carcinogenesis (Wattenberg, 1979). BHA fed to mice has been found to give significant protection against the mortality (or acute toxicity) caused by a variety of chemicals, such as ethyl methanesulphonate and monocrotaline (Cumming & Walton, 1973; Miranda *et al.* 1981). It has been suggested that the protection, when it occurred, may have been due to induction of the microsomal mixed-function oxidase system that metabolizes the carcinogen or other chemical, leading to detoxification of the chemical or to decreased binding of the carcinogen or its metabolites to DNA (Wattenberg, 1979).

Previous studies have demonstrated that BHA in the diet of mice inhibits colon carcinogenesis induced by methylazoxymethanol (MAM) acetate (Wattenberg & Sparnins, 1979). While the effect of BHA on the carcinogenicity of MAM acetate, a proximate carcinogen, has been ascribed partly to its inhibition of the activity of NAD^+-dependent alcohol dehydrogenase, which activates MAM to an ultimate carcinogenic form (Grab & Zedeck, 1977; Fernberg & Zedeck, 1980), the influence of BHA could also have been due to some other mechanism (Wattenberg & Sparnins, 1979).

For a further understanding of the mode of action of the phenolic antioxidants with respect to the carcinogenicity of the colon-specific aliphatic azoxy compound, MAM acetate, a systematic dose-response study of BHA in a single species and sex of rodent has been undertaken. This study was designed to determine the relationship of various levels of dietary BHA to the acute toxicity of MAM acetate in mice.

EXPERIMENTAL

Test compounds. BHA (a mixture of 2- and 3-*tert*-butyl-4-hydroxyanisole) was obtained from Sigma Chemical Co., St. Louis, MO, and MAM acetate from Ash Stevens, Inc., Detroit, MI.

Animals. Female CF_1 mice received from the Charles River Breeding Laboratories, Willimington, MA, at weaning were quarantined for 10 days and subjected to a series of tests to verify their health. They were then transferred to the experimental room, divided into groups and housed (ten per cage) in plastic cages with filter tops. The environment was rigidly controlled at 21°C and 50% humidity and a 12-hr light-dark cycle operated.

Diets. Two basic diets were used, the NIH-07 diet (Sontag, Page & Saffiotti, 1976) purchased from Zeigler Bros, Inc., Gardners, PA, and a semipurified diet (AIN-76; Bieri, 1980; Bieri, Stoewsand, Briggs *et al.* 1977), the ingredients of which were all purchased from ICN Pharmaceuticals, Cleveland, OH, and were as follows: casein, 20%; DL-methionine, 0·3%; corn starch, 52·0%; dextrose, 13·0%; Alphacel, 5·0%; corn oil, 5·0; mineral mix, 3·5%; vitamin mix, of revised composition with respect to vitamin K content (Bieri, 1980), 1·0%; choline bitartrate, 0·2%. This diet, except for the carbohydrate source, was based on the American Institute of Nutrition Standard Reference Diet. Its preparation and the incorporation of various

Table 1. *Effect of various levels of dietary BHA on the cumulative mortality of female CF_1 mice treated with methylazoxymethanol (MAM) acetate*

Dietary level of BHA (ppm)	No. of mice/group	Cumulative mortality (% of group) at day*: 0	1	2	3	14
NIH-07 diet						
0	120	0	0	56	89	92
300	120	0	0	13	40	42
1000	100	0	0	10	21	22
3000	100	0	0	2	5	7
6000	80	0	0	0	0	0
Semipurified diet (AIN-76)						
0	100	0	0	47	76	80
6000	100	0	0	1	1	1

*No. of days after administration of the first of two doses of 20 mg MAM acetate/kg body weight given by ip injection on day 0 and day 4.

levels of antioxidant into both diets were carried out with a Patterson-Kelly V Blender and Hobart Mixer. BHA was added to the semipurified diet at a level of 6000 ppm (at the expense of corn starch), and to the NIH-07 diet at 300, 1000, 3000 and 6000 ppm, and was monitored in all batches of experimental diet by gas–liquid chromatography (Association of Official Analytical Chemists, 1980) to ensure uniform distribution. Aliquots of samples taken from three different areas of the blender were extracted and analysed quantitatively for BHA. Fresh experimental diets were prepared every week and BHA was found to be distributed uniformly in all batches.

Test procedure. The two control and five BHA diets were fed *ad lib.* to groups of 90–130 mice from the age of 5 wk. After 2 wk on these diets (at 7 wk of age) all but ten mice of each group were given an intraperitoneal injection of 20 mg MAM acetate/kg body weight dissolved in normal saline. A second injection of MAM acetate was given 4 days after the first. The remaining ten mice in each group were given two injections of normal saline (0·1 ml). Numbers of deaths were recorded over a 14-day period following the first dose of MAM acetate or saline. Body weights were measured weekly for 3–4 wk. Animals that died or that were killed when moribund or as scheduled were autopsied. All organs were examined grossly and histologically. Tissues were fixed in 10% neutral buffered formalin and embedded in paraffin and the sections were stained with haematoxylin and eosin.

Liver microsomal preparation. To study the effects of the various levels of BHA on liver cytochrome *P*-450 and cytochrome b_5 activities, five of the ten control mice in each dietary group were killed 14 days after the saline vehicle treatment (i.e. after 4 wk on the experimental diets). The final body weights were recorded. The livers were quickly excised, rinsed in ice-cold normal saline, blotted dry on cheese cloth and weighed. Microsomal pellets were prepared by a modification of the method of Goldfarb & Pitot (1972). Liver from each animal was finely minced with scissors and homogenized in 3·5 vols ice-cold buffer (50 mM-potassium phosphate buffer, pH 7·3, containing 0·25 M-sucrose, 10 mM-EDTA and 10 mM-glutathione) using a Brinkmann polytron. The liver homogenates were centrifuged at 10,000 **g** for 20 min at 4°C in a Sorvall RC-2B centrifuge. The supernatant was freed from the floating layer of fat and the 10,000 **g** post-mitochondrial supernatant was centrifuged at 4°C in a Beckman ultracentrifuge at 105,000 **g** for 1 hr. The resulting microsomal pellet was suspended in 0·2 times the original volume of homogenization buffer. Microsomal cytochrome *P*-450 and b_5 were determined according to the procedures described by McClean & Day (1974) and Omura & Sato (1964), respectively, using an Aminco DW-2a recording spectrophotometer. Microsomal protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

The data were analysed statistically using the chi-square method and Student's *t* test.

RESULTS

In general, animals that were treated with MAM acetate (after 2 wk on the experimental diets) gained

Table 2. *Body and liver weights of vehicle-treated female CF_1 mice fed different levels of BHA for 4 wk*

Dietary level of BHA (ppm)	Body weight (g)	Liver weight (g)	Relative liver weight (g/100 g body weight)
NIH-07 diet			
0	22·6 ± 1·0	1·14 ± 0·1	5·05 ± 0·2
300	22·8 ± 1·2	1·21 ± 0·1	5·31 ± 0·3
1000	24·2 ± 1·3	1·53 ± 0·1*	6·32 ± 0·3*
3000	23·8 ± 1·0	1·63 ± 0·1*	6·85 ± 0·6*
6000	24·4 ± 0·9	1·77 ± 0·2*	7·22 ± 0·5*
Semipurified diet			
0	20·6 ± 0·8	1·04 ± 0·1	5·07 ± 0·3
6000	21·2 ± 1·5	1·55 ± 0·1*	7·32 ± 0·3*

Values are means ± SEM for groups of five animals selected at random from the ten mice in each group used as vehicle controls for the MAM acetate treatment. Those marked with an asterisk differ significantly ($P < 0·05$) from the corresponding control value.

little or no weight, irrespective of their dietary treatment. Body-weight gains in the vehicle-treated mice were slight and did not differ between the groups fed the different levels of BHA.

Mortality

The effect of MAM acetate-induced mortality of feeding various levels of BHA is shown in Table 1. Deaths were recorded for 14 days after the MAM acetate injection. In most of the groups, death, if any, occurred within 3 days of the first MAM acetate treatment. The mortality in mice fed the semipurified diet containing 6000 ppm BHA was 1%, whereas the mortality in animals fed semipurified diet without BHA was 76% by day 3 after MAM acetate administration. The mortality rate was inversely related to the amount of BHA in the NIH-07 diet. After the first MAM acetate injection, none of the mice died in the group fed the NIH-07 diet containing 6000 ppm BHA, whereas 89% of animals died in the group fed the diet without BHA. The mortality rates were reduced with increasing levels of BHA in the diet. Few deaths occurred in any dietary group after the second injection of MAM acetate. None of the animals died in the groups fed 6000 ppm BHA, even after the second MAM acetate injection.

Liver weight and cytochrome levels

The effects of 4-wk antioxidant ingestion on liver weights and liver cytochrome *P*-450 and b_5 are summarized in Tables 2 and 3. As mentioned above, feeding of different levels of BHA had no effect on body weights (Table 2). Liver weights, actual and expressed as a percentage of body weight, were significantly higher in animals fed the diets containing 1000, 3000 or 6000 ppm BHA than in those fed diets containing 0 or 300 ppm BHA; there was no difference in liver weights between the latter groups. The addition of 6000 ppm BHA to semipurified diet was associated with a significant increase in hepatic cytochrome *P*-450 and b_5 levels (Table 3). A slight but not statistically significant increase in cytochrome *P*-450 and b_5 levels was observed in mice fed NIH-07 diet with 300 ppm BHA compared to those in mice fed NIH-07 diet alone. Marked increases in cytochrome *P*-450 and b_5 levels were observed when 1000, 3000 or 6000 ppm BHA were included in the NIH-07 diet. In general, cytochrome *P*-450 and b_5 levels were higher in mice fed the NIH-07 diet than in those fed the semipurified diet.

Histopathology

The mice treated with MAM acetate and fed 0 or 300 ppm BHA showed more severe pathological changes than those treated with MAM acetate and fed the higher levels of BHA. In the former, the thoracic or abdominal cavity contained clear yellow fluid. Sometimes, moderate focal haemorrhage was observed on the mucosa of the small intestine, just below the opening orifice of the bile duct, and the liver showed a tan colour around the edges of the lobes and reddish foci on the lobes.

Figure 1 shows the necrotic changes in the livers of mice treated with MAM acetate and fed 0, 1000, 3000 or 6000 ppm BHA. Histologically, haemorrhagic necrosis of the hepatocytes was observed in the centri-

Table 3. *Effects of dietary BHA on hepatic cytochrome* P-450 *and* b_5 *levels in female CF$_1$ mice*

Dietary level of BHT (ppm)	Microsomal protein		Cytochrome *P*-450			Cytochrome b_5		
	mg/g liver	mg/liver weight/ 100 g body weight	nmol/mg microsomal protein	nmol/g liver	nmol/liver weight/ 100 g body weight	nmol/mg microsomal protein	nmol/g liver	nmol/liver weight/ 100 g body weight
				NIH-07 diet				
0	17·1 ± 1·4	86 ± 7	0·58 ± 0·06	9·9 ± 1·0	50 ± 5	0·30 ± 0·03	5·0 ± 0·5	25 ± 2
300	16·9 ± 0·7	90 ± 7	0·73 ± 0·04	12·4 ± 1·1	66 ± 8	0·36 ± 0·02	6·1 ± 0·5	33 ± 4
1000	18·0 ± 1·3	113 ± 9*	0·85 ± 0·19*	15·2 ± 1·0*	96 ± 6*	0·42 ± 0·01*	7·6 ± 0·5*	48 ± 3*
3000	18·3 ± 1·2	124 ± 8*	0·83 ± 0·26*	15·1 ± 1·0*	103 ± 8*	0·44 ± 0·07*	8·1 ± 0·05*	55 ± 3*
6000	20·0 ± 1·3	144 ± 12*	0·85 ± 0·45*	16·9 ± 1·2*	122 ± 8*	0·53 ± 0·03*	10·6 ± 1·3*	75 ± 7*
				Semipurified diet				
0	13·6 ± 1·1	68 ± 4	0·43 ± 0·03	5·9 ± 0·9	30 ± 3	0·22 ± 0·02	3·0 ± 0·4	15 ± 2
6000	14·2 ± 0·9	105 ± 10*	0·68 ± 0·09*	9·8 ± 1·7*	72 ± 13*	0·40 ± 0·05*	5·8 ± 1·0*	43 ± 8*

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

lobular zones. In mice treated with MAM acetate and fed the diet without BHA, the liver showed severe haemorrhagic necrosis and most of the hepatocytes, except some in the periportal area, showed necrotic changes, whereas in mice treated with MAM acetate and fed a diet containing 1000 or 3000 ppm BHA, the haemorrhagic necrosis of the liver ranged from moderate to mild, and a relatively moderate to small number of hepatocytes showed necrotic changes. Very few of the mice treated with MAM acetate and fed 6000 ppm BHA showed any necrotic changes in the liver or remarkable changes in other organs.

DISCUSSION

This study provides evidence that the phenolic antioxidant, BHA, protects against MAM acetate-induced toxicity in mice. The degree of protection obtained with BHA at concentrations of 1000–6000 ppm, as evidenced by a reduction in the number of deaths, was very marked and was dose related. BHA also sharply reduced the MAM acetate-induced necrotic changes in the liver. Again the effect was dose related, as was the induction of hepatic cytochrome *P*-450 and b_5 levels in mice fed diet containing 1000–6000 ppm BHA but not treated with MAM acetate.

These results confirmed other observations that this antioxidant causes an increase in liver weight and hepatic cytochrome *P*-450 and b_5 levels in mice (Cha & Bueding, 1979; Speier & Wattenberg, 1975). Although the mechanism has not been established, several observations have suggested that the protective effect of BHA in mice is probably related to an alteration of the hepatic microsomal mixed-function oxidase system (Benson, Batzinger, Ou *et al.* 1978; Speir & Wattenberg, 1975; Wattenberg, 1979; Weisberger *et al.* 1977). The compound's protective effect against chemical carcinogenesis and the hepatotoxicity and acute toxicity of carcinogenic chemicals has been related to some action of BHA on the metabolic activation and detoxification of these chemicals (Creaven, Davies & Williams, 1966; Cumming & Walton, 1973; Kahl & Wulff, 1979; Miranda *et al.* 1981; Slaga & Bracken, 1977; Wattenberg, 1979). While the involvement of the mixed-function oxidase system in the metabolism of MAM has not yet been demonstrated, it may play a role in the detoxification of this carcinogen. However, previous work has demonstrated that BHA inhibits NAD^+-dependent alcohol dehydrogenase activity (Wattenberg & Sparnins, 1979) and this enzyme has been implicated in the activation of MAM (Fernberg & Zedeck, 1980; Grab & Zedeck, 1977). Thus, the protective effect of BHA in MAM acetate-induced toxicity could be related to the inhibition of alcohol dehydrogenase activity as well as to the induction of the mixed-function oxidase system.

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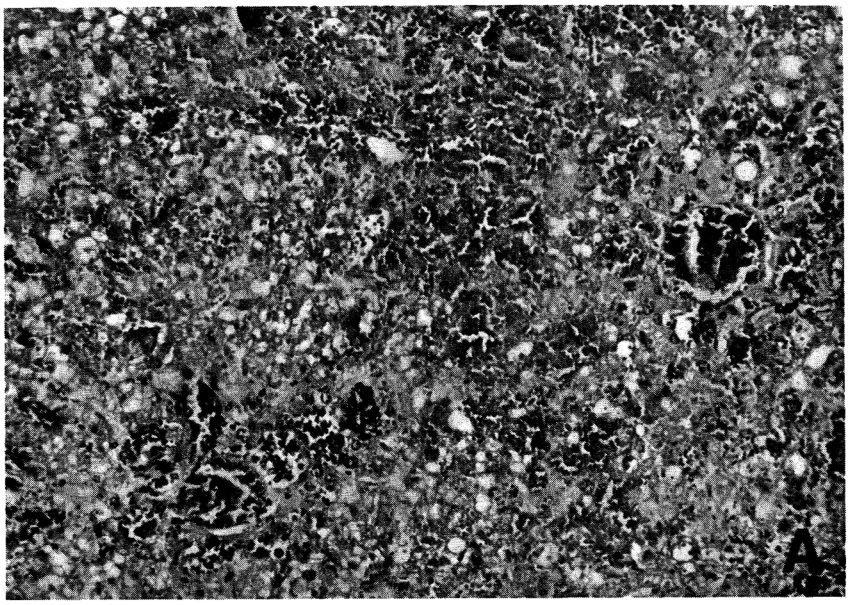
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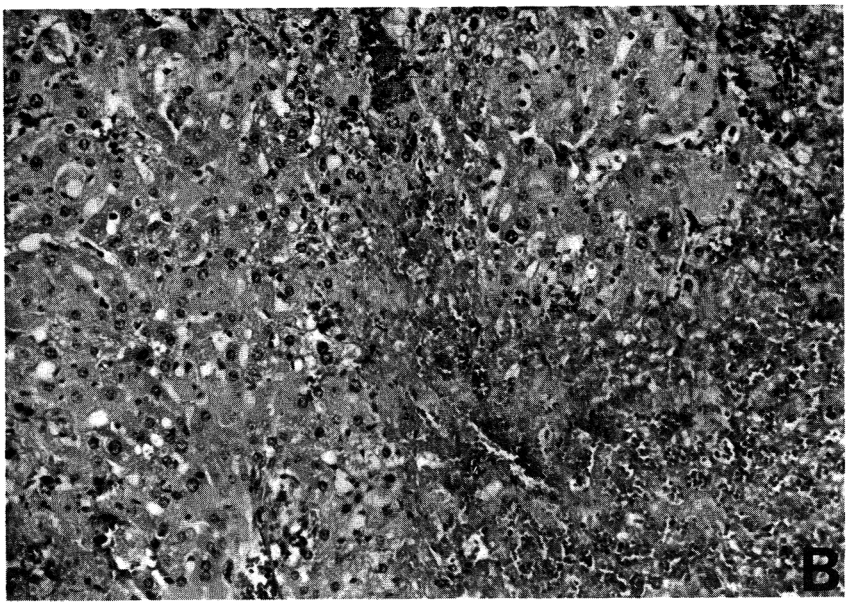
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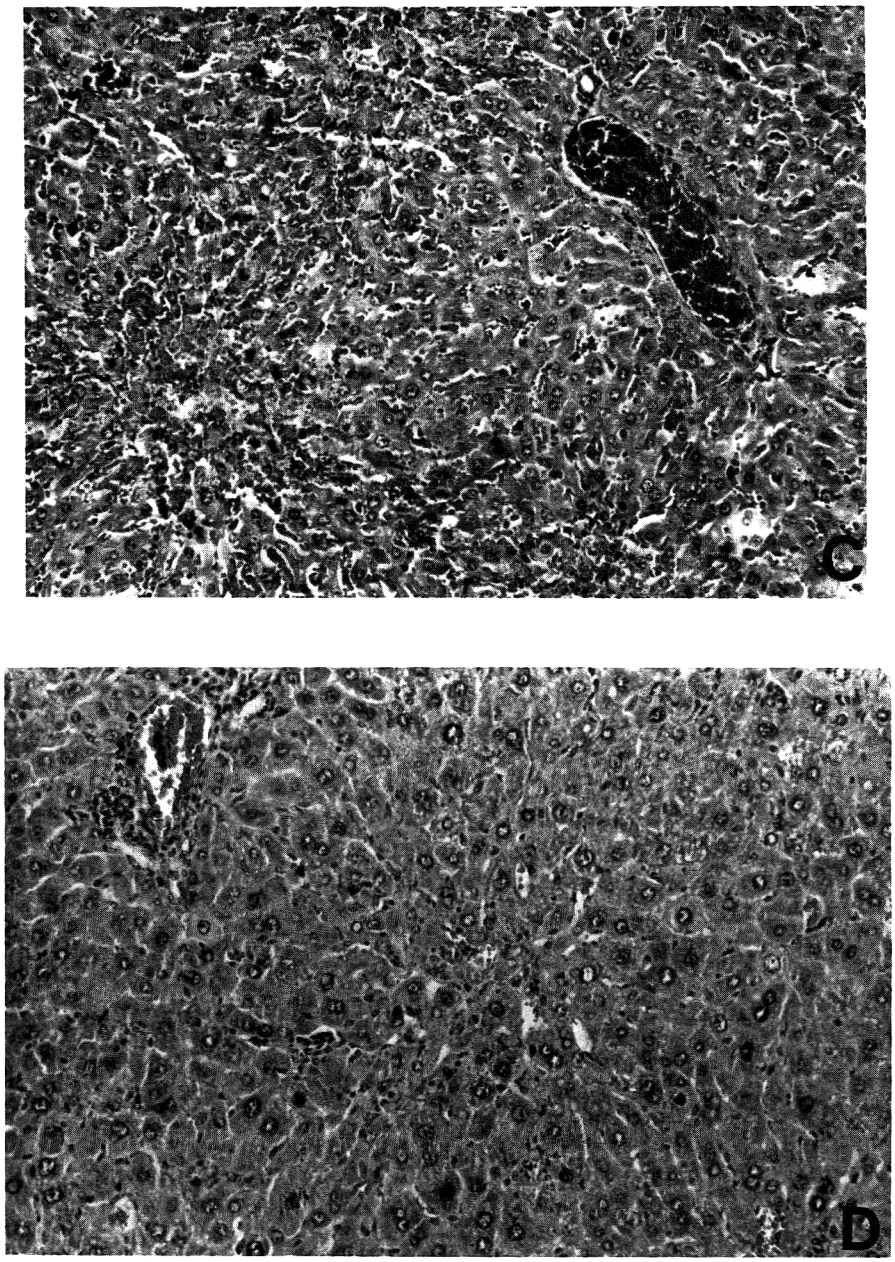

Fig. 1. MAM acetate-induced changes in the livers of female CF_1 mice fed NIH-07 diet containing (A) no BHA (showing severe haemorrhagic necrosis involving a majority of the hepatocytes), (B) 1000 ppm BHA (moderate haemorrhagic necrosis, with necrotic changes in about half of the hepatocytes), (C) 3000 ppm BHA (mild haemorrhagic necrosis, with a small number of hepatocytes showing necrotic changes) and (D) 6000 ppm BHA (no haemorrhagic necrosis and only a few hepatocytes showing mild degenerative changes). Haematoxylin and eosin × 125.

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LACK OF CARCINOGENICITY OF BUTYLATED HYDROXYTOLUENE ON LONG-TERM ADMINISTRATION TO B6C3F$_1$ MICE

T. SHIRAI, A. HAGIWARA, Y. KURATA, M. SHIBATA, S. FUKUSHIMA and N. ITO

First Department of Pathology, Nagoya City University Medical School, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467, Japan

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Abstract—Groups of approximately 50 male and 50 female B6C3F$_1$ mice were given butylated hydroxytoluene (BHT) at concentrations of 200, 1000 or 5000 ppm in their diet for 96 wk followed by a basal diet for 8 wk and were then killed. Similar groups of male and female controls were given basal diet throughout the 104 wk. Females given 1000 or 5000 ppm BHT and males given 5000 ppm showed reduced weight gain. Neither survival rates nor food consumption differed between experimental and control groups. No significant changes attributable to BHT treatment were found in the haematological examinations or serum and urine analyses. Tumours were found in many organs, especially the lungs, liver, lymph nodes and spleen, in both the experimental and control groups, but none were related to BHT treatment. Thus this experiment provided no evidence of BHT carcinogenicity in mice.

INTRODUCTION

The antioxidant butylated hydroxytoluene (BHT) has been widely used as a food additive in beverages, candies and baked goods. Recently much attention has been paid to antioxidants, because several, including BHT, have been found to inhibit chemical carcinogenesis when given before and/or with a carcinogen, such as 1,2-dimethylhydrazine (Clapp, Bowles, Satterfield & Klima, 1979), *N*-2-fluorenylacetamide (Ulland, Weisburger, Yamamoto & Weisburger, 1973) and some polycyclic hydrocarbons (Wattenberg, 1972). On the other hand, BHT was found to enhance tumour formation in the livers of rats that had been pretreated with 2-acetylaminofluorene (Peraino, Fry, Staffeldt & Christopher, 1977) and in the lungs of mice pretreated with urethane (Witschi, Williamson & Lock, 1977). Although BHT has been reported not to be carcinogenic to F344 rats or to B6C3F$_1$ mice (Department of Health, Education, and Welfare, 1979), significant numbers of lung tumours developed in female mice given a low dose of BHT but not in those given a high dose. However, no evidence of BHT carcinogenicity was found in Wistar rats of either sex (Hirose, Shibata, Hagiwara *et al.* 1980). In the work reported here, the carcinogenicity of BHT fed at levels of 200, 1000 and 5000 ppm in the diet was re-evaluated in B6C3F$_1$ mice.

EXPERIMENTAL

Animals and diet. Male and female B6C3F$_1$ mice, aged 5 wk, were obtained from Charles River Japan Inc., Kanagawa. The animals were kept on wood chips in plastic cages (five/cage) in an air-conditioned room controlled for temperature (23 ± 1°C) and humidity (55 ± 2%) and with a 12-hr light-dark cycle. Animals were given commercial diet (CE-2, CLEA Japan Inc., Tokyo) before the experiment. BHT (food-additive grade), obtained from Wako Pure Chemical Industries Ltd, Osaka, was mixed with CE-2 diet in the appropriate concentrations and made into pellets. Fresh diet was prepared every 12 wk. The stability of BHT in the diet was measured by gas-liquid chromatography on eight occasions during the feeding period.

Treatment. Groups of 51 or 52 mice of each sex were placed on diets containing BHT at concentrations of 200, 1000 or 5000 ppm from the age of 6 wk. A further 50 animals of each sex were given control diet from the same age. The animals were weighed weekly and their food consumption was measured at less frequent but regular intervals. After 96 wk on these diets, all the animals were fed the control diet for a further 8 wk, and were then killed under ether anaesthesia. Terminal blood samples were collected from the inferior vena cava of 31 mice of each sex in each group for microscopic examination, red and white cell counts, platelet counts and determination of haemoglobin, haematocrit, glutamic–oxalacetic transaminase (GOT), glutamic–pyruvic transaminase (GPT), alkaline phosphatase, total bilirubin, total cholesterol, total protein, the albumin/globulin ratio, blood urea nitrogen and glucose. Urine samples for analysis were collected by forced urination, from the same numbers of animals as were used for the blood samples, just before they were killed. The specific gravity and pH and the presence or absence of protein, glucose, ketones, bilirubin, occult blood and urobilinogen were determined. After macroscopic examination, the lungs, liver, kidneys, brains, hearts, spleens, salivary glands, testes, ovaries and pituitary glands were weighed, and all resected organs were fixed in 10% buffered formalin and processed for histological examination. Mice that died or became moribund during the experiment were also autopsied.

Statistical analyses. The data were subjected to analyses of variance and the differences between the

means were tested by Student's t test. The incidences of tumours in different groups were compared by the chi-square test.

RESULTS

Dietary levels of BHT

Analysis of food samples on eight occasions showed that the actual levels of BHT in diets initially containing 200, 1000 and 5000 ppm were about 200, 800 and 4000 ppm, respectively.

Food consumption, body weights and survival

There were no consistent deviations from normal in the rates of food consumption by males or females in any group during the experiment. No persistent decrease in food consumption was observed in any group given BHT.

However, the mean body weights of females given BHT at a level of 1000 or 5000 ppm were consistently less than the control value (Fig. 1) and the differences were statistically significant ($P < 0.05$) from wk 12 to wk 104 for the 5000-ppm group and from wk 48 to wk 84 for the 1000-ppm group. Females given the lowest dose of BHT showed sporadic decreases in body-weight gain until wk 28 but not thereafter. Males given 5000 ppm showed a statistically significant ($P < 0.05$) reduction in weight gain throughout almost all the experiment, but their body weights increased to the level of the controls by wk 104, at which time BHT feeding had been discontinued for 8 wk. No statistically significant reduction in body-weight gain was seen in males given 200 or 1000 ppm BHT except in wk 8 and 9.

Several mice of both sexes drowned as a result of inefficient functioning of their water-supply system in the early weeks of the experiment (Fig. 2). Most of the deaths occurring later in all groups were due to malignant lymphomas. The numbers of deaths among mice of both sexes treated with BHT were similar to those in the control groups.

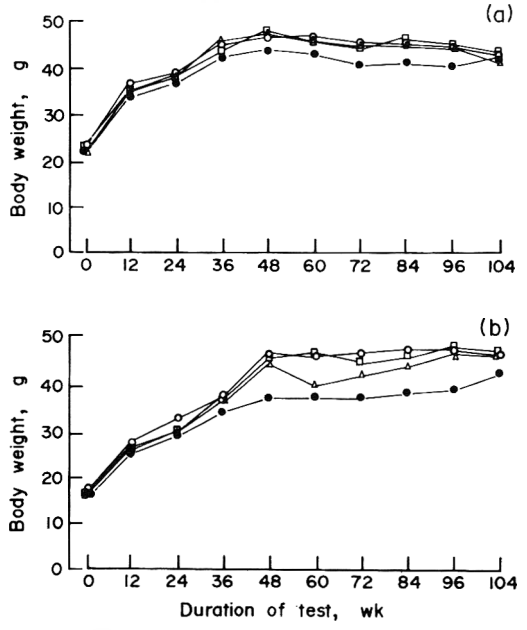

Fig. 1. Mean body weights of (a) male and (b) female mice given control diet (—○—) or diet containing 200 (—□—), 1000 (—△—) or 5000 (—●—) ppm BHT. The animals were weighed weekly throughout the study.

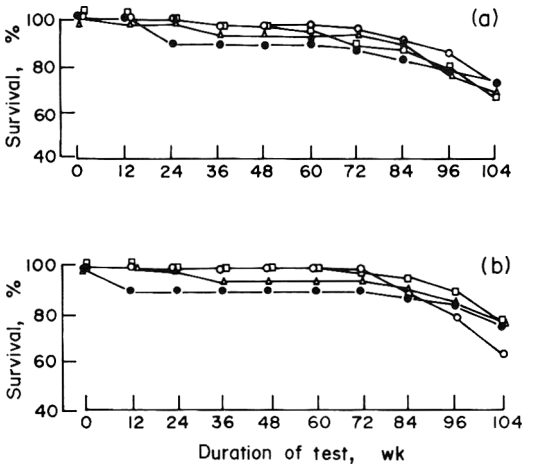

Fig. 2. Survival of (a) male and (b) female mice given control diet (—○—) or diet containing 200 (—□—), 1000 (—△—) or 5000 (—●—) ppm BHT.

Organ weights

Statistically significant decreases were seen in the mean absolute weights of the brains of males given 1000 ppm BHT, the salivary glands of females given 1000 or 5000 ppm BHT, the hearts of males given 5000 ppm BHT and the kidneys of females given 5000 ppm, as well as in the relative weights of the salivary glands of females given 1000 ppm and the hearts of males given 5000 ppm. When expressed relative to body weight, the brain weight of females given 5000 ppm BHT was significantly higher than that of controls. However, it seems unlikely that these statistically significant decreases or increases in weight of some organs were attributable to a toxic effect of BHT, because there were no detectable pathological changes in these organs and no dose-response was evident in the effect.

Blood and urine analyses

There was a statistically significant ($P < 0.05$) decrease in the red blood cell count in females given 1000 ppm BHT. Since this was observed only in the intermediate-dose group it was not considered to be related to BHT administration.

No statistical differences from control values were found in the urine analyses in any BHT-treated group.

In males, the serum levels of GOT and GPT were slightly higher in the experimental groups than in the controls, but the difference was statistically significant ($P < 0.05$) only for GOT in the group given 5000 ppm BHT. Sporadically significant increases or decreases were found in the serum values for alkaline phosphatase, total protein and the albumin/globulin ratio in the experimental groups, but the changes did not show a dose-response and were therefore unlikely to be attributable to BHT.

Table 1. *Incidence of neoplastic lesions in B6C3F$_1$ mice given diets containing 0–5000 ppm BHT*

Organ/lesion	Dietary level* (ppm)...	Incidence of lesion†							
		Males				Females			
		0	200	1000	5000	0	200	1000	5000
Harderian gland	*No. examined...*	*44*	*45*	*26*	*30*	*34*	*32*	*34*	*37*
Adenoma		3	4	1	2	1	5	1	0
Skin	*No. examined...*	*48*	*48*	*50*	*46*	*45*	*47*	*46*	*44*
Papilloma		0	1	0	0	0	0	0	0
Fibroma		2	1	1	1	0	0	0	0
Fibrosarcoma		1	0	2	1	1	0	1	0
Malignant fibrohistiocytoma		2	1	2	3	2	0	0	0
Haemangiosarcoma		0	0	0	0	1	0	0	0
Leiomyosarcoma		0	0	2	1	0	0	0	0
Lung	*No. examined...*	*48*	*48*	*50*	*47*	*47*	*47*	*46*	*44*
Adenoma		8	8	9	8	7	3	2	2
Adenocarcinoma		3	6	2	1	3	0	1	2
Haemangioma		0	0	1	0	0	0	0	0
Nasal cavity	*No. examined...*	*48*	*48*	*49*	*45*	*47*	*46*	*46*	*43*
Adenocarcinoma		0	0	0	0	1	0	0	0
Pituitary	*No. examined...*	*32*	*35*	*28*	*32*	*38*	*38*	*36*	*35*
Adenoma		0	0	0	0	1	1	2	1
Haemangioma		0	0	0	0	1	0	0	0
Thyroid	*No. examined...*	*43*	*43*	*41*	*42*	*42*	*39*	*37*	*33*
Follicular adenoma		0	0	1	0	1	0	0	0
Parathyroid	*No. examined...*	*18*	*21*	*19*	*25*	*13*	*20*	*14*	*15*
Adenoma		0	1	0	0	0	0	0	0
Adrenal	*No. examined...*	*43*	*43*	*50*	*40*	*44*	*46*	*44*	*42*
Phaeochromocytoma		0	3	2	1	0	0	0	0
Cortical fibroma		0	0	0	0	0	0	0	1
Forestomach	*No. examined...*	*45*	*47*	*48*	*46*	*47*	*46*	*46*	*44*
Papilloma		1	3	3	3	2	0	4	3
Squamous-cell carcinoma		0	0	0	0	0	0	0	1
Glandular stomach	*No. examined...*	*45*	*47*	*48*	*46*	*47*	*46*	*46*	*44*
Leiomyosarcoma		0	1	0	0	0	0	0	0
Small intestine	*No. examined...*	*45*	*46*	*49*	*47*	*43*	*47*	*45*	*44*
Adenocarcinoma		0	1	0	0	0	0	0	0
Large intestine	*No. examined...*	*47*	*47*	*49*	*46*	*43*	*47*	*45*	*43*
Leiomyosarcoma		0	0	0	0	1	0	0	0
Pancreas	*No. examined...*	*47*	*47*	*49*	*46*	*45*	*46*	*46*	*43*
Islet-cell adenoma		0	1	1	0	1	0	1	0
Liver	*No. examined...*	*48*	*48*	*50*	*47*	*47*	*47*	*46*	*44*
Hyperplastic nodule		14	10	13	16	2	3	5	5
Hepatocellular carcinoma		11	13	12	10	2	2	1	3
Haemangioendothelioma		4	5	2	2	0	0	0	0
Fibrosarcoma		1	0	0	0	0	0	0	0
Sarcoma (unclassified)		1	0	0	0	0	0	0	0
Thymus	*No. examined...*	*16*	*16*	*17*	*18*	*22*	*11*	*36*	*24*
Malignant lymphoma		1	0	0	0	3	1	0	1
Spleen	*No. examined...*	*47*	*48*	*50*	*47*	*47*	*47*	*46*	*44*
Haemangioma		0	2	0	0	1	1	0	0
Haemangioendothelioma		0	2	0	0	0	0	0	0
Haemangiosarcoma		0	0	0	0	1	0	0	0
Malignant lymphoma		5	2	7	3	13	17	4	10
Lymph node	*No. examined...*	*44*	*42*	*43*	*39*	*42*	*39*	*43*	*36*
Malignant lymphoma		7	3	10	4	14	16	12	9
Kidney	*No. examined...*	*48*	*48*	*50*	*47*	*47*	*47*	*46*	*44*
Transitional-cell carcinoma		1	0	0	0	0	0	0	0
Urinary bladder	*No. examined...*	*42*	*47*	*49*	*46*	*39*	*46*	*46*	*42*
Haemangioma		0	1	0	0	0	0	0	0
Ovary	*No. examined...*	—	—	—	—	*38*	*37*	*37*	*40*
Teratoma						1	0	0	0
Cyst adenoma		—	—	—	—	0	0	0	1
Granulosa-cell tumour		—	—	—	—	0	0	0	1
Uterus	*No. examined...*	—	—	—	—	*44*	*46*	*46*	*43*
Haemangioma		—	—	—	—	0	0	0	1
Mammary gland	*No. examined...*	*0*	*0*	*1*	*0*	*26*	*31*	*28*	*23*
Fibroadenoma		0	0	1	0	0	0	0	0
Adenocarcinoma		0	0	0	0	0	2	1	1
Abdominal cavity	*No. examined...*	*48*	*48*	*50*	*47*	*47*	*47*	*46*	*47*
Leiomyosarcoma		0	1	0	0	0	0	0	0

*Analysis of test diets at intervals throughout the study showed that diets initially containing 200, 1000 and 5000 ppm BHT actually contained about 200, 800 and 4000 ppm, respectively.

†No. of mice affected among the stated number examined.

Gross and histopathological findings

No pathological lesions were clearly related to BHT treatment. Among the non-neoplastic lesions, the incidence of lymphatic infiltration of the lung in females and of the urinary bladder in both sexes was significantly higher ($P < 0.05$) at the highest BHT level than in the controls, but the findings in the other BHT-treated groups did not suggest a dose-related trend. In females, cystic dilation of the uterine cavity accompanied by varying degrees of endometrial hyperplasia was noted in high incidence (36–62%) in both BHT-treated and control groups, but showed no relationship to BHT treatment.

The incidence of neoplastic lesions is shown in Table 1. Tumours were found in the lung, nasal cavity, forestomach, glandular stomach, small intestine, large intestine, pancreas, liver, lymph node, thymus, spleen, kidney, urinary bladder, Harderian gland, skin mammary gland, pituitary, thyroid, parathyroid, adrenal, ovary, uterus and abdominal cavity. The difference in incidence between the BHT-treated groups and the controls was not statistically significant for any type of tumour. The tumours that occurred in relatively high incidence were adenomas of the lung, hyperplastic nodules and hepatocellular carcinomas of the liver and malignant lymphomas. There was no statistically significant difference between BHT-treated and control groups in the total incidence of lung tumours (adenomas, adenocarcinomas and haemangiomas) or of liver tumours (hyperplastic nodules, hepatocellular carcinomas, haemangioendotheliomas, fibrosarcomas and other sarcomas). Thus, none of the tumours observed in this experiment were considered to be related to treatment with BHT at any dose.

DISCUSSION

The mean body weights of females given 1000 and 5000 ppm BHT and of males given 5000 ppm BHT were lower than those of the controls during the treatment period. The body weight of females given 5000 ppm BHT was more than 20% less than that of the controls, indicating that this level of BHT was the maximum tolerable dose for female B6C3F$_1$ mice. In spite of these decreases in body-weight gain, there were no clear gross or histopathological changes in any organ and there were no abnormal results in the haematology or the urine and serum analyses that could be related to administration of BHT. Moreover, survival was not affected adversely by BHT treatment and was more than 64% at the end of the experiment in mice of both sexes in all groups including the controls. The problem with the water supply, which killed some mice in the early weeks of the study, involved only animals caged on the periphery of the system.

Consequently, it may be concluded that chronic administration of BHT in the diet, even at a dose of 5000 ppm, does not induce specific tissue damage in any organs. This finding of low toxicity for BHT is consistent with results in previous long-term experiments (Deichmann, Gables, Clemmer *et al.* 1955; Department of Health, Education, and Welfare, 1979). Acute toxic injury, however, has been observed in the lungs of mice after injection of a large dose of BHT (Adamson, Bowden, Cote & Witschi, 1977; Marino & Mitchell, 1972).

In the present study, no significantly higher incidence of any tumour was observed in BHT-treated groups than in controls. Therefore, it was concluded that BHT at a level of 200, 1000 or 5000 ppm in the food had no carcinogenic effect in B6C3F$_1$ mice of either sex.

A previous study on BHT in our laboratory gave no evidence of carcinogenicity in Wistar rats of either sex at levels of 2500 and 10,000 ppm in the diet (Hirose *et al.* 1980). Moreover from a bioassay conducted by the US National Cancer Institute it was concluded that BHT was not carcinogenic to F344 rats or B6C3F$_1$ mice of either sex (Department of Health, Education, and Welfare, 1979). Furthermore, in an earlier 2-yr experiment on male and female rats, administration of BHT at levels of 2000, 5000, 8000 and 10,000 ppm in the diet induced no pathological lesions (Deichmann *et al.* 1955). The absence of tumorigenic activity in either sex of BALB/c mice fed BHT at a dietary level of 7500 ppm has also been reported (Clapp *et al.* 1979; Clapp, Tyndall, Satterfield *et al.* 1978). However, there has been one report that long-term administration of BHT in the diet increased the incidence of lung tumours in CF$_1$ mice (Brooks, Hunt, Thorpe & Walker, 1977). Strain differences in susceptibility probably contributed to this positive result on the carcinogenicity of BHT.

There have been many studies on the effects of BHT on tumour initiation, promotion and protection. BHT has been reported to enhance the induction of lung tumours in Swiss–Webster mice when given by multiple ip injections after a single ip injection of urethane (Witschi *et al.* 1977). When given in the diet after tumour initiation with a carcinogen, BHT enhanced the induction of rat-liver tumours in a long-term experiment (Peraino *et al.* 1977), but it did not enhance the appearance of preneoplastic lesions in rat liver in a short-term experiment (Ito, Tatematsu, Nakanishi *et al.* 1980). Moreover, BHT inhibited tumour development in many organs when given to animals simultaneously with a carcinogen or before administration of a carcinogen. Thus it inhibited the development of forestomach tumours in female but not male BALB/c mice treated with *N*-nitrosodiethylamine (Clapp *et al.* 1978), of forestomach tumours in mice and mammary tumours in rats treated with 7,12-dimethylbenz[*a*]anthracene (Wattenberg, 1972), of colonic tumours in female but not male BALB/c mice treated with 1,2-dimethylhydrazine (Clapp *et al.* 1979) and of liver tumours in rats given 2-acetylaminofluorene or *N*-hydroxy-2-acetylaminofluorene (Ulland *et al.* 1973). In addition, BHT showed no promoting activity in two-stage mouse-skin tumorigenesis (Berry, DiGiovanni, Juchau *et al.* 1978) and had neither an enhancing nor an inhibitory effect on dimethylhydrazine-induced colon carcinogenesis in rats (Barbolt & Abraham, 1979). The promoting and inhibiting actions of BHT may result from its induction of hepatic microsomal enzymes, as seen in experiments with phenobarbital (Peraino, Fry & Staffeldt, 1971; Shirai, Lee, Wang & King, 1981). These data also indicate that BHT may promote tumour induction only after appropriate initiation has been accomplished. This would explain why, in our study, long-term adminis-

tration of BHT alone did not enhance the incidence of any types of tumours.

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AN INVESTIGATION OF THE GENETIC TOXICOLOGY OF IRRADIATED FOODSTUFFS USING SHORT-TERM TEST SYSTEMS. III—*IN VIVO* TESTS IN SMALL RODENTS AND IN *DROSOPHILA MELANOGASTER*

H. W. RENNER

Institute of Biochemistry, Federal Research Centre for Nutrition, Engesserstrasse 20, D-7500 Karlsruhe 1, Federal Republic of Germany.

U. GRAF and F. E. WÜRGLER

Institute of Toxicology, Swiss Federal Institute of Technology and University of Zurich, CH-8603 Schwerzenbach, Switzerland.

H. ALTMANN

Institute of Biology, Research Centre Seibersdorf, Österreichisches Forschungszentrum Seibersdorf GmbH, A-2444 Seibersdorf, Austria

and

J. C. ASQUITH and P. S. ELIAS*

International Food Irradiation Project, Federal Research Centre for Nutrition, Postfach 3640, D-7500 Karlsruhe 1, Federal Republic of Germany

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Abstract—Six *in vivo* genetic toxicity tests were carried out on irradiated or unirradiated cooked chicken, dried dates and cooked fish. The tests were as follows: sex-linked recessive lethal mutations in *Drosophila melanogaster* (dried dates only), chromosome aberrations in bone marrow of Chinese hamsters, micronucleus test in rats, mice and Chinese hamsters, sister-chromatid exchange in bone marrow of mice and Chinese hamsters and in spermatogonia of mice, and DNA metabolism in spleen cells of Chinese hamsters. None of the tests provided any evidence of genetic toxicity induced by irradiation. However, dried dates, whether irradiated or not, showed evidence of some genetic toxicity in their effect on DNA metabolism in spleen cells and SCE induction in bone marrow. Feeding irradiated fish affected DNA metabolism in the spleen cells of Chinese hamsters. This effect could be interpreted as an induction of an immunoactive compound, although it could also be explained by the persistence of an immunoactive compound due to the removal by irradiation of spoilage organisms that would normally degrade it.

INTRODUCTION

The treatment of food with ionizing radiation for preservation purposes can give rise to complex chemical changes which are dependent on food composition and irradiation conditions (for a review see Elias & Cohen, 1977). The production of mutagenic and/or carcinogenic compounds cannot be excluded. Therefore genetic toxicity screening using a variety of short-term test systems has to be included in a thorough toxicological evaluation of irradiated food. The previous papers in this series (Phillips, Kranz & Elias, 1980a; Phillips, Kranz, Elias & Münzer, 1980b) have described the *in vitro* short-term tests applied to irradiated foodstuffs including an *in vitro* digestion procedure (Phillips & Elias, 1978) designed to overcome the special problems presented by using food, composed as it is of complex macromolecules and other smaller chemical moieties, as a test substrate. Since *in vitro* tests alone cannot form a total genetic toxicity screen, especially for a material such as food, *in vivo* investigations were also carried out at three European laboratories, in collaboration with the International Food Irradiation Project's own laboratory at Karlsruhe. These studies, which cover six short-term tests in four different species, complete the genetic toxicity screening of irradiated chicken, dates and fish as representatives of three different classes of food, and the results are presented in this paper.

Abbreviations: BudR = Bromodeoxyuridine; CPA = cyclophosphamide; IFIP = International Food Irradiation Project; MMS = methyl methanesulphonate; SCE = sister-chromatid exchange.

*To whom requests for reprints should be addressed.

†The names of the individual research workers involved are given in Table 1.

EXPERIMENTAL

Because the investigations were carried out in three different Institutes† on several animal species, some

Table 1. *Summary of the test methods and species used in investigations* in vivo *of the genetic toxicity of dried dates, cooked chicken and cooked fish*

Test no. (names of researchers involved)	Test method	Species used*	Foodstuff tested		
			Chicken	Dates	Fish
1 (U. Graf & F. E. Würgler)	Sex-linked recessive lethal mutations	*Drosophila melanogaster* (*Berlin K* wild type and *Basc*)	ND	+	ND
2 (H. Altmann)	Chromosome aberration in bone-marrow cells	Chinese hamsters	+	+	+
3 (H. W. Renner)	Micronucleus test in bone-marrow erythrocytes	Sprague–Dawley rats	+	+	+
		NMRI mice	+	+	+
		Chinese hamsters	+	+	+
4 (H. W. Renner)	SCE in bone-marrow cells	NMRI mice	+	+	+
		Chinese hamsters	+	+	+
5 (H. W. Renner)	SCE in spermatogonia	NMRI mice	+	+	+
6 (H. Altmann)	DNA metabolism in spleen cells	Chinese hamsters	+	+	+

+ = Test carried out ND = Not done SCE = Sister-chromatid exchange

*The Chinese hamsters used in tests 2 and 6 were obtained from Shell Toxicology Laboratory, Sittingbourne, Kent, UK and those used for tests 3 and 4 were out-bred stock bred at the Institute of Biochemistry, Federal Research Centre for Nutrition, Karlsruhe, FRG. The Sprague–Dawley rats and NMRI mice were obtained from the laboratory's own inbred stock.

small differences in food preparation, as well as in the experimental procedures, should be noted. Table 1 summarizes the tests used and the species in which they were conducted for all three foodstuffs. While the sex-linked recessive lethal test in Drosophila could be performed only with dried dates, the remaining tests (chromosome aberration, micronucleus, sister-chromatid exchange, DNA metabolism) were carried out on all three foodstuffs. A summary of the source of food, irradiation type, cooking procedure and subsequent handling of the irradiated and unirradiated diets is given in Table 2. In all cases, test animals were provided only with the test diets during the experimental period, and concurrent controls given a normal laboratory diet were always included. Positive control groups using known mutagens were included in most of the experiments; Table 3 lists the positive controls used in the various tests. In general, the tests were conducted using well-established procedures. These are described below.

Test 1. Sex-linked recessive lethal mutations in Drosophila melanogaster. Dried dates, irradiated with 1 kGy or unirradiated, were stored at 4°C until mutagenicity assays were carried out between 2 and 15 wk after irradiation of the dates. Digests prepared from uncooked dates according to the procedure described by Phillips & Elias (1978) were stored at −20°C until used. For each mutagenicity experiment six test tubes with unirradiated and six test tubes with irradiated material were prepared in the following ways.

(1) Whole dates: 10–12 dates were cut into small pieces and homogenized with 50–60 ml distilled water in a porcelain mortar. About 10 ml of the resulting paste was put into each test tube.

(2) Date digests: for each experiment a bottle containing 75–100 ml of date digest was thawed at room temperature. Ten millilitres of Instant Drosophila Medium (Formula 4-24, Carolina Biological Supply Company, Burlington, NC, USA) was put into each test tube and 10 ml of date digest was added.

(3) Controls: test tubes containing standard cornmeal–sugar–agar medium were used for the concurrent negative controls in all experiments.

A small drop of baker's yeast suspension was added to the surface of all of the test tubes in order to stimulate egg deposition. *Berlin-K* wild-type flies were allowed to oviposit for 24 hr in the test tubes. The special nature of the test material enabled it to be used directly as the food for the larvae forming the parental generation throughout their development, instead of treating only the adult flies as is the more common procedure. For the actual test the procedure described by Würgler, Sobels & Vogel (1977) was used. At eclosion, the flies were removed from the date medium, thus ensuring that at the end of treatment the germ cells of the males to be tested were spermatogonia, with a few early spermatocytes. Each male was mated sequentially with three *Basc* virgin females (*Basc*: In (1) $sc^{S1}l\,sc^{8R}$ + S, $sc^{S1}\,sc^{8}w^{a}B$) for 3 days (A brood), 2 days (B brood) and 2 days (C brood). Heterozygous females from the F_1 generation were then individually mated with *Basc* males. The F_2 tubes were scored for recessive lethals between days 10 and 14. Tubes containing no progeny but two live parents were classed as sterile. Tubes containing one or more wild-type males were classed as normal. Tubes containing no wild-type males were retested to confirm the presence of a recessive lethal. Clustering of recessive lethals could be confirmed by the numbering of the parental generation males.

Test 2. Chromosome aberrations in bone marrow. Chinese hamsters, aged 12–14 wk, were used for this test. A total of 40 male and 40 female animals, equally divided between eight experimental groups were used. All of the animals were housed separately for 1 wk

Table 2. *Sources of food, irradiation type, and preparation of foodstuffs for use as test diets*

Test No.*	Type and source of food	Irradiation type and dose	Storage time and temperature before use	Cooking method	Cooking time	Comments
				Chicken		
2, 6	Frozen, bought locally	^{60}Co, 7·0 kGy	5–8 days, −23 C	Pressure cooked	15 min	After 18 hr starvation, 8–14 g flesh was offered to all animals daily for 6 days. After cooking, stored at 4°C in plastic bags.
3, 4, 5	Frozen, bought locally	10 MeV electrons, 7·0 kGy	NR	Boiled	NR	After 8 hr starvation, flesh offered to all animals for 4 days for micronucleus test, 3 days for SCE tests.
				Dried dates		
1	Whole, stoned, and digests from IFIP	10 MeV electrons, 1·0 kGY	Whole 4 C, digests −20 C	Not cooked	—	10–12 stoned dates homogenized with 50–60 ml water to produce a paste. Date digests mixed 1:1 (v/v) with Instant Drosophila medium.
2, 6	Whole, from IFIP	^{60}Co, 1·0 kGy	Whole, 4 or 18°C	Not cooked	—	After 18 hr starvation, 6–8 g stoned dates offered to all animals daily for 6 days. Stones removed before irradiation.
3, 4, 5	Previous harvest from Iran	10 MeV electrons, 1·0 kGy	NR	Not cooked	—	After 8 hr starvation, stoned dates offered to all animals for 4 days for micronucleus test, 3 days for SCE tests. Stones removed before irradiation.
				Fish (Cod)		
2, 6	Fresh fillets, bought locally	^{60}Co, 2·5 kGy	7–10 days, −23°C	Pressure cooked	5 min	After 18 hr starvation, 11–14 g flaked fish offered to all animals daily for 6 days. After cooking stored at 4°C in plastic bags.
3, 4, 5	Frozen fillets bought locally	10 MeV electrons, 2·5 kGy	NR	Boiled	NR	After 8 hr starvation, offered to all animals for 4 days for micronucleus test, 3 days for SCE tests.

NR = Not reported IFIP = International Food Irradiation Project SCE = Sister-chromatid exchange

*Numbers refer to tests listed in Table 1 and text.

Table 3. *Positive control chemicals used in the tests*

Test diet	Test no.*	Species used: Drosophila	Chinese hamster	Rat	Mouse
Chicken	2, 6	—	CPA†	—	—
	3	—	CPA‡	NI	CPA‡
	4	—	MMS§	—	NI
	5	—	—	—	CPA‖
Dried dates	1	NI	—	—	—
	2, 6	—	CPA†	—	—
	3	—	CPA†	NI	CPA‡
	4	—	MMS§	—	NI
	5	—	—	—	CPA‖
Fish	2, 6	—	CPA†	—	—
	3	—	CPA‡	NI	CPA‡
	4	—	MMS§	—	NI
	5	—	—	—	CPA‖

CPA = Cyclophosphamide MMS = Methyl methanesulphonate
NI = Not included

*Numbers refer to tests listed in Table 1 and text.
†100 mg CPA/kg body weight injected ip 24 hr before killing.
‡Two ip injections of 20 mg CPA/kg body weight 30 and 6 hr before killing.
§1, 5 or 10 mg MMS/kg body weight injected ip 24 hr before killing.
‖5, 10 or 20 mg CPA/kg body weight injected ip 24 hr before killing.

before and during the 6-day period of feeding the experimental diets. Fresh food was offered daily and the food consumption of all of the animals was measured, with allowance made for drying of the diet.

Positive control animals, fed on a standard laboratory diet, were injected ip with cyclophosphamide (CPA, 100 mg in 10 ml physiological saline/kg body weight) 24 hr before they were killed by cervical dislocation. All of the animals were injected ip with 3·3 mg colchicine/kg body weight 2 hr before they were killed. Immediately after killing, bone marrow was washed from both femurs of each animal using Hanks balanced salt solution. Hypotonic treatment (0·075 M-KCl with 2·5 U hyaluronidase/ml for 20 min at 37°C) was followed by 24 hr fixation in methanol–acetic acid (3:1, v/v). Two slides were prepared from each animal and stained with 5% Giemsa solution. Scoring was carried out by two investigators, each of whom scored 100 metaphases on one slide from each animal. Cell selection was based on a well-spread metaphase containing at least 18 chromosomes and a diploid or tetraploid chromosome set. Classification was by the recommendations of Buckton & Evans (1973).

Test 3. Micronucleus test. Male and female adolescent Sprague–Dawley rats, NMRI mice and Chinese hamsters were used. Each of the six groups fed the test diets comprised 15 animals of each sex. For controls, 11 rats, ten mice and ten hamsters were used while the positive control groups contained eight mice and four hamsters. The animals were given the test diets for 4 days. Positive control animals were injected ip with 20 mg CPA/kg body weight 30 and 6 hr before they were killed. Bone-marrow slides were prepared from the femurs of all animals by the methods described by Schmid and co-workers (Schmid, 1975; Schmid, Arakiki, Breslau & Culbertson, 1971; Von Ledebur & Schmid, 1973). One slide was prepared per animal and 1000 polychromatic (immature) erythrocytes and 1000 mature erythrocytes (for control purposes) were examined per slide and per animal. The numbers of micronucleus-containing erythrocytes, but not the numbers of micronuclei per erythrocyte, were scored from coded slides by two investigators working independently.

Test 4. Sister-chromatid exchange in bone marrow. Adolescent NMRI mice and Chinese hamsters, weighing 30 ± 3 g, were divided into six groups of three male and three female animals for administration of the unirradiated and irradiated diets. There were eight mice or ten hamsters (equal numbers of males and females) in the control groups given standard laboratory diet and the three positive control groups each contained three hamsters of each sex. The animals were fed on the test diets for 3 days before they were killed. All of the animals had 50 mg bromodeoxyuridine (BudR) tablets implanted sc 20 hr before they were killed. The three positive control groups were injected ip with 1, 5 or 10 mg methyl methanesulphonate/kg body weight 24 hr before they were killed. Colcemid (1 mg/kg body weight) was injected ip into all of the animals 2 hr before they were killed. After death, bone marrow was removed from the femurs. Hypotonic treatment of bone marrow at 30°C for 20 min was with 1% sodium citrate for hamsters and 0·075 M-KCl for mice.

After fixation, with two changes, in ethanol–acetic acid (2·5:1, v/v) the cells were stored overnight at 4°C. The fixative was then again changed and slides prepared. Staining was by the fluorescence (Hoechst 33258/254 nm UV-irradiated) plus Giemsa technique (Renner, 1979). Twenty-five well-spread diploid metaphases were counted per animal.

Test 5. Sister-chromatid exchange in spermatogonia. Attempts to perform this test in mice and hamsters by the methods of Allen & Latt (1976) and Kanda &

Kato (1979) were unsuccessful, therefore a modification of the BudR-implant method described above was used. This was successful only in mice.

Groups of six adult male mice were used for each test diet. A control group of eight mice and three positive control groups of three mice per dose level were included. After feeding the test diets for 3 days BudR tablets (25 mg) were implanted sc 54 and 46 hr before the mice were killed. Feeding of the test diets was continued until the mice were killed. CPA (5, 10 or 20 mg/kg body weight) was injected ip into positive control animals 24 hr before they were killed. Colcemid (5 mg/kg body weight) was injected ip into all of the mice 24 hr before they were killed. Immediately after death, the tunica were removed and the testes were minced with sharp scissors. After washing in Ca^{2+}- and Mg^{2+}-free Dulbeccos saline, tissue fragments were incubated at 37°C for 10 min in 0·1% trypsin solution. Free cells were then spun down and treated with a hypotonic solution (0·075 M-KCl) for 10 min. The fixation, slide preparation and staining were carried out as for SCE preparations from bone marrow (see above). Scoring was carried out on 25 well-spread metaphases per animal.

Test 6. DNA metabolism in spleen cells of Chinese hamsters. Chinese hamsters aged 9–13 wk were fed the experimental diets for 7 days. Positive control animals were injected ip with CPA and killed 24 hr later. There were six animals per experimental group. Immediately after the hamsters had been killed by cervical dislocation, the spleens were removed, and were either pooled (experiment A) or treated individually (experiment B) chopped, then carefully suspended using a Potter homogenizer and washed twice with Hanks balanced salt solution. The cell concentration was counted after staining with Trypan blue and the concentration was adjusted to $1·9 \times 10^6$ cells by further addition of Hanks balanced salt solution. These cell suspensions were used for the measurement of semiconservative DNA synthesis, unscheduled DNA synthesis, and for the investigation of repair of supercoiled DNA.

Semiconservative DNA synthesis was determined by adding to the cell suspensions described above 5 μCi [^{3}H]thymidine (New England Nuclear GmbH, Vienna, Austria; specific activity 50 Ci/mmol) per ml, and incubating at 37°C. Incorporation of [^{3}H]thymidine into DNA was stopped by the addition of icecold perchloric acid after 120 min. The cell sediment was then washed three times with 6% perchloric acid and hydrolysed for 30 min at 90°C. After hydrolysis the amount of DNA and radioactivity were determined in aliquots of the supernatant after centrifugation at 600–800 g. The specific activities (counts/min/μg DNA) were expressed as percentages.

Unscheduled DNA synthesis was estimated by incubating the above cell suspensions with phosphate-buffered saline containing 10 μCi [^{3}H]thymidine/ml and being 10^{-2} M with respect to hydroxyurea at 37°C for 90 min. Cells were washed several times after addition of excess cold thymidine and fixed in methanol–acetic acid (3:1, v/v). Cells were spread on slides and autoradiograms were prepared using Kodak NTB 3 Nuclear Track Emulsion. One thousand cells were evaluated for each sample and the number of

Table 4. *Weight changes in rodents fed on the different test diets for periods similar to those used in the genetic toxicity tests*

Species	Feeding period (days)	Feeding period similar to test (no.)†	Sex	Weight changes (g) compared with day 0‡ in animals fed					
				Control diet	Chicken	Irradiated chicken	Dates	Irradiated dates	Fish	Irradiated fish
Hamsters	7	2	M	−0·76(5)	+0·56(5)	+0·88(5)	−3·96(5)	−4·68*(5)	+0·58(5)	−0·06(5)
			F	−2·20(5)	−0·94*(5)	+1·54*(5)	−4·60(5)	−5·24*(5)	+0·26(5)	−0·26(5)
	4	3, 4	M	ND	+2·5(4)	ND	−0·3(5)	ND	+2·3(4)	ND
			F	ND	+2·3(4)	ND	+1·5(4)	ND	+2·0(4)	ND
Rats	4	3	M	ND	+17·0(2)	ND	−8·0(3)	ND	+2·5(2)	ND
			F	ND	−11·5(2)	ND	+0·5(3)	ND	0(2)	ND
Mice	4	3, 4, 5	M	ND	+0·4(3)	ND	−2·0(3)	ND	+0·5(3)	ND
			F	ND	+0·5(3)	ND	−2·0(3)	ND	0(2)	ND

ND = Not done

†Numbers refer to tests listed in Table 1 and text.
‡Values are means for the number of animals given in brackets and those marked with an asterisk differ significantly [Scheffs test (Sachs, 1974)] from the corresponding values for the animals given the control diet ($P < 0·05$).

labelled cells were expressed as a percentage of the total lymphocytic cell population.

The repair of supercoiled DNA was estimated by incubating spleen-cell suspensions, prepared as outlined above, for 0, 30, 60, 120 and 180 min at 37 C to allow DNA repair or the regaining of the supercoiled structure. After centrifugation at 600–800 **g** cells were overlayered on a 15–30% sucrose gradient (1·95 M-NaCl, 0·001 M-EDTA, 0·01 M-Tris, pH 8·0) and lysed in a mixture of 1·95 M-NaCl, 0·1 M-EDTA, 2 mM-Tris and 0·5% Triton X-100 for 20 min. DNA peaks were determined after 60 min of centrifugation at 30,000 rpm (132,030 **g**: Beckman L5 Ultracentrifuge, SW40 Ti Rotor) and at 20 C by measuring the extinction at 254 nm in a flow-photometer (Cook & Brazell, 1975 & 1976).

RESULTS

Preliminary feeding studies

In order to carry out *in vivo* tests on irradiated food, the animals must be shown to accept for a reasonable period the test diets to be used. Table 4 shows weight changes in groups of animals fed for either 4 or 7 days on chicken, dates or fish. The results indicate that the test diets were accepted by all three species, no feeding group showing any severe loss of weight during the selected feeding period.

Test 1. Sex-linked recessive lethal test in Drosophila

Four replicate tests were carried out on each of the test media and the control medium. From each test-tube containing the date medium at least 15 males could be collected. They showed normal vitality and fertility throughout the 7 days of brooding. In each of the series of experiments with date medium between 76 and 90 treated parental males were tested, whereas in the control series about 120 males were tested. An average of 23 recessive lethal tests per male were performed in each brood in each of the five series. The combined results from all four tests are presented in Table 5. It can be seen that no increased rate of sterility was found in any of the five treatment groups. The rates of sex-linked recessive lethals varied between 0·11 and 0·17% when the results were pooled over the three broods. There was no statistically significant difference between the unirradiated and the irradiated series or between the control series and the date series.

Test 2. Chromosome aberrations in bone marrow

The data from all animals scored is summarized in Table 6. Significant differences in aberration yields and chromosome numbers shown in this table were obtained by a χ^2-test. Where no differences are indicated, none were found using this test. While the positive control (CPA) group was significantly different from all other groups, both in chromosome aberration yields and chromosome number distribution, only a few significant differences were found between the negative control and test-diet groups.

There was a significantly higher occurrence of isochromatid gaps in the negative control than any of the test-diet groups. A significantly greater number of tetraploid (4N = 44) cells were found in the animals fed on unirradiated fish than in the control: however, there was no significant difference between the unirradiated and irradiated fish diets in the number of tetraploids counted. The number of intact cells with a diploid (2N = 22) chromosome complement was significantly greater in the group given irradiated

Table 5. *Sex-linked recessive lethal test in* Drosophila melanogaster, *following feeding of parental males throughout larval development on irradiated or unirradiated dates or date digests (Test 1)*

Test medium	Brood	Total no. of cultures	Cultures not tested	No. of tests	No. of steriles	No. fertile	No. of recessive lethals
Control	A	3866	221	3645	1(0·03 ± 0·03)	3644	10(0·27 ± 0·09)
	B	3236	133	3103	0(0·0)	3103	0(0·00)
	C	2460	125	2335	1(0·04 ± 0·04)	2334	5(0·21 ± 0·10)
	Total...	9562	479	9083	2(0·02 ± 0·02)	9081	15(0·17 ± 0·04)
Whole dates, unirradiated	A	2182	75	2103	3(0·14 ± 0·08)	2103	3(0·15 ± 0·08)
	B	2092	48	2044	0(0·0)	2044	1(0·05 ± 0·05)
	C	2086	134	1951	1(0·05 ± 0·05)	1950	3(0·15 ± 0·09)
	Total...	6360	257	6101	4(0·07 ± 0·03)	6097	7(0·11 ± 0·04)
Whole dates, irradiated	A	2096	60	2036	0(0·0)	2036	2(0·11 ± 0·07)
	B	1942	27	1915	0(0·0)	1915	2(0·10 ± 0·07)
	C	1920	89	1822	1(0·05 ± 0·05)	1821	5(0·27 ± 0·12)
	Total...	5958	176	5773	1(0·02 ± 0·02)	5772	9(0·16 ± 0·05)
Date digest, unirradiated	A	2112	48	2064	0(0·0)	2064	5(0·24 ± 0·11)
	B	2136	37	2099	0(0·0)	2099	2(0·10 ± 0·07)
	C	2079	53	2025	1(0·05 ± 0·05)	2024	2(0·10 ± 0·07)
	Total...	6327	138	6188	1(0·02 ± 0·02)	6187	9(0·15 ± 0·05)
Date digest, irradiated	A	2021	69	1952	0(0·0)	1952	4(0·20 ± 0·10)
	B	2128	36	2092	2(0·10 ± 0·07)	2090	3(0·14 ± 0·08)
	C	2229	54	2175	0(0·0)	2175	0(0·00)
	Total...	6378	159	6219	2(0·3 ± 0·02)	6217	7(0·11 ± 0·04)

Values are the combined results of four replicate tests and those in brackets are percentages ± standard error of the proportion.

Table 6. *Chromosome aberrations in bone-marrow cells of Chinese hamsters given diets of irradiated or unirradiated chicken, dates or fish for 6 days (Test 2)*

Diet or treatment	No. of cells analysed	No. of cells with aberrations (other than gaps) of the				No. of cells with			No. of cells with a chromosome number of						
		Chromosome type	Chromatid type	Chromosome and chromatid types	Total	Chromatid gaps	Isochromatid gaps	Total	18–19	20	21	(2N) 22	23–25	(4N) 44	Not assessable
Control	1900	0	4	0	4	13	6†	19	45	77	103	1667	4	4	0
Chicken:															
unirradiated	2000	1	5	0	6	14	1	15	35	83	104	1770	3	5	0
irradiated	2000	1	5	0	6	14	2	16	22	46	107	1821§	1	3	0
Dates:															
unirradiated	2000	0	1	0	1	5	0	5	54	74	84	1782	0	6	0
irradiated	2000	0	2	0	2	11	1	12	63	63	116	1753	2	3	0
Fish:															
unirradiated	2000	0	4	0	4	16	0	16	53	66	124	1738	4	15‡	0
irradiated	2000	0	2	0	2	12	0	12	52	70	120	1748	3	7	0
CPA (positive control)	1800	8*	193*	11*	212*	28*	13*	39*	91	80	143	1318‖	12	17*	139

CPA = Cyclophosphamide

*Significantly higher than the mean of the other groups.
†Significantly higher than the values for the groups given irradiated or unirradiated chicken, dates or fish.
‡Significantly higher than the control, but not significantly different from the value for the group given irradiated fish.
§Significantly different distribution of chromosome number from the control group and the group given unirradiated chicken.
‖Significantly different distribution of chromosome number from all of the other groups (less cells with 2N, and more not assessable).
Statistically significant differences ($P < 0.05$) between groups were determined by the chi square test. The positive control animals were injected ip with 100 mg CPA/kg body weight 24 hr before they were killed.

Table 7. *Mean number of micronuclei-containing immature (polychromatic) and mature (normochromatic) erythrocytes per thousand cells examined in rats, mice and hamsters given diets of unirradiated or irradiated chicken, dates or fish for 4 days (Test 3)*

Diet or treatment		Mean* no. of micronuclei-containing cells/1000 cells among					
		Rat erythrocytes		Mouse erythrocytes		Hamster erythrocytes	
		Normochromatic	Polychromatic	Normochromatic	Polychromatic	Normochromatic	Polychromatic
Control		3·3(1–6)	3·5(0–6)	2·9(1–5)	3·3(0–6)	2·9(1–6)	3·1(1–6)
Chicken:	unirradiated	2·8(0–4)	2·8(0–5)	2·4(1–4)	2·8(1–5)	2·7(1–4)	3·0(1–5)
	irradiated	2·7(0–5)	2·5(0–5)	2·5(1–4)	2·9(1–7)	2·8(0–4)	3·1(2–5)
Dates:	unirradiated	3·0(1–6)	2·7(1–4)	2·5(1–4)	2·7(1–7)	3·0(1–6)	2·9(1–5)
	irradiated	2·7(1–5)	2·7(0–6)	2·5(1–4)	2·7(1–5)	2·9(2–5)	2·6(0–6)
Fish:	unirradiated	3·1(2–4)	3·1(0–5)	2·5(1–5)	3·0(1–6)	3·2(1–4)	3·9(2–7)
	irradiated	3·1(1–5)	2·9(0–6)	2·4(1–5)	3·0(1–6)	2·4(0–4)	3·3(1–7)
CPA (positive control)		ND	ND	2·5(1–5)	12·1(7–17)	2·0(0–4)	11·0(7–15)

CPA = Cyclophosphamide ND = Not done

*Values are means (range in brackets) for groups of 15 animals given the chicken, dates or fish diets, groups of 11 rats, ten mice and ten hamsters given the control diet and groups of eight mice and four hamsters (positive controls) injected ip with 20 mg CPA/kg body weight 30 and 6 hr before they were killed.

chicken than in either the negative control group or the group given unirradiated chicken.

Test 3. Micronucleus test

The results of the micronucleus test are summarized in Table 7. The counts of micronucleated erythrocytes per thousand were similar in the control and experimental groups and the means fell in the range 2·4 to 3·9. Thus the level of micronucleus formation was independent of animal species, sex, test diet and irradiation status of the test diet. As expected the positive control groups of mice and hamsters treated with CPA both showed an increased incidence of micronuclei in polychromatic erythrocytes, but not in normal mature erythrocytes.

Tests 4 and 5. SCE in bone marrow and spermatogonia

The results of the SCE test in the bone marrow of mice and Chinese hamsters, summarized in Table 8, show that the numbers of SCEs per cell were the same for the control groups and those fed unirradiated or irradiated chicken and fish, irrespective of species or sex. The groups of animals fed on dates, both irradiated and unirradiated, all showed an increase in the number of SCEs per cell compared to the other experimental and control groups. The investigations with dates were therefore repeated using a new batch of unsulphurized dates and sulphurized dates. The results (not presented here) confirmed that raised SCE levels also occurred with those two new batches of dates.

In the spermatogonial SCE test in mice (Table 9), there was no increase in the number of the SCEs per cell when any of the test diets were fed to these animals, the numbers of SCEs in all of the test groups being very similar to that in the control group. The number of SCEs per cell increased with increasing CPA concentration in the positive control groups thus confirming that for a pure chemical this method can be used for the measurement of SCEs *in vivo*.

Test 6. DNA metabolism in spleen cells

The results of the measurement of semiconservative DNA replication are summarized in Table 10. Only in date-fed animals was there a significant loss in body weight compared to controls during the 2-day treatment period. Food consumption was also considerably lower in date-fed animals but significantly higher in chicken- and fish-fed groups when compared with controls. DNA synthesis was markedly inhibited in the date-fed test groups independently of irradiation. The degree of suppression was similar to that caused by CPA at 100 mg/kg body weight. With both chicken and fish, (irradiated and unirradiated), the rate of DNA synthesis was markedly increased compared to control values. With chicken-fed animals and the individual spleens of fish-fed animals, no difference between irradiated and unirradiated diets was seen. Only with the pooled spleens from fish-fed animals was the rate of DNA synthesis significantly greater in the groups given the irradiated diet than in the group given unirradiated fish.

Autoradiographs exposed for 14 days showed no evidence of unscheduled DNA synthesis, even in the positive control (CPA-treated animals), although evidence of incisions in DNA had been seen in sedimen-

Table 8. *Sister-chromatid exchanges in the bone marrow of mice and Chinese hamsters given diets of irradiated or unirradiated chicken, dates or fish for 3 days (Test 4)*

Diet or treatment		Mean (±1 SD)† no. of sister-chromatid exchanges/cell in bone marrow of			
		Mice		Chinese hamsters	
		M	F	M	F
Control		4·56 ± 0·17	4·51 ± 0·09	3·83 ± 0·18	3·80 ± 0·12
Chicken:	unirradiated	4·62 ± 0·16	4·57 ± 0·16	3·91 ± 0·06	3·91 ± 0·15
	irradiated	4·49 ± 0·06	4·54 ± 0·02	3·85 ± 0·02	3·91 ± 0·18
Dates:	unirradiated	5·94 ± 0·37*	5·80 ± 0·41*	4·12 ± 0·12*	4·11 ± 0·07*
	irradiated	5·54 ± 0·26*	5·24 ± 0·07*	4·05 ± 0·25*	4·06 ± 0·06*
Fish:	unirradiated	4·58 ± 0·10	4·56 ± 0·19	3·76 ± 0·14	3·77 ± 0·06
	irradiated	4·68 ± 0·19	4·48 ± 0·04	3·79 ± 0·04	3·85 ± 0·16
MMS (positive control):					
1 mg/kg body weight		ND	ND	5·36 ± 0·55	ND
5 mg/kg body weight		ND	ND	8·44 ± 0·26	ND
10 mg/kg body weight		ND	ND	9·81 ± 0·12	ND

MMS = Methylmethanesulphonate ND = Not done

†There were three male and three female animals in each of the positive control groups and each of the groups given the unirradiated or irradiated chicken, dates or fish. There were four mice and five hamsters of each sex in the control groups. Twenty-five well-spread metaphases were examined per animal. The values marked with asterisks are significantly higher (Student's t test) than the control values or those values for groups fed chicken or fish ($P < 0·01$).
The positive control groups were injected with MMS 24 hr before they were killed.

tation profiles of nucleoids (see Fig. 1). In addition to the hydroxyurea-treated cells, autoradiographs were also prepared from cells not treated with hydroxyurea to determine the number of cells in S phase. These values are given in Table 11. These results show that although the dates reduce the overall rate of DNA synthesis (Table 10), they have no effect on the number of cells synthesizing DNA. Feeding chicken or fish caused an increase in the number of cells synthesizing DNA. While similar increases were caused by irradiated and unirradiated chicken, irradiated fish caused a far greater increase in the number of S-phase cells than did unirradiated fish. This finding is in agreement with the effect of fish diets on both semiconservative DNA synthesis (Table 10) and sedimentation of DNA (Fig. 1). CPA-treated animals showed a considerable reduction in DNA synthesizing cells compared to untreated controls.

Table 9. *Sister-chromatid exchanges in spermatogonia of mice given diets of irradiated or unirradiated chicken, dates or fish for 3 days (Test 5)*

Diet or treatment		Mean (± 1 SD)* no. of SCE/cell
Control		3·03 ± 0·11
Chicken:	unirradiated	3·03 ± 0·08
	irradiated	3·05 ± 0·11
Dates:	unirradiated	3·03 ± 0·11
	irradiated	3·06 ± 0·09
Fish:	unirradiated	3·08 ± 0·05
	irradiated	3·06 ± 0·10
CPA (positive control):		
5 mg/kg body weight		4·37 ± 0·18
10 mg/kg body weight		5·73 ± 0·23
20 mg/kg body weight		7·26 ± 0·10

CPA = Cyclophosphamide

*There were six mice in each of the groups given the irradiated or unirradiated test diets. There were eight mice in the control group and three in each of the positive control groups. Twenty-five well-spread metaphases were examined per animal. The positive control groups were injected ip with CPA 24 hr before they were killed.

The sedimentation profiles of supercoiled DNA after different incubation periods are shown in Fig. 1. These results show a similar pattern to that seen for semiconservative DNA synthesis, in that with the positive control chemical and both irradiated and unirradiated date diets, the DNA sedimentation velocity is considerably lower than the control value with no significant difference between irradiated and unirradiated date diets. An incubation period of 180 min was required for the DNA sedimentation rate, following date-feeding, to return to the control level. With both chicken and fish diets, no effect was seen if nucleoid sedimentation was measured without incubation. With 30 min or more incubation, a time-dependent significant increase in nucleoid sedimentation was seen with the fish diet. This effect was significantly greater with irradiated than with unirradiated fish. With chicken, significant increases in nucleoid sedimentation rates, compared to control values were seen only after incubation for 120 and 180 min.

DISCUSSION

The results presented here, drawn from the reports submitted to IFIP, complete a screen of three irradiated and unirradiated foodstuffs for genetic toxicity. The essentially negative outcome of all reported investigations confirms the earlier assessment, based on the findings of short-term tests *in vitro* and animal feeding studies of the same foods using extracts and *in*

Table 10. *Semiconservative DNA synthesis in spleen cells of Chinese hamsters given diets of irradiated or unirradiated chicken, dates or fish for 7 days (Test 6)*

Diet or treatment	Semiconservative DNA synthesis (% of control) in	
	Pooled spleens	Single spleens
Unirradiated chicken	256 ± 43·5*	241 ± 117·8*
Irradiated chicken	271 ± 34·1*	312 ± 176·5*
Control	100 ± 7·5	100 ± 25·4
CPA (positive control)	36 ± 5·6*	22 ± 10·1*
Unirradiated dates	28 ± 2·3*	29 ± 3·1*
Irradiated dates	30 ± 7·8*	31 ± 1·6*
Control	100 ± 11·2	100 ± 21·4
CPA (positive control)	21 ± 1·9*	35 ± 6·0*
Unirradiated fish	370 ± 33·7*	236 ± 95·8*
Irradiated fish	929 ± 53·0*†	357 ± 181·4*
Control	100 ± 15·6	100 ± 23·8
CBP (positive control)	42 ± 6·0*	19 ± 8·8*

CPA = Cyclophosphamide

Values are means ± 1 SD for three determinations on pooled-spleen samples and six determinations on single-spleen samples, from groups of six hamsters. Values marked with an asterisk differ significantly (Student's *t* test) from the corresponding control value (*$P < 0.05$), and the value marked with a dagger differs significantly (Student's *t* test) from the corresponding value for the group given unirradiated fish (†$P < 0.05$). Positive control animals were injected ip with 100 mg CPA/kg body weight 24 hr before they were killed.

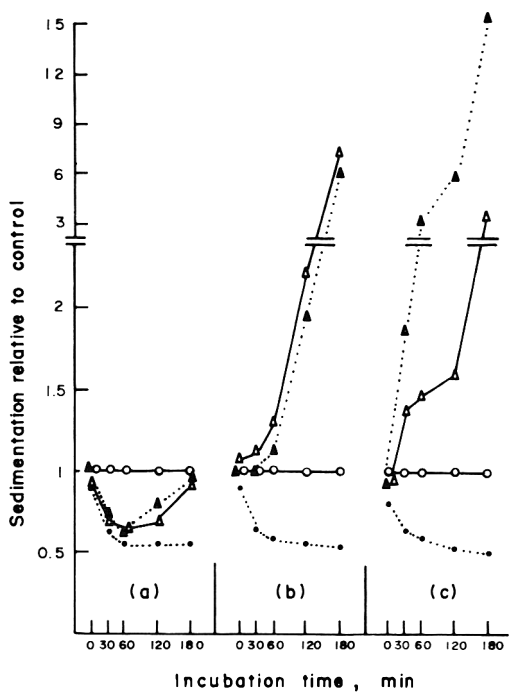

Fig. 1. Sedimentation profiles of supercoiled DNA in spleen-cell suspensions from Chinese hamsters fed unirradiated (△) or irradiated (▲) (a) dates, (b) chicken meat or (c) fish for 7 days. The values are given relative to the control (○, = 1), and values are also shown for positive controls (●) given ip 100 mg cyclophosphamide kg body weight 24 hr before they were killed.

vitro digests, that irradiation at the dose levels used does not induce any detectable mutagenic or carcinogenic activity in the treated foodstuffs. In only one case in the nine tests described in this report and in two previous papers (Phillips *et al.* 1980a, b) was an effect seen that could be attributed to an irradiated foodstuff. This was with irradiated fish in the DNA metabolism test.

The feeding of dried dates significantly suppressed semiconservative incorporation of [^{3}H]thymidine during DNA synthesis (Table 10), irrespective of any irradiation treatment, and caused breaks in the DNA strands. However it did not adversely affect DNA repair or the number of S-phase cells (Table 11). The nutritional effects of exposing Chinese hamsters for 7 days to a diet consisting entirely of dried dates were evidenced by a significant reduction in food intake and, consequently, a significant loss of body weight. This, together with the absence of dietary lipids, possibly may have affected the stability of lysosomal membranes and have caused the release of lysosomal DNAase. Subsequent synthesis of poly(ADP-Rib) may have inhibited DNA polymerase α without affecting DNA polymerase β. Therefore semiconservative DNA synthesis may have been suppressed without affecting DNA-repair synthesis and the efficient rejoining of broken DNA strands.

A diet of chicken for 7 days irrespective of irradiation treatment increased semiconservative DNA synthesis (Table 10) and the number of S-phase cells (Table 11) without inducing unscheduled DNA synthesis or affecting the DNA-repair process. The increased DNA synthesis in turn appears to lead to a faster sedimentation of supercoiled DNA.

Table 11. *The incidence of S-phase cells in the spleens of Chinese hamsters given diets of unirradiated or irradiated chicken, dates or fish for 7 days (Test 6)*

Diet or treatment	Incidence of S-phase cells/1000 cells (% of control)
Unirradiated chicken	165*
Irradiated chicken	156*
CPA (positive control)	9*
Unirradiated dates	106
Irradiated dates	104
CPA (positive control)	25*
Unirradiated fish	273*
Irradiated fish	818*†
CPA (positive control)	27*

CPA = Cyclophosphamide

Values are for samples from the pooled spleens from six hamsters per treatment group. Those marked with an asterisk differ significantly ($*P < 0.05$) from the control value and that marked with a dagger differs significantly ($\dagger P < 0.05$) from the value for the group given unirradiated fish. Positive control animals were injected ip with 100 mg CPA/kg body weight 24 hr before they were killed.

Fish protein, *per se*, appeared to have antigenic properties. Minor antigens can cause allergic reactions and stimulate sub-populations of lymphocytes (Lindner, 1974), as the fish diets have done here, as shown by the increased rate of semiconservative DNA synthesis (Table 10), the greater sedimentation velocity of DNA (Fig. 1) and the increased incidence of S-phase cells (Table 11), all of which indicate lymphocyte stimulation. The irradiated fish diet has apparently caused an even greater immunological response than unirradiated fish. It should be noted that this effect of the fish diets was the reverse of the findings in the positive control—CPA caused a decrease in all parameters—and hence no genotoxic activity is involved here; the effect of irradiation of the fish is either to increase an immune response normally elicited by this foodstuff, or more probably, to remove from fresh fish spoilage organisms that normally degrade the immunoactive protein, and thus to maintain an effect that is normally lost during the processing, storage and distribution of this food.

Since this was the only difference found between an irradiated and unirradiated food, the results of nine tests presented in this series of papers on the genetic toxicity of irradiated chicken, dates and fish have shown no evidence of the presence of mutagenic compounds. While the balance of probability suggests that this is because no mutagens were present in these foods, the possibility of a mutagen remaining undetected must be considered. Given the special nature of the materials being investigated, that is the complex chemical mixtures of macromolecules, small organic and inorganic molecules rather than the pure chemicals that are usually investigated by the methods described, this possibility must be higher than is usually the case. Firstly, it is possible that the putative mutagen may be produced by radiochemical alteration of the structure of a minor food component. The resulting mutagen or promutagen would then be present only in trace quantities, below the sensitivity thresholds of the tests applied. Secondly, a mutagen could have been produced in detectable quantities but then have disappeared as a result of reactions with macromolecules other than DNA. Since such compounds are by implication reactive species, the possibility must be envisaged that during the period between irradiation and testing, they have become deactivated by reacting with neighbouring macromolecules. With chicken and fish, cooking provides a further possibility for the destruction of any chemically reactive mutagens present. Phillips *et al.* (1980b) showed that the number of SCEs induced in Chinese hamster ovary cells in culture by CPA added to fish, was decreased compared with that induced by an equivalent amount of CPA added to the culture medium. The incidence of SCEs was further decreased by extraction or, most markedly, by artificial digestion *in vitro* of the fish after the addition of the CPA. There are thus three ways in which a mutagen present in food could be inactivated before it reached the systemic circulation of a test animal: by reaction with other food components, by cooking and by digestion.

That genotoxic activity can be detected in a food irrespective of irradiation treatment is clearly demonstrated by the results presented in this paper. Feeding Chinese hamsters and mice on dried dates has caused a significant increase in the SCEs found in bone-marrow cells and also produced changes similar to those caused by CPA in the DNA metabolism of the spleen cells. These results are unlikely to be false positives since the experiments were carried out on the same material in two laboratories in different countries and the increase in SCEs was observed again when a new sample of dried dates was used. However, this activity is not caused by irradiation, since identical results were obtained (Tables 8 & 10; Fig. 1) with untreated dates, chemically treated dates and irradiated dates. Another possible cause for this genotoxicity may have been the drying process applied to the dates because commercially dried fruits such as raisins, prunes, bananas, figs and apricots have recently been shown to have clastogenic activity (Stich, Rosin, Chiu & Powrie, 1981). Water extracts of these fruits caused chromosome aberrations in cultured Chinese hamster ovary cells, the incidence of aberrations being decreased if extracts were pretreated with S-9 mix.

No increases in SCEs were found in tests *in vitro* (Phillips *et al.* 1980b) or in an SCE test *in vivo* on mouse spermatogonia (Table 9). The failure to detect an increase in SCEs with dried dates *in vitro* may be due to some component process of physiological digestion, not mimicked by the simplified *in vitro* digestion procedure used (Phillips & Elias, 1978), that is involved in the release or activation of the genotoxic compound. Alternatively, the rat-liver S-9 activation system used may not have affected a promutagen if one were present. In the case of the spermatogonial SCE method there are three possible explanations for the absence of an increase in SCEs. Firstly, some tissue-specific activation system present in haemopoietic tissue but absent in testicular tissue could be involved; secondly there could be a concentration of the mutagen in blood-forming tissues and less accessibility to testicular tissue; finally, the spermatogonial SCE test could be less sensitive than the bone-

marrow SCE test. As different positive controls were used in these two SCE detection systems (MMS in bone marrow, CPA in spermatogonia) no definite explanation can be offered. However, qualitatively, these positive results with dried dates are important, because they demonstrate that genotoxic activity can be detected in a foodstuff by the use of the short-term tests described. Comparison of the results of tests using extracts with those of tests using digests may provide additional data to help in the assessment on the basis of these experimental findings of risks related to food irradiation.

Conclusions

In the wide range of *in vivo* tests described, no evidence of genotoxic activity caused by the irradiation of chicken, dates and fish was observed. While it is possible that this was caused by the relative insensitivity of the methods used, this seems unlikely, since in two of the tests dried dates, irrespective of irradiation, were shown to contain a compound that had genotoxic properties. Similarly, although a clear effect of irradiation on fish could be demonstrated in standard test systems designed to investigate DNA metabolism, it provided evidence for the absence of genotoxic potential in fish so processed.

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NO VOLATILE *N*-NITROSAMINES DETECTED IN BLOOD AND URINE FROM PATIENTS INGESTING DAILY LARGE AMOUNTS OF AMMONIUM NITRATE

G. ELLEN and P. L. SCHULLER
National Institute of Public Health, P.O. Box 1, 3720 BA Bilthoven

and

P. G. A. M. FROELING and E. BRUIJNS
St. Radboud University Hospital, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands

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Abstract—Whole blood and urine from 23 patients ingesting ammonium nitrate daily in amounts varying from 2·5–9 g were analysed for volatile *N*-nitrosamines. By contrast with reports in the literature *N*-nitrosodimethylamine and *N*-nitrosodiethylamine were not found in blood (limit of detection 0·1 μg/kg) but a trace of *N*-nitrosopyrrolidine was found in the blood of one subject. Small amounts of *N*-nitrosomorpholine in some blood samples and in one blank were believed to be formed during the analysis since morpholine had been added to the samples to indicate artefactual nitrosamine formation. Traces of *N*-nitrosodimethylamine (0·1 μg/kg) were found in six of the 23 urine samples.

INTRODUCTION

The presence of volatile *N*-nitrosamines in human blood after ingestion of bacon and spinach has been reported (Fine, Ross, Rounbehler *et al.* 1977). Recently, the conclusion that these *N*-nitrosamines were formed *in vivo* has been questioned (Fine, Challis, Hartman & van Ryzin, 1982) as they might have been present in the beer which was also ingested by the subject. Several other investigators have purported to show that volatile *N*-nitrosamines, notably *N*-nitrosodimethylamine (NDMA) but also *N*-nitrosodiethylamine (NDEA) are present in human blood (Kowalski, Miller & Sen, 1980; Lakritz, Simenhoff, Dunn & Fiddler, 1980; Melikian, LaVoie, Hoffman & Wynder, 1981; Yamamoto, Yamada & Tanimura, 1980), but in these studies the ingestion of meals had no distinct effect on *N*-nitrosamine levels. *N*-nitrosamines formed *in vivo* may also show up in urine and faeces. No volatile *N*-nitrosamines or only trace amounts have been found in the urine of healthy subjects (Castegnaro, Bourgade & Chernozemsky, 1980; El-Merzabani, El-Aaser & Zakhary, 1979; Hicks, Gough & Walters, 1978; Kakizoe, Wang, Eng *et al.* 1979). The presence of *N*-nitrosamines in human faeces reported by Wang, Kakizoe, Dion *et al.* (1978) has also been questioned and attributed to artefactual formation during analysis (Archer, Lee & Bruce, 1982) but confirmed by others (Suzuki & Mitsuoka, 1981). Eisenbrand, Spiegelhalder & Preussmann (1981) have been unable to detect NDMA in the urine, blood and faeces of healthy volunteers, except in the urine of an individual who had consumed a litre of beer that contained NDMA.

In our own earlier studies (Ellen, Schuller, Bruijns *et al.* 1982; Stephany & Schuller, 1978) trace amounts of NDMA were found only occasionally in the urine and saliva of healthy volunteers before and after administration of large amounts of nitrate; urine from patients who ingested gram amounts of ammonium nitrate daily for a long time occasionally contained higher levels of NDMA. As nitrate may play a key role in the *in vivo* formation of *N*-nitrosamines, we considered it of interest to analyse samples of blood from patients who took daily amounts of ammonium nitrate up to 9 g to prevent the redevelopment of calcium phosphate renal stones (Bruijns, 1982; Froeling, 1978). Samples of urine from the same patients were also analysed to check for any possible correlation between *N*-nitrosamine levels in blood and in urine.

Abbreviations: GC–TEA = Gas chromatography–thermal energy analysis; NDEA = *N*-nitrosodiethylamine; NDMA = *N*-nitrosodimethylamine; NMOR = *N*-nitrosomorpholine; NDPA = *N*-nitroso-di-*n*-propylamine; NPYR = *N*-nitrosopyrrolidine.

EXPERIMENTAL

Blood sampling. Blood samples (20 ml) from 23 patients (10 men and 13 women, aged 31–63 yr; mean age 45 yr) who were on a maintenance treatment with ammonium nitrate, were taken when they visited the clinic for a routine check. At the time of the experiment all of the patients were in good general health. Their ammonium nitrate intakes (see Table 1), the composition of their two most recent meals and their use of medicines were recorded. Commercially available evacuated blood-collection tubes were used to collect the blood samples; the rubber stoppers of these tubes were analysed for volatile *N*-nitrosamines and found to be negative. The samples were stored

Table 1. *Nitrate intake by patients and volatile N-nitrosamines in blood and urine*

Patient no.	Daily NH_4NO_3 intake (g)	Time between most recent NH_4NO_3 intake and sampling (hr)	NMOR in blood* (μg kg)	NDMA in urine* (μg kg)
1	2 × 3	2	—	—
2	2 × 1·5	4	—	0·1
3	2 × 3	14	0·7	
4	3 × 2	2·5	Tr	—
5	3 × 2·25	5	0·8	—
6	3 × 3	22	—	0·1
7	2 × 1·5	17	—	0·1
8	2 × 2·25	19	—	0·1
9	3 × 1·5	3	—	0·1
10	3 × 1·5	5	Tr	—
11	2 × 1·5	13	—	—
12	2 × 3	4·5	—	0·1
13	2 × 4·5	4	Tr	—
14	2 × 3	19	—	—
15	2 × 2·25	24	1·2	—
16	1 × 6	16	—	—
17	3 × 2·25	2·5	Tr	—
18	3 × 1·5	3	—	—
19	2 × 3	3	—	—
20	1·5 + 2·25	17	—	—
21	1 × 2·5	13	—	—
22	3 × 1·5	3	—	-
23	1 × 3	12	Tr	—

NDMA = *N*-nitrosodimethylamine NMOR = *N*-nitrosomorpholine
Tr = Trace, <0·5 μg/kg

*No other nitrosamines were detected in either blood or urine at detection limits of 0·1–0·5 μg kg (depending on the structure of the nitrosamine) except for trace amounts of *N*-nitrosopyrrolidine in the blood of patient no. 5.

until analysis in a set of 25 numbered 100-ml round-bottomed flasks (to be used for vacuum distillation of *N*-nitrosamines, see Stephany & Schuller, 1978, Fig. 1) prepared in advance. All of the flasks contained 20 ml glycerol, 10 ml distilled water, 2 ml 0·5 M-NaOH and 1 mg morpholine as an indicator of artefact formation. To the odd-numbered flasks 100 μl of a 1 mg/litre *N*-nitroso-di-*n*-propylamine (NDPA) standard solution were also added as an internal standard. Flasks numbered 24 and 25 also contained 20 ml distilled water and were used as analytical blanks and analysed in the same way as the samples. The flasks were swirled around to homogenize the contents, sealed with screw caps and stored at −20°C until use. The screw caps contained rubber inlays with a teflon coating; analysis of these inlays showed that they did not contain volatile *N*-nitrosamines. A few hours before each patient came to the clinic the corresponding flask was thawed. The blood sample was transferred into the flask immediately after collection, the contents were homogenized and the flask was stored at −20°C until analysis. The flasks were weighed before and after the addition of the samples whose weights varied from 18·4 to 20·9 g, mean 20·0 g. Samples were collected from November 1981 to January 1982, and were analysed in February 1982.

Urine sampling. Urine samples were produced by the patients during the same visit to the clinic as when blood was collected. Samples were collected in 100-ml plastic bottles, containing 1 ml 1 M-NaOH, and were stored at −20 C until analysis.

N-nitrosamine determinations. *N*-nitrosamines were determined by a standard procedure normally used in our laboratory (Eisenbrand, Ellen, Preussmann *et al.* 1982; Stephany, Schuller, Egmond *et al.* 1979). The method consists of three main steps:

(a) low-temperature distillation of *N*-nitrosamines from an alkaline slurry of water, test portion and glycerol,
(b) extraction of the acidified distillate with dichloromethane and concentration of the extract to about 250 μl,
(c) analysis of the extract (10 μl injections) with a gas–liquid chromatograph interfaced with a Thermal Energy Analyzer® Model 502 (Thermo Electron Corp., Waltham MA), known as GC–TEA analysis. A glass column (2·0 m × 2 mm) packed with 10% Carbowax 20 M on Chromosorb WHP, 80–100 mesh, programmed from 120–170°C was used.

The round-bottomed flasks containing the blood samples were cooled to −70°C and attached to the distillation unit. From the bottles containing urine 20-g test portions were weighed into round-bottomed flasks. Glycerol (20 ml), 2 ml 0·5 M-NaOH and 10 ml distilled water were added to all of the flasks and to the even-numbered samples 100 μl of a 1-mg/litre NDPA standard solution were added. After mixing the contents, the flask was cooled to −70°C and fitted to the distillation unit. The minimum confidence level of detection for NDMA and NDEA in blood and urine was 0·1 μg/kg.

RESULTS AND DISCUSSION

Recovery of added NDPA varied from 67 to 92% (mean 83%) for blood samples and from 79 to 93% (mean 86%) for urine. Recovery of NDMA added at a level of 1 μg/kg to a 20-ml blood sample from a volunteer was 85% (in the duplicate portion of this sample no NDMA was detected), so there was a good conformity between the recovery of NDMA and of the internal standard NDPA. The results of the *N*-nitrosamine determinations in blood and urine are summarized in Table 1. In none of the blood samples nor in the two blanks were NDMA or NDEA detected, thus if they were present, their levels were less than 0·1 μg/kg. In one sample a trace amount (<0·5 μg/kg) of *N*-nitrosopyrrolidine (NPYR) was found. Three blood samples contained measurable amounts of *N*-nitrosomorpholine (NMOR), 0·7–1·2 μg/kg, and in five more samples traces of NMOR (<0·5 μg/kg) were found. No other TEA-positive compounds were detected in the blood. The presence of NMOR in human blood has not previously been described in the literature. Since we added the easily nitrosatable amine, morpholine to the samples as an indicator of artefact formation, we believe that NMOR was not present in the blood originally, but was formed during analysis. This hypothesis is supported by the finding that one of the two blanks also contained NMOR at levels comparable with those in the blood samples. The highest level of NMOR detected (1·2 μg/kg) corresponds with only $2{\cdot}4 \times 10^{-3}$% conversion of the added morpholine (1 mg). Traces of NMOR were found most frequently in samples with NDPA added as internal standard, so it is possible that NMOR might be formed by transnitrosation from NDPA. Ville & Mestrezat (1907) found that the nitrate content of human saliva is not constant but rises sharply after ingestion of nitrate, that saliva sampled directly from the salivary glands contains nitrate but no nitrite and that in the oral cavity a part of the salivary nitrate is reduced to nitrite by microorganisms (Ville & Mestrezat, 1907). These findings have since been confirmed by several investigators (Goaz & Biswell, 1961; Harada, Ishiwata, Nakamura *et al.* 1975; Ishiwata, Boriboon, Nakamura *et al.* 1975; Ishiwata, Tanimura & Ishidate, 1975; Spiegelhalder, Eisenbrand & Preussmann, 1976; Stephany & Schuller, 1975; Suchomel, 1979; Tannenbaum, Sinskey, Weisman & Bishop, 1974; Tannenbaum, Weisman & Fett, 1976). From these studies and our recent results with volunteers who took a single large dose of nitrate (Ellen *et al.* 1982) it is clear that after nitrate intake, nitrate and nitrite levels in saliva and nitrate levels in blood remain enhanced for several hours. As the patients in our study ingested large amounts of nitrate daily and most of them took 1–6 g NH_4NO_3 a few hours before the samples were taken, the nitrite burden of these persons was much higher than normal. Twenty-one patients consumed meat and four consumed fish as part of their two most recent meals before sampling, giving rise to the presence of amino compounds in their gastro-intestinal tracts. Nonetheless no volatile *N*-nitrosamines were detected in the blood of these patients, showing either that no *in vivo* formation of such compounds had taken place or that the metabolism of *N*-nitrosamines is so fast that measurable amounts did not build up in the blood. Our findings, like those of Eisenbrand *et al.* (1981) that *N*-nitrosamines are not present in human blood, demonstrate that reports of positive findings should be considered with caution. False positive results may be caused not only by artefact formation but also by inadequate storage of samples, i.e. samples and standards stored in the same refrigerator. According to the calculations of Tannenbaum (1980), the implications of reported NDMA blood levels of up to about 1 μg/kg (Lakritz *et al.* 1980; Yamamoto *et al.* 1980) or even 2·5 μg/kg (Melikian *et al.* 1981) would be considerable: a constant blood-level of 1 μg NDMA/kg corresponds with daily exposure to 670 μg NDMA from endogenous formation and a blood level of 2·5 μg/kg corresponds with daily exposure to 1700 μg NDMA formed endogenously. Thus human exposure to NDMA resulting from endogenous formation could be three orders of magnitude higher than that resulting from food ingestion, which has been determined to be *c.* 1 μg/24 hr (Gough, Webb & Coleman, 1978; Spiegelhalder, Eisenbrand & Preussmann, 1980; Stephany & Schuller, 1980).

NDMA was detected in some of the human urines examined but even then it was only at trace levels (0·1 μg/kg). This result, which is in agreement with that of our previous study (Ellen *et al.* 1982), shows that volatile *N*-nitrosamines are not normally present in human urine and when they are present, it is only at trace levels. These trace amounts might be formed in the bladder or at any other site in the body.

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THE INFLUENCE OF FOOD FLAVONOIDS ON THE ACTIVITY OF SOME HEPATIC MICROSOMAL MONOOXYGENASES IN RATS

M. H. SIESS and M. F. VERNEVAUT

Station de Recherches sur la Qualité des Aliments de l'Homme, Institut National de la Recherche Agronomique, Dijon, France

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Abstract—The effects of several food flavonoids on the activities of some drug-metabolizing enzymes in rat-liver microsomes were determined *in vivo*. When quercetin, chrysin, hesperetin and (+)-catechin were injected ip at doses of 150 mg/kg body weight/day for 3 days, quercetin and chrysin stimulated aminopyrine demethylase. When quercetin, rutin, hesperetin and (+)-catechin were fed as components (0·1%) of purified diets administered to rats for 4 wk, rutin and (+)-catechin increased microsomal cytochrome b_5 levels and quercetin and (+)-catechin increased aminopyrine demethylase activity. On the other hand rutin, quercetin and hesperetin inhibited NADH-cytochrome *c* reductase. Aniline hydroxylase was not affected by any of the flavonoids administered by either route. The results show that each flavonoid affects different components of the monooxygenase system. The fact that some flavonoids give different effects according to the route and/or the duration of administration is discussed.

INTRODUCTION

Many flavonoids are naturally occurring constituents of edible plants. They have been the subject of extensive nutritional and pharmacological studies for many years. These studies have demonstrated that both *in vivo* and *in vitro* flavonoids can exert a wide range of biological and physiological effects (Kühnau, 1976; Mabry & Ulubelen, 1980) the mechanisms of which largely remain unclear.

Several studies have shown that synthetic or natural flavonoids affect the activity of cytochrome *P*-450-mediated monooxygenases. Thus, the addition of 7,8-benzoflavone inhibited the oxidation of benzo[*a*]pyrene *in vitro* in hepatic microsomes from male rats treated with methylcholanthrene. In contrast, 7,8-benzoflavone stimulated this process in liver microsomes from newborn rats (Wiebel, Leutz, Diamond & Gelboin, 1971; Wiebel & Gelboin, 1975). Further studies have shown that the stimulatory or inhibitory effects of the compound depended on the type of cytochrome *P*-450 used in a reconstituted monooxygenase system (Huang, Johnson, Muller-Eberhard *et al.* 1981).

Other studies have demonstrated that 5,6-benzoflavone, flavone, and two naturaliy occurring polymethoxylated flavones (tangeretin and nobiletin) are active inducers of benzo[*a*]pyrene hydroxylase (Cantrell & Bresnick, 1971; Wattenberg, Page & Leong, 1968). It has been shown using human liver microsomes that flavonoids possessing hydroxyl groups (e.g. chrysin, hesperetin and quercetin) inhibit the hydroxylation of benzo[*a*]pyrene and the metabolic activation of aflatoxin B_1 to mutagenic products, whereas flavonoids lacking hydroxyl groups (7,8-benzoflavone, flavone, tangeretin, nobiletin) accelerate these transformations (Buening, Chang, Huang *et al.* 1981). These results suggested that naturally occurring flavonoids could have different specific actions in the regulation of the monooxygenase system.

In the present study, we examined the effects of dietary flavonoids on the activities of drug-metabolizing enzymes of rat-liver microsomes. First we tested quercetin, chrysin, hesperetin and (+)-catechin injected ip. These molecules represented different levels of oxidation of the flavane nucleus. In a second experiment we determined the effects of quercetin, rutin, hesperetin and (+)-catechin incorporated into the diet. These flavonoids possessed the same hydroxylation pattern (5,7,3′,4′OH) but had different substituents in positions 3 and 4. The chemical structures of these compounds are given in Table 1.

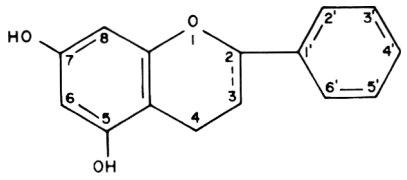
EXPERIMENTAL

Chemicals. Rutin and quercetin were purchased from Merck Inc. (Darmstadt, FRG) and chrysin, hesperetin, (+)-catechin from Sigma Chemical Co. (St. Louis, MO).

Animals, diets and treatments. In the first experiment, 40 6-wk-old SPF male Wistar rats, each weighing about 75 g, were housed individually in cages. For 4 wk, they were fed a diet consisting of a mixture of purified casein (23%), starch (38%), sucrose (28%), corn oil (5%), salt mixture (4%), agar (2%) and a vitamin mixture made to a semi-liquid consistency by mixing with 40% water. They were then divided into five groups. In four of the groups, the rats received daily ip injections for 3 days of 150 mg/kg of a flavonoid (quercetin, chrysin, hesperetin or (+)-catechin) dissolved in 30% aqueous polyethylene glycol 300. Control animals were injected with an equivalent volume of polyethylene glycol. The rats were killed 24 hr after the last injection.

In the second experiment, 40 6-wk-old rats were divided into five groups. In each group they were fed the same semi-liquid purified diet for 4 wk and in four groups 0·1% of a flavonoid (quercetin, rutin, hesperetin or (+)-catechin) was incorporated into the diet.

Table 1. *Chemical structures of the flavonoids tested*



Name	Position 3	Position 4	Hydroxylation pattern
Quercetin (flavonol)	—OH	=O	3',4' —OH
Rutin (flavonol)	—O-Gluc-Rhamnose	=O	3',4' —OH
Chrysin (flavone)	—H	=O	
Hesperetin (flavanone)	—H,H	=O	3' —OH,4' —OCH_3
(+)-Catechin (flavan-3-ol)	—H,OH	—H,H	3',4' —OH

The animals were killed at the end of the 4-wk treatment period. Rutin (the most common glycoside of quercetin in food plants) was substituted for chrysin in this second experiment since it was easier to purchase the large quantities of this compound needed for the feeding experiment.

Isolation of microsomes. Animals were killed by decapitation after 18 hr fasting. The livers were immediately excised, weighed, cut into small pieces and homogenized in three volumes 0·25 M-sucrose. The homogenate was centrifuged at 10,000 g for 20 min. An aliquot of the supernatant was centrifuged at 105,000 g for 90 min. The resulting pellet was suspended in 0·15 M-Tris-HCl pH 8·0, and centrifuged again at 105,000 g. The washed microsomal pellet that resulted was resuspended in 0·25 M-sucrose containing 0·05 M-Tris.HCl, pH 7·5, to give a concentration of approximately 10 mg microsomal protein per ml and was stored at −70° C. All operations were carried out at 4°C.

Enzyme assays. The protein contents of the microsomes were measured by the method of Lowry, Rosebrough, Farr & Randall (1951). Cytochromes *P*-450 and b_5 were determined according to Omura & Sato (1964). Aminopyrine demethylase activity was assayed by measuring aminoantipyrine formation according to Gilbert & Golberg (1965) and aniline hydroxylase activity by the production of *p*-aminophenol (Imai, Ito & Sato, 1966). The reaction system for these two enzyme assays comprised 83·5 mM-phosphate buffer, pH 7·4, 6·66 mM-$MgCl_2$, 0·5 ml microsomal suspension, an NADPH-generating system (2·5 mM-glucose-6-phosphate, 1 mM-NADP and 0·5 I.U. glucose-6-phosphate dehydrogenase), and 5 mM-aminopyrine or 10 mM-aniline, the final volume being 5 ml. The reaction was carried out at 37°C and stopped after 20 min by addition of trichloracetic acid. NADPH-cytochrome *c* reductase and NADH-cytochrome *c* reductase were assayed at 20°C, according to Phillips & Langdon (1962).

Statistical analysis. The results were evaluated by analysis of variance and Dunnett's test for multiple comparisons of means (Dagnélie, 1975).

RESULTS

Intraperitoneal injections

When quercetin, chrysin and hesperetin were injected ip, the body weights of the rats at the end of the experiment were slightly lower than those of control rats (Table 2). However relative liver weights and microsomal protein concentrations were not affected. Neither cytochrome *P*-450 and cytochrome b_5 levels nor the activities of the two associated flavoproteins, NADPH-cytochrome *c* reductase and NADH-cytochrome *c* reductase were changed. Aminopyrine demethylase activity was stimulated by quercetin (63%) and chrysin (59%) and unchanged by (+)-catechin and hesperetin. Aniline hydroxylase activity was not modified by any treatment.

Incorporation into the diet

When quercetin, rutin, hesperetin and (+)-catechin were ingested for 4 wk, body weights and relative liver weights were unaffected (Table 3). Rutin and (+)-catechin increased microsomal cytochrome b_5 content (11%). NADPH-cytochrome *c* reductase activity was not modified while NADH-cytochrome *c* reductase was inhibited by quercetin, rutin and hesperetin, the relative decreases being 21, 55 and 34% respectively. Aminopyrine demethylase activity was stimulated by quercetin (55%) and (+)-catechin (39%). Aniline hydroxylase was not affected by any of the flavonoids.

DISCUSSION

The present study shows that some naturally occurring flavonoids affect the activity of the monooxygenase system of rat-liver microsomes and some of its components when they are administered ip and/or ingested. Wattenberg *et al.* (1968) showed that flavonoids with free hydroxyl groups were not inducers of benzo[*a*]pyrene hydroxylase possibly because of their high polarity and their rapid conjugation and excretion. Our results indicate that quercetin, chrysin and (+)-catechin which contain free hydroxyl groups can stimulate the activity of aminopyrine demethylase but not that of aniline hydroxylase. Hesperetin which also has free hydroxyl groups has no effect on these two activities. It seems that the presence of free hydroxyl groups on the flavane nucleus, does not necessarily prevent these compounds from inducing some monooxygenases. Neither can we establish a relationship between the level of oxidation of the flavane nucleus and the effects of flavonoids on mixed-function oxidase activity. For example, both quercetin, the most highly oxidized of these molecules and (+)-cate-

Table 2. *The effects of flavonoids administered ip on body weights, relative liver weights and the mixed-function oxidase system in hepatic microsomes in rats*

Treatment	Control	Quercetin	Chrysin	Hesperetin	(+)-Catechin	SEM†
Body weights (g)	231	212*	208**	213*	232	4.2
Relative liver weights (g/100 g body weight)	3·9	3·8	3·7	3·8	3·9	0·07
Microsomal protein (mg/g liver)	19·5	16·4	17·1	19·2	17·0	0·89
Cytochrome *P*-450 (nmol/mg protein)	0·86	0·82	0.91	0·82	0·86	0·037
Cytochrome b_5 (nmol/mg protein)	0·246	0·236	0·237	0·222	0·252	0·011
NADPH-cytochrome *c* reductase (nmol cyt *c*/min/mg protein)	149	131	121	135	135	10·8
NADH-cytochrome *c* reductase (nmol cyt *c*/min/mg protein)	732	625	911	710	669	72·8
Aminopyrine demethylase (nmol AA/min/mg protein)	0·51	0·83**	0·81**	0·63	0·50	0·065
Aniline hydroxylase (nmol *p*-AP/min/mg protein)	0·65	0·55	0·66	0·62	0·59	0·036

AA = Aminoantipyrine *p*-AP = *p*-Aminophenol cyt *c* = Cytochrome *c* reduced

†Pooled SEM = $\sqrt{(s_E^2/n)}$ where s_E^2 is the error mean square, n is the sample size = 8.

Values are means for 8 rats: those marked with asterisks differ significantly (Dunnett's test) from the corresponding control value (*$P \leqslant 0{\cdot}05$; **$P \leqslant 0{\cdot}01$).

chin, the least oxidized molecule, stimulate aminopyrine demethylase.

The two different routes of administration produce quite different results. For instance aminopyrine demethylase and cytochrome b_5 are stimulated by (+)-catechin and NADH-cytochrome *c* reductase is inhibited by quercetin and hesperetin, only when these compounds are ingested. There are two possible explanations. First the metabolic fate of these compounds is probably quite different according to whether they are administered orally or ip. When flavonoids are injected ip they reach the liver unchanged. However, after ingestion they are first degraded by the intestinal microflora and are absorbed intact only to a limited extent (Booth, Jones & DeEds, 1958; Booth, Murray, Jones & DeEds, 1956; Das, 1969). Thus the stimulatory effect of ingested (+)-catechin on aminopyrine demethylase and cytochrome b_5, and the inhibitory effects of ingested quercetin and hesperetin on NADH-cytochrome *c* reductase, may be at least partly attributable to the metabolites of these flavonoids and not to the compounds themselves. Secondly, the inducing effect of (+)-catechin on aminopyrine demethylase is dose- and time-dependent and long-term exposure is required before a significant effect is observed. When the compound was injected, the rats received a dose of 150 mg/kg/day on three consecutive days and when it was

Table 3. *The effects of feeding flavonoids on body weights, relative liver weights and the mixed-function oxidase system in hepatic microsomes in rats*

Treatment	Control	Quercetin	Rutin	Hesperetin	(+)-Catechin	SEM†
Body weights (g)	208	213	215	214	213	4·6
Relative liver weights (g/100 g body weight)	3·9	4·0	4·0	4·0	3·9	0·07
Microsomal protein (mg/g liver)	22·8	20·0	20·6	20·2	22·8	0·81
Cytochrome *P*-450 (nmol/mg protein)	0·74	0·78	0·85	0·80	0·79	0·027
Cytochrome b_5 (nmol/mg protein)	0·211	0·231	0·235*	0·230	0·234*	0·006
NADPH-cytochrome *c* reductase (nmol cyt *c*/min/mg protein)	114	123	126	128	122	5·5
NADH-cytochrome *c* reductase (nmol cyt *c*/min/mg protein)	584	459 **	263 **	386 **	519	26·5
Aminopyrine demethylase (nmol AA/min/mg protein)	0·31	0·48**	0·38	0·41	0·43*	0·031
Aniline hydroxylase (nmol *p*-AP/min/mg protein)	0·42	0·45	0·43	0·42	0·42	0·024

AA = Aminoantipyrine *p*-AP = *p*-Aminophenol cyt *c* = Cytochrome *c* reduced

†Pooled SEM = $\sqrt{(s_E^2/n)}$ where s_E^2 = error mean square, n = sample size = 8.

Values are means for eight rats: those marked with asterisks differ significantly (Dunnett's test) from the control value (*$P \leqslant 0{\cdot}05$; **$P \leqslant 0{\cdot}01$).

ingested, they ate approximately 120 mg/kg/day for 4 wk resulting in very different cumulative doses. Most inducers (e.g. phenobarbital, methylcholanthrene) give maximal effects within 2–3 days. However, with some, the effects are very slow to appear. For instance, prolonged exposure to linalool was required before significant effects were observed (Parke, Rahman & Walker, 1974).

Our results indicate that when quercetin is ingested, NADH-cytochrome *c* reductase is inhibited while aminopyrine demethylase is stimulated. This result provides evidence that NADH-cytochrome *c* reductase is not rate-limiting in the overall scheme of oxidation *in vivo*. The role of this flavoprotein in regulating the monooxygenase system is not yet clear. De Barros, Kaplan, Duvaldestin & Berthelot (1978) have shown that phenobarbital increases the activity of NADH-cytochrome b_5 reductase in rat-liver microsomes. They postulated that such stimulation may contribute to the enhancement of drug oxidations in microsomes. It has been known for many years that NADH has a synergistic effect on cytochrome *P*-450-dependent reactions *in vitro* (Cohen & Estabrook, 1971; Correia & Mannering, 1973). Recently, Imai (1981) has shown that electron flow from NADH via NADH-cytochrome b_5 reductase and cytochrome b_5 can be used efficiently as a source of the second electron for specific reactions catalysed by reconstituted systems containing purified forms of cytochrome *P*-450. In contrast, in other reconstituted systems, NADH-cytochrome b_5 and cytochrome b_5 were not absolutely essential for functional activity (Lu, West, Vore *et al.* 1974).

Cytochrome b_5 and NADH-cytochrome *c* reductase are involved in other aspects of metabolism such as fatty acid desaturation (Oshino, Imai & Sato, 1971). Thus the increase of cytochrome b_5 after feeding rutin and (+)-catechin and the decrease of NADH-cytochrome *c* reductase after feeding quercetin, rutin and hesperetin could affect such pathways. It would be interesting to test this hypothesis.

Further studies are required to evaluate the effects of natural flavonoids or their metabolites on the activities of other monooxygenases in order to determine more precisely the mechanisms by which they affect the drug-metabolizing enzyme system in the rat.

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INACTIVATION OF AFLATOXIN B_1 MUTAGENICITY BY THIOLS*

M. FRIEDMAN, C. M. WEHR, J. E. SCHADE and J. T. MACGREGOR

Western Regional Research Center, Agricultural Research Service, US Department of Agriculture, Berkeley, CA 94710, USA

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Abstract—The mutagenicity of aflatoxin B_1 to *Salmonella typhimurium* strain TA98 decreased rapidly upon exposure of aflatoxin B_1 to various thiols in aqueous solution. Mutagenic activity was reduced to control values within 24 hr with *N*-acetyl-L-cysteine (NAC), *N*-2-mercaptopropionylglycine (MPG), mercaptoethanol, reduced glutathione or mercaptopropionic acid at pH values near 4. Mercaptoacetic acid, mercaptosuccinic acid, cysteine, acetyl-D,L-homocysteine thiolactone, cysteine methyl ester, D-penicillamine and β-mercaptoethylamine were less effective. Relatively high thiol concentrations ($\geqslant$ 0·25 M) were required to achieve complete inactivation within 24 hr with the thiols tested. The inactivation rate was strongly dependent on thiol concentration and pH, but was relatively independent of the aflatoxin concentration under the conditions examined. With MPG and NAC reaction rates were much slower at neutral pH values than at pH's between 3 and 4. HPLC and thin-layer chromatographic examination of aflatoxin B_1 solutions partially inactivated with NAC revealed the formation of a new product at a rate that correlated with the disappearance of aflatoxin B_1 and the loss of mutagenic activity. This reaction product has not yet been identified, but the evidence suggests that it is the product of an addition of the thiol at the difuran region of the aflatoxin.

INTRODUCTION

Reduction of the amounts of carcinogenic natural products present in human diets may be important for preventing cancer (Doll & Peto, 1981; Salunkhe, Wu, Do & Maas, 1980). Since carcinogenic aflatoxins and related mycotoxins sometimes occur in foods and feeds, practical methods for their inactivation might reduce exposure or help to eliminate economic losses. Since aflatoxins are transformed *in vivo* to an active 2,3-epoxide (Swenson, Lin, Miller & Miller, 1977; *cf.* however, Coles, Smith & Garner, 1979; Garner & Wright, 1973), prior treatment with a site-specific reagent to prevent formation of this genotoxic derivative might suppress the mutagenic and carcinogenic activity of these mycotoxins. Because thiols are potent nucleophiles (Friedman, 1973), they may react with the electrophilic sites of aflatoxin and thus prevent the activation of this site to the epoxy derivative. We present evidence that exposure of aflatoxin B_1 to various thiols *in vitro* prevents activation to form(s) mutagenic in the Ames Salmonella/mammalian microsome assay, probably by forming a thiol adduct at the furan ring.

Abbreviations: AFB_1 = Aflatoxin B_1; DMSO = dimethyl sulphoxide; GSH = reduced glutathione; HPLC = high-performance liquid chromatography; MPG = *N*-2-mercaptopropionylglycine; NAC = *N*-acetyl-L-cysteine; TLC = thin-layer chromatography.

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†Reference to a company and/or product named by the Department is only for the purposes of information and does not imply approval or recommendation of the product to the exclusion of others that may also be suitable.

EXPERIMENTAL

Chemicals

Aflatoxin B_1 (AFB_1), *N*-acetyl-L-cysteine (NAC), *N*-2-mercaptopropionylglycine (MPG), reduced glutathione (GSH), mercaptopropionic acid, cysteine, cysteine methyl ester, β-mercaptoethylamine, mercaptosuccinic acid, and acetyl-D,L-homocysteine thiolactone were obtained from Sigma Chemical Co. (St. Louis, MO)†. D-Penicillamine, mercaptoethanol and mercaptoacetic acid were from Aldrich Chemical Co. (Milwaukee, WI). The other reagents were of the best commercial grades available.

Mutagenicity assay

Salmonella typhimurium strain TA98 has been described elsewhere (Ames, McCann & Yamasaki, 1975). Procedures for growth, storage, and verification of the presence of the R-factor plasmid and *rfa* character were carried out according to Ames *et al.* (1975). The rat-liver homogenate fraction (S-9) metabolizing system, prepared as described by Ames *et al.* (1975), was used throughout the experiments; liver S-9 was prepared from Aroclor 1254-induced male Sprague–Dawley-derived rats (Simonsen Laboratory, Gilroy, CA) and was used at a concentration of 0·1 ml S-9 per ml of S-9 mix.

Duplicate reaction mixtures were prepared under sterile conditions by mixing equal volumes of buffer and thiol solutions made up to twice the desired final concentration. Nitrogen was bubbled through the mixture for 1 min to reduce the oxygen content. The pH was measured, and the designated level of AFB_1 in dimethylformamide, or an equal volume of di-

methylformamide (no more than 5% of the reaction mixture), was then added to each aluminium-foil-wrapped vial. Nitrogen was passed gently over the mixture prior to the final capping and vortexing, and the mixtures were incubated at room temperature (about 24 C), or, in some experiments, at a controlled temperature of 24–25 C for the designated times. Aliquots were then diluted in dimethyl sulphoxide (DMSO) for plating at 0·25 μg AFB_1/plate in the Ames Salmonella assay. Duplicate dilution tubes with 95% ethanol were used in some experiments to ensure that DMSO did not enter into the reaction. Aliquots of 0·1 ml of the diluted reaction mixture were incorporated into top agar, and plate assays were made in duplicate with tester strain TA98. A reference standard of buffer plus AFB_1 without thiol was incubated, sampled, diluted and plated in the same manner as other reaction vials, as was a negative control vial of buffer plus thiol without aflatoxin. To confirm the activity of the tester strain, 0·1 ml DMSO or 0·1 ml AFB_1 solution (0·25 μg AFB_1) in DMSO was added to control plates; this dose produced 1500–2200 revertants/plate. The mean revertant frequencies of the buffer/thiol controls (values given in tables) in no case differed significantly from those of historic or concurrent DMSO-treated TA98 controls used in this laboratory. The activity of AFB_1 in the buffers tested did not change significantly (for up to 71 days) in the absence of thiol.

Thin-layer chromatography (TLC)

The reaction mixtures were spotted onto Whatman HP-K TLC plates, then developed with chloroform–acetone (90 : 10, v/v). The dried spots were viewed under fluorescent light.

High-performance liquid chromatography (HPLC)

Aflatoxin standard. Approximately 1 mg aflatoxin B_1 (Applied Science Laboratories Inc., State College, PA) was dissolved in 25 ml methanol (HPLC grade, Fisher Scientific Co., Fairlawn, NJ). The exact concentration of aflatoxin was determined from UV absorbance of a 1 : 5 methanol dilution of this solution. The absorbance at 360 nm was measured with a Cary 14 spectrophotometer and the concentration calculated from the reported extinction coefficient at A_{360} = 21,800 (Association of Official Analytical Chemists, 1980).

Reaction rate of AFB_1 with N-acetyl-L-cysteine. NAC (815 mg) was dissolved in 2 M-acetate buffer (pH 5·0) in a 25-ml volumetric flask to give a 0·2 M-solution when diluted to volume. The buffer was flushed with N_2 before and after the addition of NAC. AFB_1 (73 μg in 1·5 ml methanol) was added to about 23 ml NAC-buffer solution, and the volume was adjusted to 25 ml with buffer to give a final concentration of 9·36 μM-AFB_1. The aluminium-foil-wrapped flask was stored at room temperature (*c.* 24°C). Aliquots (4 ml) were withdrawn after 1, 2, 3, 6 and 24 hr. Each aliquot was acidified to pH 3 by adding 1 ml 15 N-H_3PO_4 before it was extracted three times with methylene chloride (CH_2Cl_2). The first extraction was made by vigorously shaking the aliquot of AFB_1 reaction mixture with 10 ml CH_2Cl_2 for 1 min. The extraction was repeated twice with 5-ml portions of CH_2Cl_2, and the combined extracts were then dried with about 3 g granular Na_2SO_4. The Na_2SO_4 was rinsed twice with 2-ml portions of CH_2Cl_2. The combined extracts were concentrated on a steam bath to about 2 ml, then evaporated to dryness under nitrogen. This extraction procedure gives a 95% recovery

Table 1. *Inactivation of aflatoxin B_1 mutagenicity by various thiols*

Compound (0·5 M)	pK*	Mutagenic activity of AFB_1 (% of original)†	pH
N-Acetyl-L-cysteine (NAC)	9·5	0	4·0
Mercaptopropionic acid‡	10·4	<0·2	3·4
Mercaptoethanol‡	9·6	<0·3	4·5
Reduced glutathione	8·8	<0·6	3·9
N-2-Mercaptopropionylglycine	10·4	<2·6	3·8
Mercaptoacetic acid‡	10·7	7	3·2
Mercaptosuccinic acid	10·9	70	3·6
L-Cysteine	8·3	79	4·2
Acetyl-D,L-homocysteine thiolactone		85	4·2
Cysteine methyl ester	6·6	85	4·0
D-Penicillamine	7·9	94	4·3
β-Mercaptoethylamine (cysteamine)	8·3	100	4·0

AFB_1 = Aflatoxin B_1

*As reported by Lange (1972), Friedman (1973) and Pitman & Morris (1979).

†Each assay was carried out in duplicate. The value for each assay was calculated from an AFB_1/buffer reference standard, and the negative control (thiol plus buffer without AFB_1) value for each thiol was used to correct for spontaneous revertants; 0·25 μg AFB_1 plate produced approximately 1500–2200 revertants plate. The mean value for the buffer/thiol negative controls was 50 ± 12 (± 1 SD, n = 13).

‡The reaction mixture had a concentration of 580 μM-AFB_1 [200 μl of a solution of 2 mg AFB_1/ml in dimethylformamide–ethanol (40:60, v/v) in a reaction mixture of 2·2 ml] and was incubated at room temperature in 0·5 M-Tris acetate buffer for 24 hr. All other reaction mixtures had a concentration of 530 μM AFB_1 (200 μl of the same AFB_1 solution in a total reaction mixture of 2·4 ml) and were incubated under the same conditions.

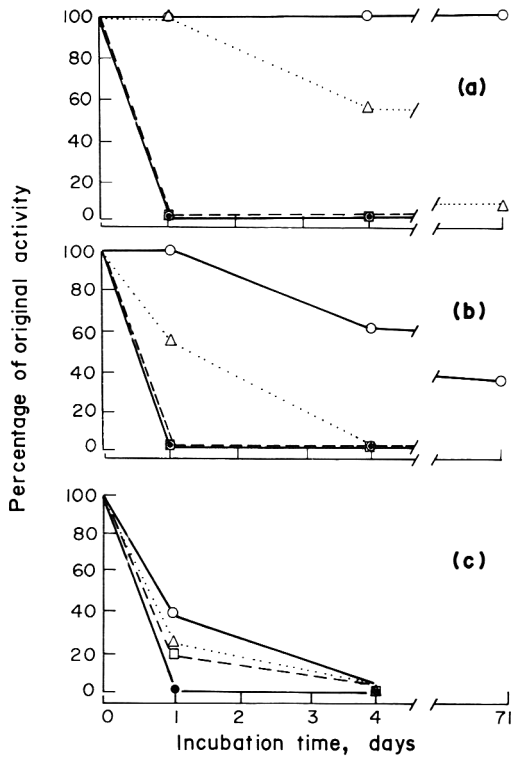

Fig. 1. Effect of (a) reduced glutathione, (b) *N*-acetyl-L-cysteine and (c) *N*-2-mercaptopropionylglycine at concentrations of 0·06 (○), 0·125 (△), 0·25 (□) or 0·5 (●) M on time of inactivation of mutagenic activity of aflatoxin B_1(AFB_1). AFB_1 at a concentration of 580 μM [100 μl of a solution of 2 mg AFB_1 ml in dimethylformamide–ethanol (40:60, v/v) in 1·1 ml reaction mixture] was incubated at room temperature in 0·5 M-Tris acetate buffer at pH 4–4·5 for the designated times. Each assay was run in duplicate. The percentage of original activity was calculated from an AFB_1 buffer reference standard and the negative control value (thiol plus buffer without AFB_1) for each thiol was used to correct for spontaneous revertants: 0·25 μg AFB_1 plate produced approximately 1500–2200 revertants plate. The mean value for the buffer/thiol negative controls was 37 ± 5 (± 1 SD, n = 20).

of AFB_1 or the NAC–AFB_1 reaction product, assuming that both compounds have the same molar extinction coefficient at 360 nm, as appears to be the case (see below).

HPLC analysis. Residues were dissolved in 2 ml of mobile phase, then filtered through a 0·45 μm millipore filter. Analysis was by a reverse-phase HPLC procedure using a 25 cm × 4·6 mm ID column of 5 μm Spherisorb ODS (Applied Science Laboratories Inc.). The HPLC system consisted of a Beckman Model 110A pump (Beckman Instruments, Berkeley, CA), a guard column packed with 37–50 μm Bondapak C_{18}/Corasil (Waters Associates, Inc., Milford, MA), an Isco Monitor Model 1840 absorbance detector (Instrument Specialties Co., Lincoln, ME), and a Model 3390A recording integrator (Hewlett-Packard, Avondale, PA). The mobile phase was water–acetonitrile–methanol (40 : 13 : 47, by vol.); the flow rate was 1 ml/min. The absorbance detector was set at 360 nm; 20 μl of the reaction mixture extract was injected into the column.

RESULTS AND DISCUSSION

The mutagenic activity of AFB_1 (5×10^{-4} M) decreased rapidly at pH 3·2–4·5 in the presence of NAC, mercaptopropionic acid, mercaptoethanol, GSH or MPG (all present at 0·5 M), reaching background values within 24 hr (Table 1). The seven other thiols tested were less effective under similar conditions. At pH 4·5, the rate of inactivation by GSH and NAC was markedly influenced by thiol concentration (Fig. 1). GSH, NAC and MPG at 0·5 M reduced AFB_1 mutagenicity to less than 1% of its original value after 24 hr. In contrast, more than 50% activity remained after 4 days with 0·06 M-GSH or -NAC. MPG was generally more effective than the other thiols at the lower concentrations (Fig. 1). The inactivation rate was apparently less dependent on the aflatoxin level. Either 640 or 64 μM-AFB_1 was inactivated at approximately the same rate by each NAC concentration from 0·06 to 0·5 M (Table 2).

The reaction of MPG with AFB_1 was markedly pH-dependent (Table 3). Within the pH range 3–7, inactivation was most rapid at lower pH values. With 0·1 M-MPG, for example, only 9% of the original activity remained after 24 hr at pH 3·7, whereas 53% remained after 18 days at pH 7·1. Inactivation by other thiols was also pH-dependent: in 1 M-acetate buffer at pH 3·2, 0·5 M-NAC inactivated AFB_1 to 6% of original activity within 1 hr; at pH 5·2, 42% remained after 4 hr (data not shown). Reaction rates were similar in acetate, succinate or phosphate buffers (Table 3), but inactivation was very slow in citrate buffer (data not shown). Citric acid has also been shown to retard AFB_1 degradation by bisulphite (Doyle & Marth, 1978).

Table 2. *Effect of N-acetyl-L-cysteine concentration on the inactivation of aflatoxin B_1**

NAC concn (M)	Time (hr)...	Mutagenic activity of AFB_1 (% of original)† at AFB_1 concn of 640 μM 4	640 μM 24	64 μM 4	64 μM 24
0·5		20	>0·1	64	>0·2
0·25		91	60	92	45
0·125		89	84	100	94
0·06		100	100	100	100

NAC = *N*-Acetyl-L-cysteine AFB_1 = Aflatoxin B_1

*Incubation of aflatoxin B_1 and *N*-acetyl-L-cysteine was carried out at 25 C in 1 M-acetate buffer, pH 4·4·5; 100 or 10 μl of a solution of 4 mg AFB_1/ml dimethylformamide was added to make a reaction mixture of total volume 2 ml. Reaction vials were bubbled with nitrogen prior to incubation.

†Each assay was carried out in duplicate. The value for each assay was calculated from an AFB_1/buffer reference standard and the negative control (thiol plus buffer without AFB_1) value for each thiol was used to correct for spontaneous revertants; 0·25 μg AFB_1/plate produced approximately 1500–2200 revertants/plate. The mean value for the buffer thiol negative controls was 78 ± 7 (± 1 SD, n = 8).

Table 3. *Effect of pH and time on the inactivation of aflatoxin B_1 by 0·1 M-N-2-mercaptopropionylglycine*

Buffer*	pH	Incubation time (days)...	Mutagenic activity of AFB_1 (% of original)† 1	7	14	18	37
0·5 M-acetate	2·8		11	—	—		—
	3·7		9	—	—		—
	4·7		33	1	—	—	—
	5·1		74	1	—	—	—
0·5 M-succinate	4·3		9	—	0		—
	5·0		32	—	0	—	—
	5·6		85	—	0	—	
	6·0		100	—	9	—	—
0·5 M-phosphate	5·8		89	—	—	2	0
	6·5		85	—	—	41	15
	7·1		97	—	—	53	26

AFB_1 = Aflatoxin B_1 — = Not determined

*Aflatoxin B_1 at a concentration of 128 μM (20 μl of a solution of 4 mg AFB_1/ml dimethylformamide in a 2-ml reaction mixture) was incubated in the appropriate buffer containing *N*-2-mercaptopropionylglycine (0·1 M) at 25°C after bubbling nitrogen through the reaction mixture.

†Each assay was carried out in duplicate. The value for each assay was calculated from its own zero-time value (mixed but plated prior to incubation) and the negative control value (thiol plus buffer without AFB_1) was used to correct for spontaneous revertants. Zero-time samples averaged 1548 revertants/plate; an equivalent amount (0·25 μg) of AFB_1 plated directly produced approximately 1500–2200 revertants/plate. The mean values for the buffer/thiol negative controls was 58 ± 11 (± 1 SD, n = 45).

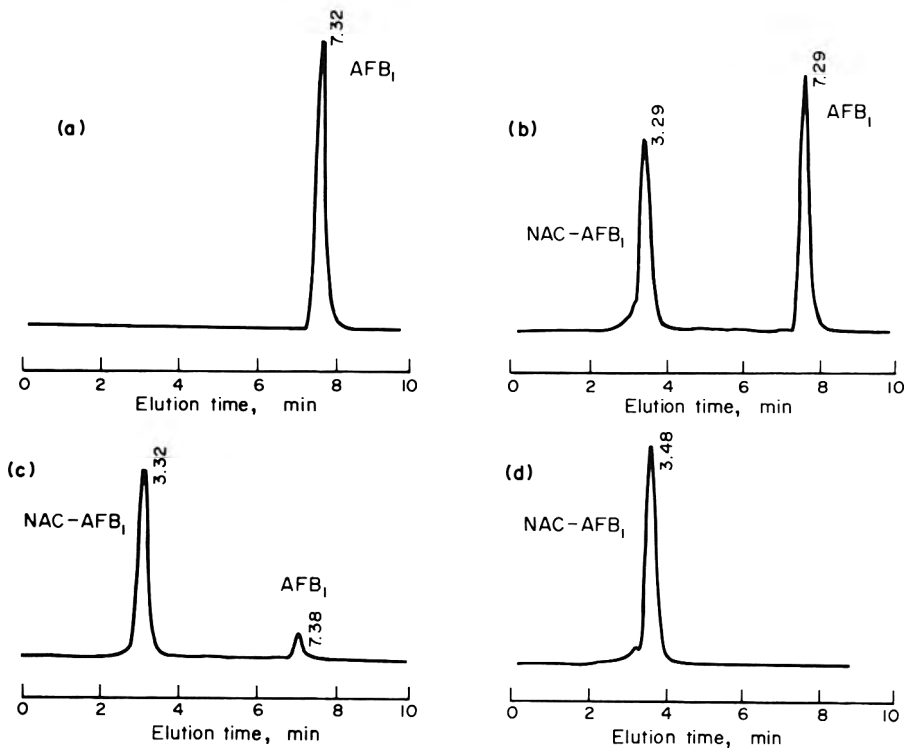

Fig. 2. Reverse-phase high-performance liquid chromatogram of aflatoxin B_1 (AFB_1) and its *N*-acetylcysteine derivative (NAC-AFB_1) after (a) 0, (b) 1, (c) 3 and (d) 24 hr reaction of AFB_1 with *N*-acetyl-L-cysteine. See Experimental section for conditions. Actual elution times in minutes are shown near each peak. Attenuation was as follows: (a), 5, (b) 4, (c) 5 and (d) 5.

HPLC studies showed that the disappearance of AFB_1 after incubation with NAC was accompanied by the appearance of a single new peak on the chromatogram (Fig. 2). Assuming that the 360-nm extinction coefficient of this product is not markedly changed from that of AFB_1 (see below), the integrated UV absorbance of this peak indicated that AFB_1 was converted nearly quantitatively to this single derivative (Fig. 3). TLC examination of partially NAC-inactivated AFB solution also showed a new fluorescent spot, which remained near the origin. The fluorescence intensity of this spot relative to that of AFB_1 correlated with the loss of mutagenicity measured by the Ames test.

Earlier work (Rosin & Stich, 1978) demonstrating the inhibitory effect of cysteine on the mutagenicity of several carcinogens has focused on the reaction of the activated AFB_1 derivative with the nucleophilic thiol. Our work demonstrates that thiols are capable of reacting directly with AFB_1, so that the reaction products are no longer capable of activation by the *in vitro* S-9 metabolizing system used.

Data in Table 1 indicate that the influences of polar and steric factors associated with the structurally different thiols would be consistent with the observed extent of inactivation of AFB_1. For instance, the SH group of NAC has a higher pK than the corresponding group in cysteine (9·5 *vs.* 8·3). The ionized SH group of NAC is therefore a better nucleophile than the corresponding group in cysteine at any pH; it is also more stable to oxidation (Snow, Finley & Friedman, 1975). These considerations suggest that NAC should be a more potent inactivator than cysteine, as was observed. However, acid-catalysed attack of the free thiol, RSH, is also a likely possibility (Gould, 1959; Pons, Cucullu, Lee *et al.* 1972). Steric factors associated with the sulphydryl compounds may also influence their effectiveness. For example, the decreased effectiveness in inactivating AFB_1 of penicillamine compared with cysteine might be due to steric hindrance by the two methyl groups of the former during the formation of the transition state (Friedman, 1977). Similarly, destabilization of the transition state by charge repulsion among the two carboxyl groups of mercaptosuccinic acid is a likely cause for its lower effectiveness compared with the monocarboxylic mercaptoacetic and mercaptopropionic acid analogues.

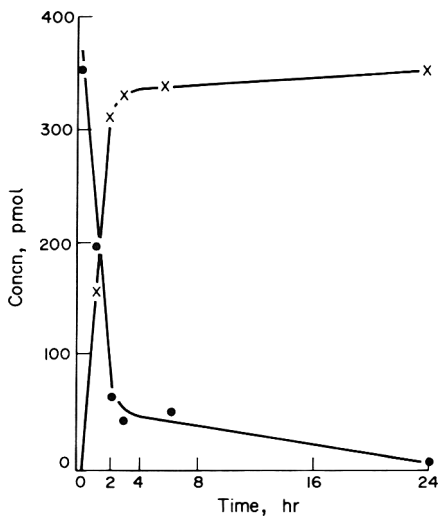

Fig. 3. Plot of the disappearance of aflatoxin B_1 (●) and the concurrent appearance of its *N*-acetyl-cysteine derivative (×) after up to 24 hr of reaction of aflatoxin B_1 with *N*-acetyl-L-cysteine (from integrated areas of a high-performance liquid chromatogram). See Experimental section for details.

Although the NAC–AFB_1 reaction product has not yet been isolated and characterized, a likely mechanism of inactivation is reaction of the nucleophilic thiol with the electrophilic double bond of the furan ring of AFB_1, by analogy with the observed hydration of the double bond to form aflatoxin B_{2a} (Heathcote & Hibbert, 1978; Pons *et al.* 1972). Aflatoxin B_{2a}, which eluted at about 5·5 min on our HPLC column compared with 3·3 min elution time for the NAC–AFB_1 reaction product, was sometimes observed in trace amounts. Cleavage of the lactone ring would lead to a major change in the 360-nm UV absorption maximum of AFB_1 (Beckwith, Vesonder & Ciegler, 1976). We found the 360-nm absorbance of AFB_1 and of NAC-inactivated AFB_1 to be similar, although the ratio of the two absorption maxima $A_{360nm}:A_{265nm}$ is almost reversed from 1·75 for AFB_1 (Association of Official Analytical Chemists, 1980) to the observed value of 0·64 for the inactivated derivative. Cleavage of the lactone ring is therefore unlikely, since the expected change in the UV spectrum was not observed. However, acid-catalysed interaction between a thiol and the acetal carbon atom (O–C–O) of the difuran ring of AFB_1 cannot be ruled out. Another pathway, involving nucleophilic addition of SH groups to the double bond of the lactone ring, though possible, would probably not be favoured energetically because conjugation would be disrupted. Additional studies are needed to elucidate the structure(s) of the thiol-aflatoxin derivative.

In summary, our results demonstrate the ability of thiols to inactive the mutagenic, and possibly the carcinogenic, potential of aflatoxins. Whether thiols in fact prove to be of practical use for inactivating aflatoxins and related compounds in contaminated foods, or whether they may inactivate aflatoxins *in vivo*, and hence be of value in treating aflatoxin-exposed individuals, awaits further study.

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THE EFFECTS OF PATULIN AND PATULIN–CYSTEINE MIXTURES ON DNA SYNTHESIS AND THE FREQUENCY OF SISTER-CHROMATID EXCHANGES IN HUMAN LYMPHOCYTES

R. COORAY*, K.-H. KIESSLING** and K. LINDAHL-KIESSLING*

*Institute of Zoophysiology, University of Uppsala, P.O. Box 560, S-751 22, Uppsala

and

**Department of Animal Nutrition, The Swedish University of Agricultural Sciences, P.O. Box 7046, S-75007, Uppsala, Sweden

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Abstract—The effects of patulin and patulin–cysteine mixtures on DNA synthesis in human blood lymphocytes were measured by assaying incorporation of [^{3}H]thymidine into cellular DNA. Patulin inhibited and patulin–cysteine mixtures stimulated the incorporation of [^{3}H]thymidine into DNA. The inhibitory action of patulin diminished with time in culture. Patulin was found to be unstable in the culture medium. The sister-chromatid exchange (SCE) frequency was significantly elevated by intermediate concentrations (0·1–0·2 μg/ml culture) of the toxin. The cells are protected from the effect at low concentrations of the toxin. There may be excessive damage at higher concentrations but only the unaffected cells go into mitosis. Therefore an increased frequency of SCEs is detected only at intermediate concentrations, or, at higher concentrations, with early harvesting. Cysteine seems to potentiate the effect of patulin on SCE frequency.

INTRODUCTION

The capacity of various fungi to produce toxic metabolites is well established, but the role that many of these toxic compounds may play in disease processes is not well understood. One fungal metabolite that has an uncertain involvement in mycotoxicoses is patulin (4-hydroxy-4*H*-furo[3,2-*c*]pyran-2(6*H*)-one). Patulin is an antibiotic furopyrone, a neutral substance which is soluble in water and most organic solvents. It is unstable in alkali but stable in acid. Its chemical structure is given in Fig. 1 (Woodward & Singh, 1949). Patulin is produced mainly by Penicillium and Aspergillus species. It is a common contaminant of fruit, especially apples and berries and products derived from them (Åkerstrand, Molander, Anderson & Nilsson, 1976; Frank, Orth & Figge, 1977; Harwig, Chen, Kennedy & Scott, 1973a; Harwig, Scott, Kennedy & Chen, 1973b). Unfermented apple juice is the most frequent source of patulin (Josefsson & Andersson, 1976; Scott, Miles, Toft & Dubé, 1972). The toxin is stable in dry corn, apple and grape juice but not in wet corn in the presence of SO_2 (Pohland & Allen, 1973; Scott & Kennedy, 1973).

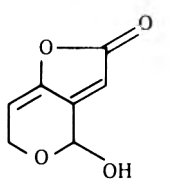

Fig. 1. Chemical structure of patulin.

Abbreviations: BudR = Bromodeoxyuridine; SCE = sister-chromatid exchange.

Patulin is toxic to most bacteria, fungi and protozoa. In tissue-culture studies it has been found to be toxic to various types of mammalian cells (for a review see Singh, 1967). In addition to acute toxic effects it is reported to have mutagenic, carcinogenic and teratogenic properties (Dickens & Jones, 1961 & 1965; Reddy, Chan & Hayes, 1978). On the cellular level patulin appears to inhibit aerobic respiration (Singh, 1967), affect some aspects of membrane permeability (Kahn, 1957), inhibit ATP-ase enzyme activity and concomitantly reduce the rate of electrogenic Na^+ transport across epithelial membranes (Phillips & Hayes, 1979).

Patulin has been considered an important mycotoxin with respect to health hazards in northern countries (Åkerstrand *et al.* 1976; Frank *et al.* 1977; Harwig *et al.* 1973a,b; Josefsson & Andersson, 1976). Therefore the mutagenic and carcinogenic properties of patulin itself as well as its reaction products with sulphydryl compounds should be evaluated more closely. We have studied the effects of patulin and patulin–cysteine mixtures on DNA synthesis and the frequency of sister-chromatid exchanges (SCEs) in cultured human peripheral blood lymphocytes.

EXPERIMENTAL

Peripheral blood lymphocytes were obtained from venous blood of healthy donors. The blood was defibrinated by shaking with glass beads. The defibrinated blood was diluted with sterile saline (1:1, v/v). Lymphoprep (Nyegard, Oslo, Norway) was added to the diluted blood, and centrifuged at 500 **g** for 35 min at room temperature. The intermediate zones between

plasma and erythrocytes were removed, washed twice in tissue culture medium RPMI 1640 (Flow Lab., Irvine, Ayrshire, UK) supplemented with 50 μg gentamycin/ml (Shering Corp., New York, USA) and 0·01 M-Hepes buffer (Sigma Chemical Co., St Louis, MO, USA). Lymphocytes were also prepared from buffy coats (the top cell layer from centrifuged citrated whole blood) obtained from the Academic Hospital, Uppsala. The blood was transferred to plastic tubes containing glass beads, and diluted with sterile saline (1:1 v/v). A solution of $CaCl_2$ (1 mmol/buffy coat) was added to remove the citrate. Defibrination was done as described above. Defibrinated blood was then centrifuged at 400 g for 30 min on Ficoll-Paque (relative density 1·077; Pharmacia Fine Chemicals, Uppsala). The intermediate zone between the supernatant and the red cells was carefully removed and washed twice in culture medium. The lymphocytes thus obtained had a viability of about 85–95% as tested by trypan blue staining.

Preparation of cultures. For the assay of DNA synthesis as indicated by the incorporation of [^{3}H]thymidine triplicate 1-ml cultures were set up in 16 × 100 mm T/A culture tubes (Corning, Medical and Scientific Co., Medfield, MA, USA). These cultures had a cell concentration of 1×10^6 cells in RPMI 1640/ml, tissue-culture medium and concentrations of 2 mM L-glutamine (Flow Lab.) and 0·4% human albumin or 10% pooled inactivated human serum. The cells were stimulated at 0 hr with phytohaemagglutinin (PHA; Wellcome, Beckenham, Kent, UK) added at a concentration of 0·5 μg/ml. Patulin (crystalline patulin, produced by *Penicillium urtica*, packed under liquid nitrogen and obtained from Makor-Chemicals, Jerusalem, Israel) and patulin cysteine (L-cysteine–HCl, Sigma Chemical Co.) mixtures were added either at 0 or 24 hr. The [^{3}H]thymidine (100 Ci/mol; Amersham International plc, Amersham, Bucks, UK) was added at a concentration of 0·5 μCi/ml 12 hr before cell harvest. The cells were harvested in a semi-automatic cell harvester (Scatron, Lierbyen, Norway). The filters were counted in a liquid scintillator (Nuclear Chicago Corp., Des Plaines, IL, USA).

For the SCE analysis 4-ml cultures were set up in 10-ml styrene vials (Cerbo, Trollhättan, Sweden) in the same way as for the DNA-synthesis assay. 5-Bromo-2′-deoxyuridine (BudR; Sigma Chemical Co.) was added at a concentration of 15 μg/ml after 24 hr. Two hours before cell harvest 62·5 ng colcemid (Sigma Chemical Co.)/ml was added. The cultures were harvested after approximately 72 hr. The cells were hypotonically treated with 0·56% KCl solution and fixed in methanol–acetic acid (3:1, v/v). A concentrated suspension of the fixed cells was allowed to run over wet, alcohol-washed, glass slides. The slides were dried at room temperature.

When cells are exposed to BudR for two rounds of replication secondary metaphase chromosomes possess one chromatid unifilarly substituted with BudR and its sister chromatid bifilarly substituted. Such chromatids stain differentially with the fluorescence plus Giemsa technique of Wolff & Perry (1974). From each culture a total of 20 complete mitoses in the secondary metaphase stage was counted for SCE. Statistically significant differences between SCE frequencies were determined using the Wilcoxon rank test (Colquhoun, 1971).

Parallel cultures were set up for SCE and DNA assays. All incubations were done at 37°C in an incubator with a 5% CO_2-humidified air atmosphere.

Stability of patulin in tissue-culture medium. High-pressure liquid chromatography (HPLC) was used to determine patulin concentrations in the tissue-culture medium. The HPLC equipment and conditions were as follows: Waters' 6000-A (Waters' Associates Inc., Milford, MA, USA) liquid chromatograph pump, Waters' model U-G-K injector, Altex model 153 ultraviolet (280 nm) detector (Altex Corp, Berkeley, CA, USA) set to 0·01 A, 10-mV recorder, 25 cm Partisial-10 column (Reeve Angel, Whatman Inc., Clifton, NJ, USA). The mobile solvent was isooctane chloroform–methanol (80:15:5, by vol.) flow rate *c.* 15 ml/min.

Known amounts of patulin were added to 1-ml aliquots of RPMI-1640. Duplicate mixtures were set up at each concentration. Half of the samples were incubated at 37 C for 30 hr. The remaining samples were immediately treated as follows. Each was acidified to pH 3·1 by the addition of two drops of 10% H_3PO_4. The patulin was extracted with ethyl acetate. Four extractions were carried out on each sample. The extracts were combined, evaporated to dryness under a stream of nitrogen and dissolved in 10 μl chloroform. The same extraction procedure was used for the samples incubated for 24 hr. Using a standard patulin solution several samples of known concentrations were prepared. A 5-μl aliquot of each patulin standard solution was injected onto the HPLC column. By plotting peak height against patulin concentration a standard curve was obtained. A 5-μl sample of each test extract was injected under the same conditions and the peak heights were measured. The concentration of patulin in each test sample was determined directly from the standard curve (Stray, 1978; Ware, 1975; Ware, Thorpe & Pohland, 1973).

RESULTS AND DISCUSSION

Our experiments show that the DNA synthesis of cultured peripheral human blood lymphocytes is inhibited by patulin. The effect of two different concentrations of patulin is illustrated in Fig. 2. At a concentration of 0·05 μg/ml the toxin retarded the incorporation of [^{3}H]thymidine into cellular DNA at the initial stages of the culture, but as the time elapsed the cells resumed normal growth. Concentrations above 1 μg/ml inhibited DNA synthesis completely. In HeLa cells treated with 3·2 μg patulin/ml inhibition of DNA synthesis and depression of RNA and protein synthesis has been found (Kawasaki, Oki, Umeda & Saito, 1972). At a much higher concentration (200 μg/ml) the initiation step of the transcriptional process seemed to be the target of inhibition in a cell-free RNA synthesizing system (Moulé & Hatey, 1977).

The viability of the cells at various time intervals after treatment with 0·05 μg patulin/ml was tested using trypan blue. The cells were 100% viable at the beginning of the experiment. The viability was reduced to 87% after 24 hr and to 72% after 48 hr. During the time that followed the percentage of viable

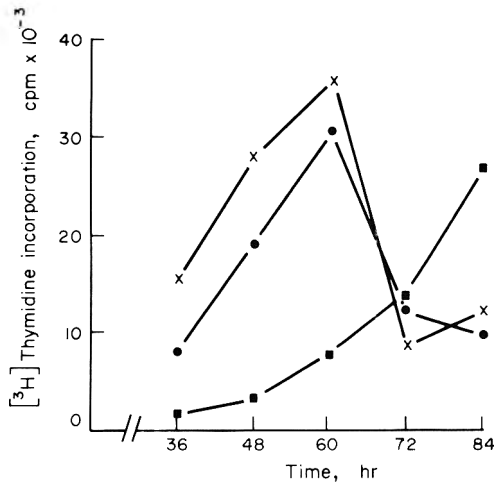

Fig. 2. The effect of 0 (×), 0·025 (●) or 0·05 (■) μg patulin/ml on DNA synthesis in human peripheral blood lymphocytes in culture. The cultures contained 1 × 10^6 cells/ml and were prepared as described in the Experimental section. Patulin was added to the cultures at 0 hr, and [^{3}H]thymidine was added at 24, 36, 48, 58 or 72 hr and the cells were harvested 12 hr later.

cells increased again. At 48 hr there was a substantial number of blasts in the culture and a strong agglutination of the cells in spite of the patulin treatment.

These observations led us to test the stability of patulin in culture medium (Table 1). HPLC analysis proved that patulin was not stable in the culture medium. Therefore, unless the cells are irreversibly damaged initially, which we observed at a high patulin concentration (1 μg/ml), the cells resume normal growth when the toxin has disappeared.

Figure 3 summarizes the results of an experiment that demonstrates that although patulin inhibits DNA synthesis in cultured human peripheral blood

Table 1. *Recovery of patulin from tissue-culture medium*

Patulin added (μg/ml)	Recovery (%)* at 0 hr	Recovery (%)* at 30 hr
0·025	67	0
0·05	75	0
0·1	77	0
0·3	78	0

*The method of recovery of patulin from the tissue culture medium is described in the Experimental section. Patulin concentration was determined by high-pressure liquid chromatography.

lymphocytes, the incorporation of [^{3}H]thymidine is increased in the presence of cysteine. In this experiment cysteine even accelerated cell growth in comparison to the control, which indicates that the medium may be deficient in SH-compounds. This enhancement has not been found in all experiments.

Patulin reacts readily with sulphydryl compounds. The mechanism of the reaction is complex and can be postulated to involve a Michael addition (a nucleophilic addition of a carboanion to an α,β-unsaturated carbonyl compound) of the sulphydryl group to the double bond of the unsaturated lactone system of patulin. In buffered solutions the reaction proceeds rapidly at pH 5·4 (Ciegler, Beckwith & Jackson, 1976).

Our experiments demonstrate that patulin, at a concentration of 1 μg/ml, completely inhibits the growth of lymphocytes when present from the initiation of the cultures. The cells are damaged beyond repair by this concentration of the toxin. Cysteine can abolish the inhibition completely. At a concentration of 0·05 μg/ml, the toxin inhibits the growth of lymphocytes during the first 60 hr but later the toxic effect is reversed. The same amount of patulin

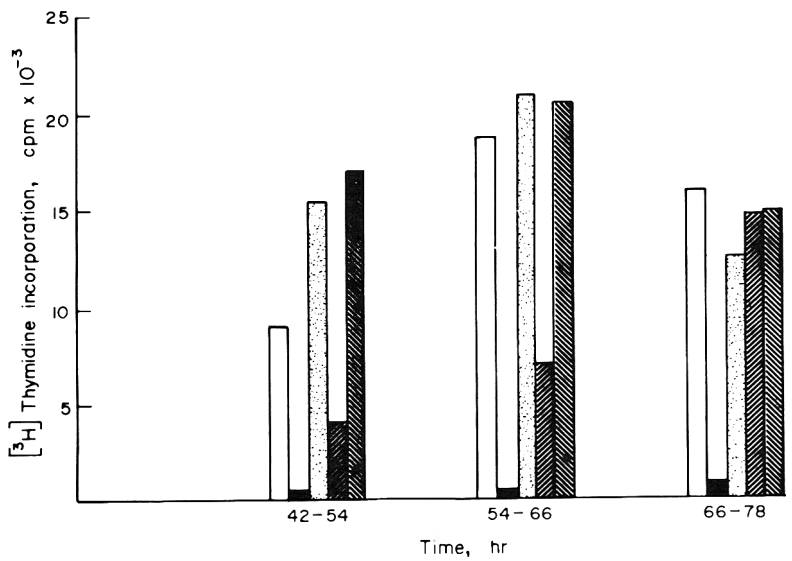

Fig. 3. The effect of cysteine on the inhibition by patulin of DNA synthesis in human peripheral blood lymphocytes. Cultures containing 1 × 10^6 cells/ml were prepared as described in the Experimental section and the following were treated at 0 hr as follows: 0 μg patulin/ml (□), 1 μg patulin/ml (■), 1 μg patulin/ml + 1 μg cysteine/ml (▩), 0·05 μg patulin/ml (▨), 0·05 μg patulin/ml + 0·05 μg cysteine/ml (▧). [^{3}H]Thymidine was added at 42, 54 or 66 hr of culture.

Table 2. *The effect of patulin on the frequency of sister-chromatid exchanges in human peripheral blood lymphocytes in culture*

Patulin concn (μg/ml)	SCEs/cell†	Percentage of cells at harvest in generation			Inhibition of [^{3}H]thymidine incorporation into DNA (%)
		1	2	3 or later	
		Experiment 1			
0	9·7 ± 3·05	45	33	22	0
0·075	11·8 ± 4·93	28	54	18	5
0·10	12·35 ± 4·28**	28	52	19	19
0·20	12·85 ± 4·43**	36	39	25	34
0·30	10·40 ± 4·23	19	64	17	22
		Experiment 2			
0	12·85 ± 4·37	13	44	43	0
0·075	11·94 ± 3·12‡	30	48	22	24
0·10	15·05 ± 3·66**	20	54	26	24
0·20	13·85 ± 4·60	24	48	28	35
0·30	13·10 ± 3·34	24	46	29	53
		Experiment 3			
0	12·40 ± 3·50	54	42	4	0
0·10	14·10 ± 3·75*	63	35	1	23
0·20	12·50 ± 3·81**	71	28	1	22
0·30	12·40 ± 3·50‡	83	17	0	44
0·45	—§	—	—	—	68

SCEs = Sister-chromatid exchanges

†Values are means ± 1 SD for 20 cells except where indicated. Values marked with asterisks differ significantly (Wilcoxon rank test) from control values (*$P < 0·05$; **$P < 0·01$).

‡18 cells only.

§No mitoses could be found.

The cells were cultured as described in the Experimental section. Patulin was added 24 hr after the culture was set up. [^{3}H]thymidine was added to a parallel culture after 60 hr and the cells were harvested after 72 hr in culture.

together with cysteine allows a normal incorporation of [^{3}H]thymidine from the beginning of the culture.

The patulin–cysteine adducts are considerably less toxic than the parent compound (Lindroth & von Wright, 1978), although they are still partially bacteriostatic to selected Gram-positive and -negative bacteria (Ashoor & Chu, 1973; Geiger & Conn, 1945). To our knowledge the carcinogenic and mutagenic properties of the sulphydryl adducts have not been tested except in a Salmonella system in which a negative response was obtained as for the parent compound (von Wright & Lindroth, 1978). However, theoretical reasons have been proposed for both an increased (Wilson, 1976) and a decreased (Rosin & Stich, 1978) ability of the patulin–cysteine adducts to attack DNA.

Tables 2 and 3 summarize the results of the SCE-frequency tests. In these experiments patulin was added 24 hr after the culture was begun in order to ensure that the toxin was present when the cells reached the phase of DNA synthesis. In most cultures the SCE frequency is not significantly elevated above

Table 3. *The effect of patulin on fast and slow growing human peripheral blood lymphocytes in culture*

Time of cell harvest (hr)	Patulin concn (μg/ml)	SCEs/cell†	Percentage of cells at harvest in generation		
			1	2	3 or later
50	0	11·6 ± 3·41	70	30	0
	0·075	11·9 ± 3·45	84	16	0
	0·3	14·25 ± 3·77**	87	13	0
72	0	11·20 ± 3·35	20	23	57
	0·075	12·50 ± 3·50	17	27	56
	0·3	13·55 ± 3·70	21	30	49

SCEs = Sister-chromatid exchanges

†Values are means ± 1 SD for 20 cells and that marked with an asterisk differs significantly (Wilcoxon rank test) from the control values (**$P < 0·01$).

The cells were cultured as described in the Experimental section. Patulin was added 24 hr after the culture was set up and the cells were harvested after 50 or 72 hr in culture.

Table 4. *The effect of patulin and patulin–cysteine mixtures on the frequency of sister-chromatid exchanges in human peripheral blood lymphocytes in culture*

Patulin concn (μg/ml)	Cysteine concn (μg/ml)	SCEs/cell†	Percentage of cells at harvest in generation			[^{3}H]Thymidine incorporation into DNA (% of control)
			1	2	3 or later	
Control	—	13·90 ± 3·04	67	22	11	100
0·03	0	13·33 ± 3·25	59	34	7	92·8
0·05	0	13·75 ± 4·76	62	33	5	88·3
0·15	0	14·80 ± 4·27	76	23	1	20·1
0	10	12·60 ± 4·24	44	31	14	100
0·03	10	15·15 ± 3·73**	74	21	5	122·17
0·05	10	15·25 ± 3·91**	45	38	17	118·38
0·15	10	16·25 ± 4·15**	86	14	0	115·97

SCEs = Sister-chromatid exchanges

†Values are means ± 1 SD for 20 cells. Those marked with asterisks differ significantly (Wilcoxon rank test) from the value for the culture containing cysteine alone (**$P < 0·01$). The result for the latter culture was compared with that of the control but did not differ significantly.

The cells were cultured as described in the Experimental section. Patulin and cysteine were added 24 hr after the culture was set up. [^{3}H]thymidine was added to a parallel culture after 60 hr and the cells were harvested after 72 hr in culture.

background level. However, at intermediate concentrations (0·1–0·2 μg/ml) the SCE frequency is significantly elevated (Table 2). At higher concentrations (0·3 μg/ml) there is a substantial inhibition of the overall DNA synthesis, but the SCE frequency is not significantly higher than in the control cultures (Table 2). These results indicate that patulin may have different effects on different cell populations. The experiment was repeated and, to reduce the exposure time, cells were also collected after 50 hr. A cell population was obtained that had an elevated SCE frequency in the presence of 0·3 μg/ml patulin. Again the more slowly growing cells that reached their second metaphase at 72 hr did not have an elevated SCE frequency at this patulin concentration (Table 3).

In HeLa cells patulin induced DNA-strand breakage and chromosomal aberrations (Umeda, Yamamoto & Saito, 1972). It has also been shown to induce abnormal metaphases and a high percentage of polyploid cells in human cell cultures (Withers, 1966) and petite mutants in *Saccharomyces cerevisiae* (Mayer & Legator, 1969). Positive results were also obtained with patulin in the *rec* assay using a recombination-deficient mutant of *Bacillus subtilis* (Ueno & Kubota, 1975). However, patulin did not induce mutations in histidine auxotroph strains of *Salmonella typhimurium* either in an Ames test or in a host-mediated assay (von Wright & Lindroth, 1978). However, these findings indicate that the precise mode of action of patulin is still uncertain; indeed it may have multiple effects on cell metabolism.

In Table 4 the results of a typical experiment showing the effect of cysteine is reported. There is a slight elevation in SCE frequency caused by patulin as in the former experiment. The increased SCE frequency is more evident in the presence of the patulin and cysteine mixture. (The results shown in Table 4 are from one of three experiments which all yielded very similar results.) According to current theories, clastogenic effects can be inhibited by trapping the reactive electrophilic species of mutagens through the use of reducing agents (Rosin & Stich, 1978). However, cysteine does not reverse the clastogenic effects of patulin in our test system. It is clear though that the inhibitory effect of patulin on DNA synthesis can be reversed by cysteine.

In conclusion patulin has a weak enhancing effect on SCE formation in human lymphocytes. This effect is apparent at intermediate concentrations of the toxin and also at high concentrations if the cells are harvested early. Cysteine strengthens this effect at concentrations at which it abolishes the toxic effects of patulin on cell proliferation. Mutagenic chemicals are usually considered to cause an elevated frequency of SCE (Kato, 1977; Wolff, 1981). Therefore our results indicate that patulin may have a mutagenic effect on mammalian cells.

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THE SUBCHRONIC TOXICITY AND TERATOGENICITY OF ALTERNARIOL MONOMETHYL ETHER PRODUCED BY *ALTERNARIA SOLANI**

G. A. POLLOCK and C. E. DISABATINO

Department of Veterinary and Comparative Anatomy, Pharmacology and Physiology, WOI Regional Program in Veterinary Medical Education, University of Idaho, Moscow, ID 83843

R. C. HEIMSCH

Department of Bacteriology and Biochemistry, University of Idaho, Moscow, ID 83843

and

D. R. HILBELINK†

Department of Veterinary and Comparative Anatomy, Pharmacology and Physiology, WOI Regional Program in Veterinary Medicine, Washington State University, Pullman, WA 99163, USA

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Abstract—Alternariol monomethyl ether (AME), a metabolite of species of Alternaria, was studied for subchronic toxicity to rats and teratogenic effects in golden hamsters. In rats no toxic effects were observed at the highest dosage tested (3·75 mg given by oral gavage daily for 30 days). However, AME was maternally toxic and foetotoxic to Syrian golden hamsters when given ip at 200 mg/kg body weight on day 8 of gestation. No such effects were observed at dose levels of 50 or 100 mg/kg.

INTRODUCTION

Alternariol monomethyl ether (AME) is a member of the dibenzo-α-pyrone group of mycotoxins which include alternariol (AOH), altenuisol and altenuene (ALT; Fig 1; Harvan & Pero, 1976). All of these compounds contain a lactone ring and are produced via the polyketide pathway (Gatenbeck & Hermodsson, 1965).

These secondary metabolites are produced by various Alternaria species which are ubiquitous in the environment and can infect a wide range of agricultural products in the field or during storage. Alternaria species have been isolated from field-weathered sorghum (Seitz, Sauer, Mohr & Burroughs, 1975a; Seitz, Sauer, Mohr *et al.* 1975b), commercial discoloured pecans (Schindler, Abadie, Gecan *et al.* 1977; Schroeder & Cole, 1977) and decaying tomatoes from the field and domestic refrigerators (Harwig, Scott, Stoltz & Blanchfield, 1979). *Alternaria solani* is a common mould associated with rot in potato tubers (R. C. Heimsch, personal communication, 1981).

However the toxicity of AME has only recently been examined. Pero, Posner, Blois *et al.* (1973) studied the cytotoxicity, acute toxicity, foetotoxicity and teratogenicity of several Alternaria metabolites including AME. AME was shown to be cytotoxic and a 1:1 mixture of AOH and AME produced a synergistic effect. AME at a dose of 400 mg/kg caused only 10% mortality whereas the same dose of AOH resulted in 30% mortality. AOH had foetotoxic effects in mice 100 mg/kg (a slightly maternally toxic dose) while AME was without significant foetotoxic or teratogenic effects at doses up to 100 mg/kg. Sauer, Seitz, Burroughs *et al.* (1978) fed diets containing AOH, AME, ALT and/or tenuazonic acid (TA) to rats and

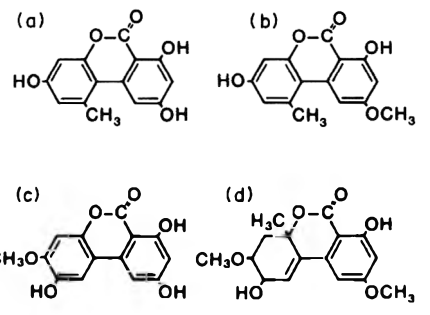

Fig. 1. Structures of three dibenzo-α-pyrone mycotoxins and a related compound produced by Alternaria species: (a) alternariol; (b) alternariol monomethyl ether; (c) altenuisol; (d) altenuene.

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†Present address: Department of Anatomy, College of Medicine, University of South Florida, Tampa, FL 33612, USA.

Abbreviations: ALT = Altenuene; AME = alternariol monomethyl ether; AOH = alternariol; TA = tenuazonic acid.

chicks for 21 days. The highest level of the dibenzo-α-pyrones was 54 μg g diet and they did not contain any TA or altertoxin I. This level was without gross or histological effects in rats and chicks. AME was reported to be mutagenic in the Salmonella mammalian microsome mutagenicity assay system (Scott & Stoltz, 1980). The mutagenic activity of AME was weak but was due to intrinsic activity (did not require metabolic activation). Such results have also been obtained by Heimsch (R. C. Heimsch, personal communication, 1978).

This relative absence of quantitative toxicological information on the dibenzo-α-pyrone Alternaria metabolites must be viewed in relation to their occurrence in the environment. Reports of the presence of AME and AOH in various commodities have been increasing in recent years. Seitz *et al.* (1975a) showed that AME and AOH interfered with the analysis of zearalenone and aflatoxin using standard analytical procedures. They later analysed samples of weathered grain sorghum from seven locations in Kansas and detected AOH and AME levels ranging from 0 to 7·9 ppm (Seitz *et al.* 1975b). Schroeder & Cole (1977) reported the presence of AME and AOH in damaged discoloured pecan kernels (pickouts). Alternaria species were frequently isolated from decaying tomatoes (Harwig *et al.* 1979). Some of these isolates produced AME when inoculated into tomatoes. These authors pointed out the need for better analytical methods for detecting the various mycotoxins in tomato products. Stinson, Bills, Osman *et al.* (1980) studied the toxin-producing ability of various Alternaria species on apple and tomato slices. They found that the cultures could elaborate TA, AME, AOH, ALT and altertoxin I on both substrates. The highest level of dibenzo-α-pyrone metabolites produced by a wild strain of Alternaria was 75 mg/100 g substrate.

The increasing number of investigations reporting significant levels of AME in various products coupled with the limited amount of information on the toxicology of AME indicated the need for further studies. The present investigation was designed to study the subchronic toxicity and teratogenicity/foetotoxicity of AME.

EXPERIMENTAL

Production and purification of AME. AME used in this study was extracted and purified from *A. solani* mycelium. *A. solani* UI-2, previously shown to produce high levels of AME, was cultured at 25 C for 52 days in the dark in 36 Roux bottles each containing 225 ml of sterile glucose–peptone broth (containing, per litre distilled water, 5·0 g peptone, 40 g glucose, 0·25 g $MgSO_4.7H_2O$ and 0·25 g K_2HPO_4) as formulated by Coombe, Jacobs & Watson (1968). The *A. solani* mycelial mass was separated from the culture fluid by vacuum filtration and then dried at 70 C in a hot-air oven.

AME was extracted and purified from the dried mycelium according to the scheme presented in Fig. 2. The dried mycelium (117 g) was combined with approximately 15 volumes (1750 ml) of methanol and the mixture was macerated in a Waring blender. The resulting slurry was boiled for 20 min on a hotplate and then filtered while hot through Whatman cellulose extraction thimbles (45 × 123 mm). The filtrate was saved and the ground mycelium residue was re-extracted with hot methanol (1750 ml) using a Soxhlet apparatus. The two extracts were combined and evaporated to approximately half of the original volume using a rotary vacuum evaporator. The concentrated extract was held at −10 C for several days to promote precipitation of AME. The crude AME precipitate was recovered by filtration onto Whatman no. 1 paper and dissolved in approximately 1 litre of tetrahydrofuran. The tetrahydrofuran preparation was clarified by filtration (Whatman no. 1 paper), the insoluble residue was discarded, and the AME-containing filtrate was evaporated to dryness in a 2-litre, round-bottomed flask. While in the flask, the AME residue was washed with 1·5 litres of hexanes at 55–60 C in order to remove some of the yellow-orange pigment(s). Portions of the AME residue were dissolved as needed in chloroform–methanol (85:15, v/v) for silica-gel column chromatography.

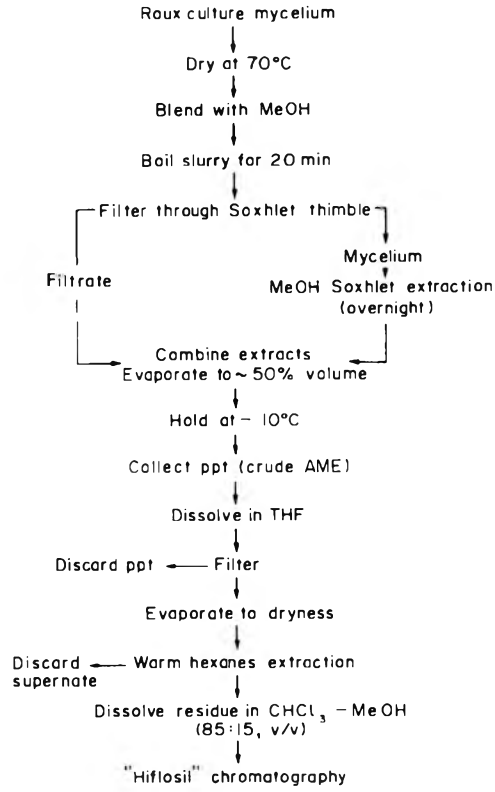

Fig. 2. Procedure for extraction and purification of alternariol monomethyl ether from *Alternaria solani* mycelium.

Approximately 40-ml portions of chloroform–methanol (85:15, v/v) saturated with crude AME were applied to 2·5 × 25–30-cm Hiflosil (Applied Science Laboratories, Inc., State College, PA) columns equilibrated to the same solvent. As chloroform–methanol (85:15, v/v) percolated through the columns, purified AME was eluted frontally as a broad band which fluoresced blue under long-wave ultraviolet light. The purified AME was collected in a flask as it eluted from the columns, was evaporated to dryness, and was used in animal toxicity trials. A total of 22 chromatographic separations yielded 4·6 g of purified

AME. Fluorodensitometric scans of the purified AME chromatographed on thin-layer plates [Absorbosil-5. Applied Science Laboratories, Inc., with chloroform–methanol (95:5. v/v)] revealed that the preparation contained approximately 93% AME and 7% AOH as the major contaminating compound.

Subchronic toxicity. Male Sprague–Dawley rats (160 ± 10 g. 5 wk old) were treated by daily oral gavage for 30 days with AME suspended in 0·1 ml of olive oil. This dosing procedure was necessitated by limited amounts of available AME. Control animals were given only olive oil. Each group (high dose, low dose and control) contained 15 animals. The high-dose animals were given an initial dosage of 23·4 mg/kg body weight (3·75 mg/rat/day) which was not adjusted subsequently for weight gain because of the limited quantity of AME available. The final dosage for this group was 12·3 mg/kg. The low-dose group was initially given 0·234 mg/kg and the final dosage was 0·116 mg/kg.

Animals were observed daily for general health, and food consumption and body weights were measured twice weekly. Blood parameters and serum enzyme levels were measured in blood samples obtained from approximately half of the animals in each group at each bleeding; animals were anaesthetized with methoxyflurane and 1 ml of blood was obtained by heart puncture. Haematocrit, white-cell, red-cell and differential blood-cell counts and haemoglobin levels were measured. Serum glutamic–pyruvic transaminase, alkaline phosphatase and glutamic–oxaloacetic transaminase activities were measured using Worthington enzyme assay kits. Serum cholinesterase was determined by a modified Ellman technique (Ellman, Courtney, Andres *et al.* 1961).

Animals were killed after 31 days and examined for any gross lesions. Liver, lungs, kidneys, testes, spleen and heart were removed, weighed and fixed in 10% buffered formalin. Brain, thymus, adrenals, stomach, small and large intestines and urinary bladder were also fixed for histological examination.

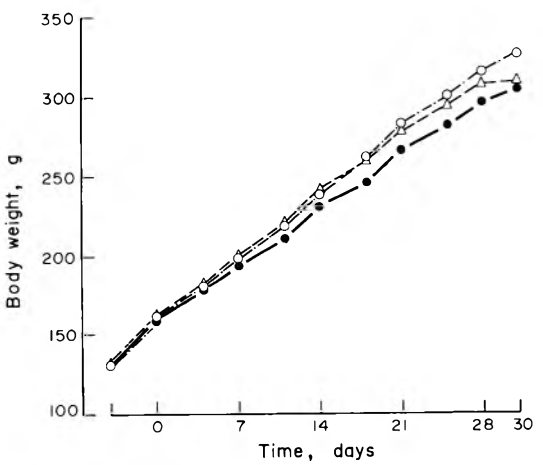

Fig. 3. Body-weight gains of rats given by oral gavage 0 (●), 0·037 (○) or 3·75 (△) mg alternariol monomethyl ether/day for 30 days. (Dosing was started at day 0.) There was a significant reduction in weight gain in the high-dose group in comparison with the controls between days 28 and 31 ($P < 0·05$ by Student's t test).

Mean values were calculated for each group and groups were compared using Student's t test.

Teratogenicity/foetotoxicity. Virgin female Syrian golden hamsters (*Cricetus auratus*) were mated during the early evening. Animals were fed Purina laboratory chow and water *ad lib.* On the morning of day 8 of gestation, the animals were divided into groups and given a single ip injection of 50, 100 or 200 mg AME (in olive oil)/kg body weight. Control animals were treated with olive oil or left untreated. The hamsters were killed on day 15 of gestation and the foetuses were removed and resorption sites were counted. The foetuses were examined for external malformations. Following fixation and weighing, each foetus was further examined for additional malformations by either razor-blade sectioning or alizarin-staining. Initially, the 200-mg/kg dose group were given the AME suspended in 0·4 ml oil but showed localized necrosis that was not apparent in the 100 mg/kg group. Therefore the volume of oil was later changed to 0·5 ml, a dose that did not cause the severe pathological lesions of the viscera. All data were statistically analysed by Student's t test using the means for each litter.

RESULTS AND DISCUSSION

The only effect seen in the subchronic toxicity study was a significant ($P < 0·05$) decrease in weight gain from day 28 to 32 (Fig. 3). During this period, the mean weight gains in the control and low-dose groups were 9·1 g and 11·5 g, respectively, whereas in the high-dose group the weight gain was only 2·2 g. Total body-weight gains over the whole study period and organ to body weight ratios did not differ significantly between the three groups. It is possible that the significant decrease in weight gain was a result of AME administration; however, this conclusion would seem to be premature. Had the study continued and the decrease in weight gain been maintained, then it would be appropriate to conclude that the animals were receiving a toxic dose of AME. The notable shift in the shape of the weight-gain curve for the high-dose group (Fig. 3) further indicates a possible toxic effect on weight gain. However, until such a study is repeated and continued for a longer period, it is only possible to conclude that the administration of 3·75 mg AME/day may have caused a decrease in weight gain. All other measurements did not reveal any significant differences between control, low- and high-dose groups.

When hamsters were given AME at 200 mg/kg in a volume of 0·4 ml olive oil, they developed severe visceral necrosis. Upon opening the peritoneum, there was a foul odour and the visceral organs appeared necrotic and coalesced. Since this effect was not seen in the group given 100 mg/kg (in 0·4 ml oil), another group of five hamsters was given 200 mg/AME/kg in 0·5 ml of oil. This dose did not produce severe necrosis but the animals displayed an initial toxic response. Within 1 to 2 min of injection, the animals became lethargic, had difficulty breathing, and their hindlimbs became flaccid. This effect lasted for approximately 45 min after which time the animals recovered and appeared normal. The results of the teratogenicity study are shown in Table 1. The high-dose group had a significant increase in the number of

Table 1. *Reproductive effects of treating Syrian golden hamsters by ip injection with alternariol monomethyl ether (AME) on day 8 of gestation*

Treatment‡ (no. of pregnant hamsters)	Total no. of implantations	Total resorptions		Total no. of foetuses		Mean (± SEM) foetal weight (g)
		No.	% of total implantations	Alive	Malformed	
AME:						
200 mg/kg (5)	58	25	43·1*	33	1	1·02 ± 0·16*
100 mg/kg (5)	75	4	5·3	71	0	1·38 ± 0·09†
50 mg kg (4)	58	2	3·4	56	0	1·53 ± 0·11
Vehicle-treated controls (10)	123	5	4·0	118	0	1·39 ± 0·05†
Untreated controls (14)	206	11	5·3	195	0	1·63 ± 0·03

‡The 200-mg AME/kg dose was administered in 0·5 ml olive oil. The two lower doses were administered in 0·4 ml olive oil. Two of the hamsters in the vehicle-treated control group were injected ip with 0·5 ml olive oil and the remainder were injected with 0·4 ml olive oil.

Values marked with an asterisk differ significantly (Student's t test) from those of the vehicle-treated and untreated control groups ($P < 0·01$). That marked with a dagger differs significantly (Student's t test) from the untreated control group ($P < 0·05$).

resorptions and a decrease in mean foetal weight. There was only one malformation, an exencephaly, in the 200-mg kg dose group. This is a common spontaneous malformation in hamsters and was probably not due to AME administration.

These results indicated that AME at a dose of 200 mg/kg was maternally toxic in the golden hamsters. In addition, this dose level was foetotoxic as evidenced by the increased number of resorptions and decreased foetal weights. None of the dose levels was teratogenic. Administration of 50 or 100 mg AME/kg did not cause any toxic responses.

The results of this study indicated that AME administered subchronically was slightly toxic to rats and foetotoxic to golden hamsters. Further subchronic studies at higher dose levels should be conducted in order to determine the mode of action (target tissue/organ) of AME and to establish a definite toxic level. Teratogenicity studies using other species, and mixtures of AME and AOH, should also be conducted. The present studies do not provide information on the long-term effects of AME exposure and these should be explored further since AME has measurable mutagenic activity.

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OCCURRENCE, STABILITY AND DECOMPOSITION OF β-*N*[γ-L(+)-GLUTAMYL]-4-HYDROXYMETHYLPHENYLHYDRAZINE (AGARITINE) FROM THE MUSHROOM *AGARICUS BISPORUS*

A. E. Ross, D. L. Nagel and B. Toth

Eppley Institute for Research in Cancer, University of Nebraska Medical Center, Omaha, NE 68105, USA

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Abstract—A chromatographic technique was developed that could clearly separate β-*N*[γ-L(+)-glutamyl]-4-hydroxymethylphenylhydrazine (agaritine) from all other components in 10–500-μl samples of mushroom extracts. Locally purchased mushrooms were found to contain mean levels of 0·4–0·7 mg agaritine g. The agaritine content of the mushrooms had decreased by 2–47% after 1 wk of storage in a domestic refrigerator and by 36–76% after 2 wk of such storage. Canned mushroom soup and canned mushrooms did not contain detectable agaritine; a sample of frozen mushrooms contained a mean level of 0·33 mg/g and a batch of fresh mushrooms lost about 32% of their agaritine content on cooking. In mice given 3 mg agaritine by gavage, agaritine was detected in all parts of the gastro-intestinal tract 15 min after dosing, but none was detectable in the gut after 3 hr. The enzyme γ-glutamyltranspeptidase derived from pig's kidney was found to be capable of decomposing agaritine to glutamic acid and 4-(hydroxymethyl)phenylhydrazine, and to have nine times such activity as an enzyme isolated from mushrooms.

INTRODUCTION

The common cultivated commercial mushroom *Agaricus bisporus* contains relatively large amounts of the hydrazide β-*N*-[γ-L(+)-glutamyl]-4-hydroxymethylphenylhydrazine (agaritine). This compound was first reported by Levenberg (1960) and subsequently Kelly, Daniels & Hinman (1962) obtained a yield of 0·4% (based on the fresh weight) and confirmed its structure. In 1964, Levenberg also found the compound in ten other Agaricus species, including *Agaricus campestris*, the familiar 'meadow' mushroom. Agaritine has been shown to be toxic to mice (Toth, Nagel, Shimizu *et al.* 1975), but not carcinogenic (Toth, Raha, Wallcave & Nagel, 1981b).

The first substituted hydrazine found to be carcinogenic was the antitubercular drug isonicotinic acid hydrazide (Juhász, Baló & Kendney, 1957). Since then, over 50 hydrazines and hydrazides have been shown to induce tumours in laboratory animals (reviewed by Toth, 1975 & 1980). Three of these are structurally related to agaritine (Fig. 1). Both *N*'-acetyl-4-(hydroxymethyl)-phenylhydrazine and 4-methylphenylhydrazine hydrochloride are lung and blood-vessel carcinogens in mice (Toth, Nagel, Patil *et al.* 1978; Toth, Tompa & Patil, 1977) and the latter also induces tumours of the subcutis in mice. The tetrafluoroborate salt of the 4-(hydroxymethyl)benzenediazonium ion induces tumours in the skin, subcutis and glandular stomach of mice (Toth, Nagel & Ross, 1982; Toth, Patil & Hwan-Soo, 1981a). This diazonium ion was detected in *A. bisporus* by Levenberg (1962) and its presence is probably due to the enzymatic breakdown of agaritine via 4-(hydroxymethyl) phenylhydrazine. Enzymes for this pathway have been isolated from *A. bisporus* (Gigliotti, 1963; Gigliotti & Levenberg, 1964).

This report records the occurrence and stability of agaritine and its metabolism by a mammalian enzyme.

EXPERIMENTAL

Chemicals. *N*-1-Naphthylethylenediamine dihydrochloride was obtained from Eastman Organic Chemi-

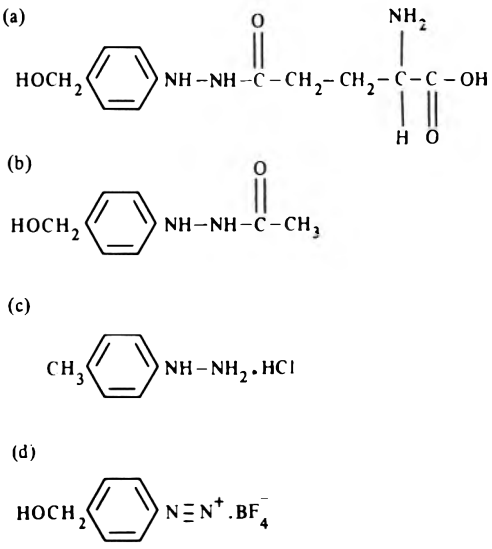

Fig. 1. Structures of (a) agaritine, (b) *N*'-acetyl-4-(hydroxymethyl)phenylhydrazine, (c) 4-methylphenylhydrazine hydrochloride and (d) 4-(hydroxymethyl)benzenediazonium tetrafluoroborate.

cals (Rochester, NY), sodium glyoxylate and γ-glutamyltranspeptidase were purchased from Sigma Chemical Co. (St. Louis, MO) and 2N Phenol Reagent (Folin Ciocalteau) was obtained from Fisher Scientific Co. (St. Louis, MO). Synthetic agaritine was prepared in this laboratory (Wallcave, Nagel, Raha *et al.* 1979).

Extraction of agaritine from mushroom products. The following technique was used to extract agaritine from fresh and cooked mushrooms and mushroom products. Samples were homogenized with two volumes of methanol in a Potter Elvejhem tissue grinder for amounts of 2 g or less, or in a Waring Blender for larger amounts. The homogenate was centrifuged at the top speed of an International model HN laboratory centrifuge for 10 min. The supernatant was recentrifuged after chilling at −20 C overnight and was used directly for TLC and, after filtering through a 0·4 μm filter, for quantitative graphic assay, or was concentrated by rotary evaporation at 37 C. The recovery was found to be 60% using added synthetic agaritine.

Chromatography. Extracts were monitored for the presence and approximate amounts of agaritine by chromatography on 0·25-mm silica-gel sheets eluting first with *n*-propanol–ammonium hydroxide (7:3, v v) and then, after drying, with butanol–water–acetic acid (4:1:1, by vol.). The agaritine was visualized by spraying with Folin Ciocalteau reagent diluted with one volume of water and two volumes of methanol. After drying, the blue colour could be intensified by spraying with a 10% solution of sodium carbonate in 50% methanol. An approximate measure of the amount of agaritine present in the applied sample was obtained by comparing spot sizes and densities with standard amounts of synthetic agaritine applied to the same plate.

Quantitative assays of agaritine in mouse-gut extracts were carried out using a Waters' Associates Inc. (Milford, MA) M 6000 solvent delivery system and a μ-Porasil column (2 × 300 mm) fitted with an HC Pellosil (Whatman Inc., Clifton NJ) pre-column (2 × 60 mm) and eluted with methanol–water (80:20, v/v) at 1 ml/min; the eluate was monitored at 254 nm. The amount of agaritine in extracts (retention time 8 min) was determined by comparing peak heights with those from synthetic standards (Fig. 2).

The agaritine content of mushroom extracts could not be measured using this high-pressure liquid chromatographic system because the extract contained a UV(254 nm)-absorbing component that co-chromatographed with agaritine. Mushroom extracts were therefore chromatographed on an Aminex Q150S (Biorad, Richmond, CA) cation column in the ammonium form eluting with either 0·1 M-ammonium formate (1·7 ml/min) for quantitative analysis, or water (1·3 ml/min) for preparative samples. The eluate was monitored at 254 mm with an ISCO UA5 absorbance monitor (Lincoln, NE). Typical UV profiles of synthetic agaritine and a fresh mushroom extract are shown in Fig. 3. To ensure the purity of the component in the extract that had the same Rf as agaritine, a large sample was collected by rotary evaporation of 6 ml extract to 0·5 ml at 37°C and this was placed on the cation column and eluted with water. The peak corresponding to agaritine showed the

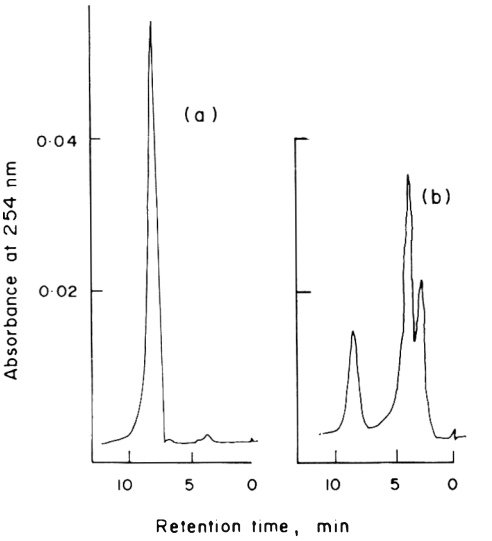

Fig. 2. High-pressure liquid chromatographic profile of (a) 12·5 μg synthetic agaritine and (b) 10 μl of a methanol extract of mouse upper small intestine and contents, containing 2·8 μg, agaritine, 5 min after giving 3 mg agaritine in 0·1 ml water by gavage.

characteristic UV absorbance spectrum of pure agaritine without any low-wavelength-absorbing contaminants and with maxima at 238 and 280 nm (Levenberg, 1964). This sample (29 ml) was rotary evaporated to dryness (37°C), lyophilized twice from 5 ml deuterium oxide, dissolved in 0·5 ml deuterium oxide and the ^{1}H nuclear magnetic resonance spectrum was determined with a Varian CFT-20 Fourier transform spectrometer using a double-pulse sequence to eliminate the residual HOO resonance.

Storage tests. The mushrooms were purchased locally and, in order to mimic typical household storage procedures, left at 4 C in a domestic refrigerator in the plastic bags supplied by the store.

In vivo *experiments.* Albino Swiss mice weighing about 30 g from our own randomly bred colony were

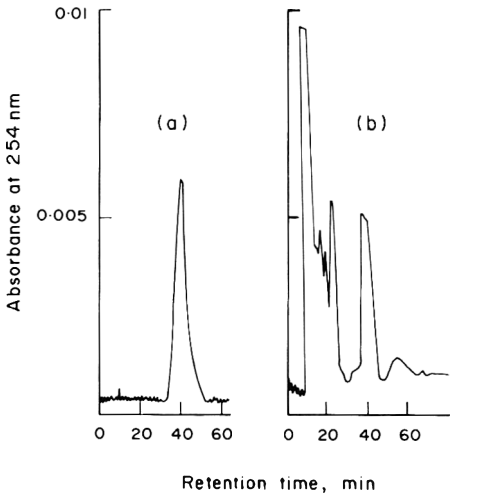

Fig. 3. Liquid chromatographic profile of (a) 10 μg of synthetic agaritine and (b) 50 μl of a methanol extract of a fresh mushroom containing 7·7 μg agaritine.

used in this study. Each was given 3 mg agaritine in 0·1 ml water by gavage. At various times after treatment, mice were anaesthetized and killed by exsanguination at the jugular vein. Blood was collected for analysis and the stomach and gut were removed and divided into five sections: (1) stomach, (2) top half of small intestine, (3) bottom half of small intestine, (4) caecum and (5) large intestine. Each section and its contents were finely chopped with scissors and the total volume was made up to 1 ml in a 15-ml polycarbonate test tube. Methanol (3 ml) was added and the mixture was homogenized for 20 sec with a Polytron tissue grinder (Brinkmann Instruments, Inc., Westbury, NY). The homogenates were then put in a freezer for at least 12 hr to precipitate most of the fats. After centrifugation in a bench-top centrifuge, samples of supernatants were examined for agaritine by thin-layer and high-pressure liquid chromatography.

Enzyme experiments. The conditions used to test whether commercially produced pig's kidney γ-glutamyltranspeptidase was capable of hydrolysing agaritine to a hydrazine were the same as those used to assay this enzyme (Orlowski & Meister, 1970), with agaritine as substrate and the addition of sodium glyoxylate to trap 4-(hydroxymethylphenyl)hydrazine as sodium glyoxylate 4-(hydroxymethyl)phenylhydrazone (ϵ_{325}, 12,500). Each mixture was of a total volume of 1 ml, was 0·11 M with respect to Tris (pH 9·0), 0·11 M with respect to $MgCl_2$ was 0·05 M with respect to sodium glyoxylate, and contained 45 μg agaritine and 50–200 μg γ-glutamyltranspeptidase. The mixtures were incubated at room temperature and the absorbance at 325 nm was measured at various times.

RESULTS AND DISCUSSION

Confirmation of the presence of agaritine in mushrooms

The ^1H nuclear magnetic resonance spectrum obtained following chromatography of the mushroom extract was identical to that from synthetic agaritine (Wallcave *et al.* 1979).

Agaritine content of mushrooms and mushroom products

Of the commercially processed mushrooms tested, only frozen mushrooms contained significant amounts of agaritine (Table 1). Presumably during processing canned mushrooms and mushroom soup are subjected to temperatures and pressures that are sufficient to decompose the agaritine. When fresh mushrooms were cooked in the manner described (Table 1) 32% of the agaritine was destroyed.

Table 1. *Agaritine in mushroom products*

Mushroom product		Agaritine concn (mg g)*
Fresh:	batch A	0·44 ± 0·08
	batch B	0·72 ± 0·4
Frozen		0·33 ± 0·04
Canned:	liquor	0
	mushrooms	0
Soup:	liquor	0
	mushrooms	0
Cooked batch A†		0·3 ± 0·06

*Values are means ± 1SD for three samples.
†The mushrooms (50 g) were sautéed in olive oil at 300°C for 7 min, were extracted with ether and then were homogenized with 100 ml methanol.

To examine the effect of storage on the agaritine content of commercial mushrooms, two batches (A and B) were purchased at different times from a local store and refrigerated in the polyethylene bags supplied by the store. At the time of purchase and 1–2 wk later, three 2 g samples from three different mushrooms were assayed for agaritine. The results are shown in Table 2. Mushrooms apparently vary greatly in their agaritine content; the range in these two batches was 0·17–1·17 mg/g. After a week, when both batches became moist, and were (subjectively) marginally edible, batch A had lost little, if any, agaritine, while batch B had lost 47% and continued to lose agaritine at a faster rate than batch A. The differences in the initial amount of agaritine and in the rate of loss on storage are presumably due mostly to the length of time after picking before purchase, although strain differences may also be involved.

Stability of agaritine in the gastro-intestinal tract of the mouse

The results of the analysis of the blood and gastrointestinal tracts of mice given 3 mg agaritine are shown in Table 3. From 15 min to 2 hr after administration, significant proportions of the dose reached all parts of the digestive tract and agaritine was thus exposed to the full spectrum of digestive enzymes and those produced by the gut bacteria. No agaritine was detected in any part of the gut 3 hr after administration. We were unable to detect agaritine in the blood at any time (the detection limit was 30 μg/ml).

Table 2. *Agaritine content of mushrooms stored in a domestic refrigerator*

Storage time (days)	Batch	Agaritine concn (mg g)*	Percentage decrease relative to day 0
0	A	0·44 ± 0·08	—
	B	0·72 ± 0·4	—
7	A	0·43 ± 0·04	2
	B	0·38 ± 0·14	47
14	A	0·28 ± 0·09	36
	B	0·17 ± 0·04	76

*Values are means ± 1SD for three samples.

Table 3. *Recovery of agaritine from the gastro-intestinal tracts of mice given 3 mg agaritine in 0·1 ml water by gavage*

Time after dosing (min)	Agaritine (mg) recovered in					Total	
	Stomach	Upper SI	Lower SI	Caecum	Large intestine	mg	Percentage of dose
0	1·7	0·67	0·27	0	0	2·64	88
5	1·41	1·18	0·25	0	0	2·84	95
15	0·15	0·7	0·31	0·9	Tr	2·06	69
30	0·15	0·15	0·49	0·8	Tr	1·59	53
60	0	0	0·34	0·1	Tr	0·44	15
120	0	0	0	0·2	0·3	0·5	0
180	0	0	0	0	0	0	0

SI = Small intestine Tr = Trace
Values are for one mouse/time interval.

Mammalian γ-glutamyltranspeptidase

The non-detection of agaritine in the blood of these mice is probably due to an inadequacy of the present analytical techniques. Without the use of radioactive agaritine, this assay system is not sensitive enough for a complete tissue distribution study. Nevertheless, it seems important to determine if agaritine is a substrate for mammalian enzymes and particularly to demonstrate whether they, like the mushroom γ-glutamyltranspeptidase, generate 4-(hydroxymethyl)-phenylhydrazine from agaritine. This hydrazine has a close structural relationship with three known carcinogens (Fig. 1).

We determined that pig's kidney γ-glutamyltranspeptidase has properties very similar to those of the mushroom enzyme. Three incubation mixtures were made up which contained 50, 100 or 200 μg enzyme protein. Fig. 4 shows the absorbance at 325 nm of these incubation mixtures. The concentration of sodium glyoxylate 4-(hydroxymethyl)phenylhydrazone increased with time, due to the generation of 4-(hydroxymethyl)phenylhydrazine from agaritine in the presence of sodium glyoxylate.

The mushroom enzyme isolated by Levenberg (1970) had an activity of 0·146 units/mg. One unit was defined as the amount that produces 1 μmol glyoxylate-phenylhydrazone (ϵ_{325}, 14,000). Based on the initial phase of the reaction, the pig's kidney enzyme had a specific activity of seven times this (0·98 ± 0·1 units/mg protein).

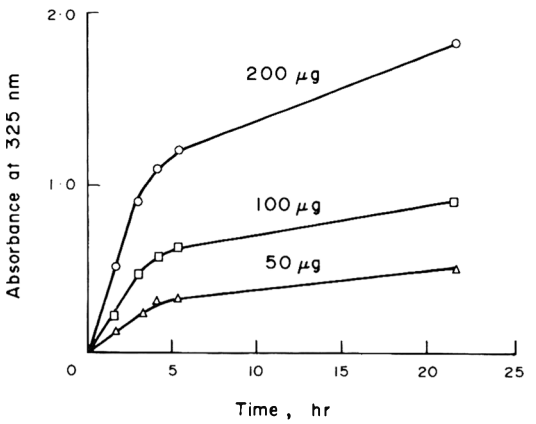

Fig. 4. The generation of 4-hydroxymethylphenylhydrazine from agaritine by hydrolysis in the presence of 50 (△), 100 (□) or 200 (○) μg mammalian γ-glutamyltranspeptidase/ml incubation medium. The reaction was monitored by the formation of sodium glyoxylate-4-hydroxymethylphenylhydrazone. For details see Experimental section.

It is very likely that human γ-glutamyltranspeptidase has similar properties and is therefore capable of generating a potentially dangerous hydrazine from agaritine which, present at concentrations of at least 0·2–1·2 mg/g fresh mushroom, is consumed in significant quantities throughout the world.

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COMPARATIVE TOXICITY OF ALKYLTIN AND ESTERTIN STABILIZERS

A. H. PENNINKS and W. SEINEN

Pathology-Toxicology Working Group, Departments of Experimental Pathology and Pharmacology-Toxicology, Faculty of Veterinary Sciences, State University of Utrecht, The Netherlands

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Abstract—In a 2-wk feeding study the oral toxicities of the estertin compounds bis-(β-carbobutoxyethyl)-tin dichloride (CBETC) and bis-(β-carbomethoxyethyl)tin dichloride (CMETC) and their hydrolysis products bis-(β-carboxyethyl)tin dichloride (CETC) were compared with those of the dialkyltin compounds di-*n*-octyltin dichloride (DOTC) and di-*n*-butyltin dichloride (DBTC). At a dietary level of 450 ppm the estertins did not affect weights, but at the relatively high level of 1350 ppm both caused growth retardation and a statistically significant decrease in the relative weight of the liver, while CBETC also reduced the relative weights of the thymus and spleen. A diminished amount of liver glycogen was the only treatment-related histopathological change observed in these organs. CETC caused no signs of toxicity up to a level of 1350 ppm. Slightly decreased body-weight gains were recorded in rats fed 50 ppm DBTC or 150 ppm DBTC or DOTC, but the main effect in dialkyltin-fed rats was a decrease in lymphoid-organ weights. In rats fed 150 ppm DBTC or DOTC for 2 wk, the relative thymus weight was decreased by more than 70% compared with the control value and there was lymphocyte depletion in the thymus, especially the thymic cortex, and in thymus-dependent lymphoid areas of the spleen. Parenteral exposure demonstrated a similar difference in toxicity between these groups: a single ip or iv injection of 2·5 mg DBTC/kg body weight caused a slight reduction in body-weight gain and severe thymic atrophy, whereas CBETC and CETC had no such effects even at 10 mg/kg. However, *in vitro* the activities of the estertins were similar to those of the dialkyltins. In lymphocyte metabolism studies, all these compounds induced a dose-dependent stimulation of glucose consumption, with maximum stimulation occurring at a level of 5 μM with DBTC and CBETC and at 120 μM with DOTC and CMETC. At higher exposure levels, glucose consumption fell sharply, oxygen consumption was reduced and lymphocyte viability was impaired. Since the dicarboxyltin compound CETC induced no signs of lymphocytotoxicity in the *in vitro* cell-metabolism, cell-viability or blast-transformation studies, hydrolysis of CBETC and CMETC to CETC in the rat is suggested as a possible explanation for the discrepancy between the lymphocytotoxic activity of the estertin compounds *in vitro* and the absence of lymphoid atrophy following *in vivo* exposure, and for the finding that in the rat CMETC and CBETC are much less toxic than the dialkyltin compounds DBTC and DOTC, which induce lymphocytotoxicity both *in vitro* and *in vivo*.

INTRODUCTION

Since organotin compounds were developed as stabilizers for PVC plastics (V. Yngve, 1940, US Patent no. 2,219,463) their use has increased considerably. Dibutyltins are used in particular for this purpose, but dioctyl- and dimethyltin compounds are most commonly used in PVC intended for food packaging. Relatively recently, another class of organotin compounds, the estertins, appeared on the stabilizer market. Whereas the toxicity of dialkyltins has been studied extensively (for reviews see: Barnes & Stoner, 1959; Luyten, 1972; Piver, 1973), no data on the toxicity of the estertins appear to have been published.

This report describes the results of 2-wk feeding studies and various *in vitro* tests on two estertins, bis-(β-carbobutoxyethyl)tin dichloride (CBETC) and bis-(β-carbomethoxyethyl)tin dichloride (CMETC), their hydrolysis product bis-(β-carboxyethyl)tin dichloride (CETC) and two dialkyltins, di-*n*-butyltin dichloride (DBTC) and di-*n*-octyltin dichloride (DOTC). Since the nature of the anionic group is of only minor importance in the toxicity of the dialkyltin compounds, and probably also of the estertins, the chlorides of both types of compound have been used throughout these comparative studies. Moreover, the fact that the chloride derivatives of these organotins are more water soluble than the commercial stabilizers is an advantage for the *in vitro* studies. In these *in vitro* experiments, the cytotoxic activities of dialkyltin and estertin compounds were compared in rat lymphocytes. For the dialkytlins, DBTC and DOTC, a selective lymphocytotoxicity has already been shown (Penninks & Seinen, 1980; Seinen, 1981; Seinen & Penninks, 1979). As a consequence of this lymphocytotoxic action, these dialkyltins cause atrophy of the lymphoid system (Seinen, Vos, van Spanje *et al.* 1977b; Seinen & Willems, 1976). The resulting suppression of immune reactivity (Seinen & Penninks, 1979; Seinen, Vos, Brands & Hooykass, 1979; Seinen, Vos, van Krieken *et al.* 1977a), is the most sensitive criterion for their toxicity in rats.

The studies reported here indicate that among the estertins, CBETC especially shows a particularly high degree of lymphocytotoxicity *in vitro*, its activity

Abbreviations: CBETC = Bis-(β-carbobutoxyethyl)tin dichloride; CETC = bis-(β-carboxyethyl)tin dichloride; CMETC = bis-(β-carbomethoxyethyl)tin dichloride; Con A = concanavalin A; DBTC = di-*n*-butyltin dichloride; DOTC = di-*n*-octyltin dichloride; LPS = *Escherichia coli* lipopolysaccharide; PHA = phytohaemagglutinin.

being comparable to that of DBTC and DOTC. However, in contrast to the dialkyltins, the estertin compounds do not induce lymphocytotoxicity when administered *in vivo*.

EXPERIMENTAL

Test compounds. The alkyltin compounds used in this study:

DBTC —$CH_3 \cdot [CH_2]_3 \cdot Sn(Cl_2) \cdot [CH_2]_3 \cdot CH_3$

and

DOTC—$CH_3 \cdot [CH_2]_7 \cdot Sn(Cl_2) \cdot [CH_2]_7 \cdot CH_3$

were kindly provided by Dr E. J. Bulten, Institute for Organic Chemistry, TNO, Utrecht. The estertin compounds:

CBETC—$CH_3 \cdot [CH_2]_3 \cdot OOC \cdot [CH_2]_2 \cdot Sn(Cl_2) \cdot [CH_2]_2 \cdot COO \cdot [CH_2]_3 \cdot CH_3$

CMETC—$CH_3 \cdot OOC \cdot [CH_2]_2 \cdot Sn(Cl_2) \cdot [CH_2]_2 \cdot COO \cdot CH_3$

and

CETC—$HOOC \cdot [CH_2]_2 \cdot Sn(Cl_2) \cdot [CH_2]_2 \cdot COOH$

were supplied by Dr R. S. Talbot, Akzo Chemie UK Ltd, Manchester, England.

Animals and diets. Specified pathogen-free Wistar-derived rats (WU–CPB) from the Central Institute for the Breeding of Laboratory Animals, TNO, Zeist, were housed in plastic cages maintained at a room temperature of 23 ± 2 C and a relative humidity of 50–60%, with a 12-hr light dark cycle. Diet (Muracon, from Trouw & Co., Putten) and tap-water were constantly available. For the oral studies, test diets were prepared by mixing the compounds thoroughly with the commercial ration.

In vivo *experiments*

Oral treatment with organotin compounds. Randomly allocated groups of ten weanling male rats, weighing 40–45 g, were fed CBETC, CMETC or CETC at dietary levels of 0, 150, 450 or 1350 ppm for 2 wk. DOTC and DBTC were each fed at dietary levels of 0, 50 or 150 ppm. Animals were weighed on days 0 and 7 and terminally on day 14. At autopsy the thymus, spleen, liver, kidneys and adrenals were weighed, fixed in neutral buffered 10% formalin and processed by conventional means for histopathological examination. Sections were stained with haematoxylin and eosin.

Parenteral treatment with organotin compounds. The vehicle used for the parenteral administration of the various organotin compounds was a mixture of absolute ethanol, Tween 80 and saline (5:2:93, by vol.). The test compounds were first dissolved in ethanol and then mixed with Tween 80; finally, while the mixture was swirled on a whorl mixer, the saline was added slowly. The various test compounds were administered parenterally to groups of six male rats, weighing 60–65 g, at a dose level of 0, 2·5 or 10 mg/kg body weight, by means of a single ip or iv injection of 0·2 ml of the test solution. Animals were killed 4 days after the injection. Body weights and the weights of the thymus, spleen, liver, kidneys and adrenals were recorded, and these organs were sampled for histopathological examination, as above.

In vitro *experiments*

Cell preparation. For all the *in vitro* experiments, male rats weighing 125–150 g were used. Immediately after decapitation of the rats, the thymus glands (carefully trimmed free from adjoining lymph nodes) and the spleens were removed under aseptic conditions, and sampled in cold phosphate-buffered saline or in cold RPMI-1640 medium, supplemented with 50 U penicillin and 50 μg streptomycin ml (all from Gibco-Biocult, Paisley, Scotland) and 2 mM-L-glutamine (Schwarz/Mann, Orangeburg, New York, USA), for the preparation of rat-lymphocyte suspensions. The tissues were minced with scissors in fragments, which were gently pressed through a nylon sieve with a pore diameter of 220 μm. To remove cell clumps, the suspensions were drawn through a 25-gauge needle, and the cells were washed twice. All processing was carried out in the cold throughout.

Lymphocyte viability studies. The tin compounds were dissolved in absolute ethanol just before their addition to the cell suspensions, for all *in vitro* experiments. The final ethanol concentration was always 0·5%, a concentration that did not affect the test system. Duplicate cultures, each of 2 million cells in 0·5 ml bicarbonate-buffered RPMI-1640, were incubated in loosely capped polystyrene culture tubes of 12 × 75 mm (Falcon Plastics, Oxnard, CA, USA). The medium supplemented with 15% heat-inactivated (56 C for 30 min) foetal calf serum (Difco Laboratories, Detroit, MI, USA). Cultures were incubated at 37 C in a humid atmosphere of 5% CO_2 in air for periods up to 24 hr. Cell numbers were counted with an electronic particle counter (Coulter Counter, model ZF; Coulter Electronics, Dunstable, Beds., England). Cell viability was determined in haemocytometers with a 0·05% nigrosin solution in saline (Merck AG, Darmstadt, FRG). At the start of the culture period, cell viability was always more than 90%. Numbers and viability of the cells were recorded after incubation for 0, 4, 8, 16 and 24 hr.

Lymphocyte transformation studies. Thymus or spleen cells (4×10^5 cells in 0·5 ml of medium) were cultured in wells of U-form microtitre plates with loosely fitting lids (type M 220-24 AR, C.A. Greiner & Söhne, Nürtingen, FRG). The bicarbonate-buffered RPMI 1640 medium was supplemented with penicillin, streptomycin, L-glutamine and foetal calf serum as described above. The plates were wrapped in plastic adhesive kitchen film to prevent evaporation from the wells at the edge of the plate (Du Bois, Huismans, Schellekens & Eysvogel, 1973) and were incubated for 72 hr at 37 C in a humid atmosphere of 5% CO_2 in air. Dose-response curves for mitogens showed the following doses per well to be optimal: 5 μg phytohaemagglutinin (PHA-P, Difco Laboratories), 2 μg concanavalin A, crystallized twice (Con A; Nutritional Biochemicals Corp., Cleveland, OH, USA) and 0·25 μg *Escherichia coli* lipopolysaccharide (LPS 0 127: B8, Westphal method; Difco Laboratories). After the cultures had been maintained for 48 hr, 0·25 μCi [*Me*-^{3}H]thymidine ([^{3}H]TdR, specific ac-

tivity 0·5 Ci/mmol; The Radiochemical Centre, Amersham, Bucks., England) in 20 μl medium was added to each well. After incubation for an additional 24 hr, the cells were harvested on to glass-fibre filters using a multiple cell-culture harvester (Skatron, Lierbyen, Norway). Filters were dried and transferred to scintillation vials containing 5 ml scintillation fluid consisting of 5 g 2,5-diphenyloxazole (PPO) and 20 mg 2,2'-*p*-phenylene-bis-(5-phenyloxazole (POPOP) per litre reagent-grade toluene (Merck AG) and counted in a Mark II (Nuclear Chicago) scintillation counter. The mean uptake of [^{3}H]TdR in triplicate cultures was calculated and the results were expressed as percentages of [^{3}H]TdR incorporation in the control cultures. Standard deviations for the triplicate cultures never exceeded 20%.

Metabolism studies. For these studies, 2 ml aliquots of a suspension of 5×10^7 thymocytes/ml phosphate-buffered saline were incubated in 15-ml Warburg vessels of a respirometer (Gilson Medical Electronics, Villiers-le-Bel, France) for 4 hr at 37°C. The side arm of each vessel contained 1 ml 6 mM-glucose solution (Boehringer Mannheim GmbH, Mannheim, FRG) in phosphate-buffered saline, as substrate for the cells. After preincubation for 10 min for temperature equilibration, the glucose solution was added to the cells to give a final concentration of 2 mM. The centre wells of the vessels contained 200 μl 20% potassium hydroxide for carbon dioxide trapping. Oxygen consumption was measured, with air as the gas phase, during the whole incubation period and was expressed in μmol/10^8 cells per 4 hr.

Both at the start and at the end of the incubation period, a sample of each cell suspension was mixed with an equal volume of ice-cold 0·66 M-perchloric acid (Merck AG). After 15 min in the cold the mixture was centrifuged at 400 g for 10 min. In the neutralized supernatant, glucose, lactate and pyruvate concentrations were measured by standard enzymatic procedures (Bergmeyer, 1974). The mean consumption or production of the respective compounds in triplicate incubations was calculated from the concentrations before and after the experiments. All results are expressed in μmol/10^8 cells per 4 hr and are the means of at least three experiments. The number and viability of the thymocytes were checked immediately after the end of the incubation.

Statistical analyses

The data from both *in vivo* and *in vitro* studies were analysed for significance of differences by Student's *t* test (De Jonge, 1960).

RESULTS

Oral treatment

Body weights and relative organ weights of control rats and of rats fed 150, 450 or 1350 ppm of the three estertin compounds in the diet for 2 wk are presented in Table 1. Rats fed the highest dose level of CBETC

Table 1. *Body weight and relative organ weights of male rats fed an estertin compound (CBETC or CMETC) or the hydrolysis product (CETC) at dietary levels up to 1350 ppm or a dialkyltin compound (DBTC or DOTC) at 50 or 150 ppm for 2 wk*

Dietary level (ppm)	Terminal body weight (g)	Relative organ weights (g/100 g body weight)				
		Liver	Thymus	Spleen	Kidneys	Adrenals
			CBETC			
0	95·6 ± 8·3	5·02 ± 0·30	0·30 ± 0·05	0·30 ± 0·03	0·94 ± 0·06	0·021 ± 0·005
150	98·9 ± 12·6	4·99 ± 0·30	0·31 ± 0·06	0·28 ± 0·04	0·93 ± 0·04	0·023 ± 0·003
450	92·5 ± 7·9	4·91 ± 0·29	0·30 ± 0·05	0·26 ± 0·04	0·93 ± 0·05	0·022 ± 0·005
1350	79·0 ± 9·5**	4·42 ± 0·38*	0·22 ± 0·05*	0·25 ± 0·03*	0·94 ± 0·04	0·025 ± 0·004
			CMETC			
0	91·6 ± 4·9	4·80 ± 0·40	0·32 ± 0·05	0·30 ± 0·04	0·97 ± 0·05	0·026 ± 0·011
150	94·2 ± 6·1	4·91 ± 0·30	0·35 ± 0·05	0·32 ± 0·03	0·97 ± 0·04	0·024 ± 0·010
450	92·1 ± 6·1	4·73 ± 0·27	0·31 ± 0·06	0·31 ± 0·04	0·96 ± 0·05	0·023 ± 0·007
1350	76·7 ± 5·8**	4·01 ± 0·34**	0·34 ± 0·05	0·29 ± 0·04	0·97 ± 0·04	0·030 ± 0·010
			CETC			
0	89·9 ± 4·8	4·59 ± 0·26	0·35 ± 0·07	0·25 ± 0·03	0·96 ± 0·07	0·022 ± 0·006
150	84·7 ± 8·4	4·75 ± 0·25	0·35 ± 0·07	0·28 ± 0·03	0·97 ± 0·04	0·021 ± 0·006
450	92·7 ± 3·3	4·82 ± 0·29	0·33 ± 0·05	0·31 ± 0·03	0·98 ± 0·05	0·021 ± 0·004
1350	87·2 ± 7·7	4·71 ± 0·46	0·34 ± 0·08	0·31 ± 0·03	0·96 ± 0·04	0·021 ± 0·004
			DBTC			
0	115·3 ± 3·9	4·25 ± 0·09	0·38 ± 0·02	0·36 ± 0·02	1·07 ± 0·02	0·025 ± 0·003
50	107·7 ± 2·4**	4·29 ± 0·07	0·17 ± 0·01**	0·30 ± 0·01**	1·04 ± 0·02	0·021 ± 0·004
150	92·1 ± 4·5**	4·93 ± 0·10**	0·10 ± 0·01**	0·24 ± 0·01**	1·06 ± 0·03	0·022 ± 0·004
			DOTC			
0	109·2 ± 1·8	5·16 ± 0·06	0·37 ± 0·02	0·34 ± 0·02	1·03 ± 0·02	0·027 ± 0·005
50	109·9 ± 3·4	5·18 ± 0·07	0·23 ± 0·02**	0·30 ± 0·01**	1·02 ± 0·02	0·028 ± 0·004
150	103·9 ± 5·1*	5·00 ± 0·09**	0·11 ± 0·01**	0·27 ± 0·02**	1·04 ± 0·02	0·029 ± 0·009

CBETC = Bis-(β-carbobutoxyethyl)tin dichloride CMETC = Bis(β-carbomethoxyethyl)tin dichloride CETC = Bis-(β-carboxyethyl)tin dichloride DBTC = Di-*n*-butyltin dichloride DOTC = Di-*n*-octyltin dichloride

Values are means ± 1 SD for groups of ten rats except for the group fed 150 ppm DBTC, in which there were only eight survivors; those marked with asterisks differ significantly (Student's *t* test) from the corresponding control value: *$P < 0·01$; **$P < 0·001$.

Table 2. *Body and relative organ weights of male rats killed 4 days after a single intravenous or intraperitoneal injection of various concentrations of DBTC, CBETC or CETC*

Dose (mg/kg)	Compound	Body weight (g)	Relative organ weights (g/100 g body weight)			
			Liver	Thymus	Spleen	Kidneys
Intravenous injection						
0	—	78·6 ± 3·0	5·08 ± 0·36	0·39 ± 0·02	0·46 ± 0·08	1·06 ± 0·06
2·5	DBTC	72·4 ± 5·3*	4·92 ± 0·44	0·23 ± 0·03***	0·42 ± 0·12	0·99 ± 0·05
	CBETC	80·3 ± 5·1	5·13 ± 0·30	0·36 ± 0·05	0·54 ± 0·07	1·04 ± 0·09
	CETC	78·7 ± 4·2	5·04 ± 0·29	0·37 ± 0·07	0·45 ± 0·08	1·04 ± 0·09
0	—	84·5 ± 5·0	4·53 ± 0·25	0·33 ± 0·03	0·33 ± 0·06	0·92 ± 0·03
10	CBETC	76·7 ± 4·3	5·07 ± 0·45	0·31 ± 0·05	0·32 ± 0·03	0·93 ± 0·01
	CETC	82·7 ± 3·6	4·95 ± 0·46	0·33 ± 0·05	0·31 ± 0·06	0·94 ± 0·05
Intraperitoneal injection						
0	—	81·3 ± 4·1	5·14 ± 0·47	0·41 ± 0·05	0·35 ± 0·04	0·98 ± 0·06
2·5	DBTC	70·2 ± 9·1*	5·27 ± 0·44	0·20 ± 0·04***	0·29 ± 0·06	0·98 ± 0·04
	CBETC	85·9 ± 7·4	5·28 ± 0·16	0·38 ± 0·06	0·46 ± 0·08**	1·01 ± 0·04
	CETC	79·1 ± 6·0	5·22 ± 0·47	0·39 ± 0·05	0·42 ± 0·08	0·99 ± 0·06
0	—	88·4 ± 4·9	5·00 ± 0·45	0·35 ± 0·05	0·36 ± 0·05	0·93 ± 0·04
10	CBETC	84·5 ± 4·4	4·81 ± 0·19	0·33 ± 0·06	0·36 ± 0·08	0·94 ± 0·05
	CETC	88·1 ± 4·8	5·07 ± 0·36	0·33 ± 0·02	0·33 ± 0·08	0·93 ± 0·03

DBTC = Di-*n*-butyltin dichloride CBETC = Bis-(β-carbobutoxyethyl)tin dichloride
CETC = Bis-(β-carboxyethyl)tin dichloride

Values are means ± 1 SD for groups of six rats: those marked with asterisks differ significantly (Student's *t* test) from the corresponding control value: *$P < 0.05$; **$P < 0.02$; ***$P < 0.001$.

or CMETC showed significant ($P < 0.001$) growth retardation compared with the controls. With this dose level of CBETC, slight but statistically significant decreases in relative liver, thymus and spleen weights occurred. With CMETC at this level a statistically significant decrease was found only in the relative liver weight. Microscopic examination showed that the glycogen content of the hepatocytes was diminished. No other treatment-related histopathological changes were observed. Up to a dietary level of 450 ppm neither CBETC nor CMETC induced body- or organ-weight changes. None of the dietary levels of CETC, the hydrolysis product of the estertin compounds, affected any of the parameters tested.

The dialkyltin compounds, DBTC and DOTC, were fed only at levels of 50 and 150 ppm (Table 1), since 450 ppm killed young rats within 2 wk. In the 150-ppm DBTC group two animals died during wk 2 (showing signs of severe jaundice). Body weights showed a dose-related decrease in rats fed DBTC. DOTC caused growth retardation only at the highest dose level and the difference from the controls, while statistically significant, was less than that with DBTC. The relative weights of lymphoid organs (thymus and spleen) were decreased in a dose-related manner by both compounds. The decrease in thymus weight was the more pronounced and amounted to more than 70% in rats fed 150 ppm DBTC or DOTC for 2 wk. The relative liver weight was increased in the 150-ppm DBTC group, whereas a slight but again statistically significant decrease was observed in the 150-ppm DOTC group. Liver changes were detected microscopically only in the rats fed 150 ppm DBTC. Severe proliferation of bile-duct epithelial cells and bile ductules, associated with pericholangitis, periportal fibrosis and accumulation of bile pigment in the hepatocytes, was observed in the animals that died and in two others. The livers of these rats showed a yellowish discoloration. A slight bile-duct proliferation was seen in another four animals of this group. However, the most prominent histopathological feature in all DBTC- and DOTC-treated animals was lymphocyte depletion. This was seen particularly in the thymic cortex, but also in the splenic periarteriolar lymphocyte sheets.

Parenteral treatment

Four days after a single iv injection of 2·5 mg DBTC/kg body weight the relative thymus weight had already decreased by more than one third of the corresponding control value (Table 2). There was also a reduction in body-weight gain. An iv dose of 5 mg/kg induced more pronounced effects on the thymus, but also caused liver pathology and some deaths, while a dose of 10 mg DBTC/kg was always fatal. Histopathological examination showed lymphoid atrophy.

The estertin compound CBETC and its hydrolysis product CETC did not affect the thymus; even at a dose level of 10 mg/kg no effect was seen on thymus or other organ weights.

After ip injection the effects of DBTC on body weight and lymphoid organs were similar to those after iv injection (Table 2), and again neither CBETC nor CETC induced overt signs of toxicity. The only change noted with these compounds was a slight increase in spleen weight in rats given 2·5 mg CBETC/kg.

Cell viability studies

The effects of the various organotin compounds on the survival of rat thymocytes *in vitro* are presented in Fig. 1. In the presence of graded amounts of DBTC or DOTC, thymocyte survival decreased in a dose-related and time-dependent fashion when compared with the thymocyte loss in the control cultures. DBTC acted the more rapidly, but both compounds

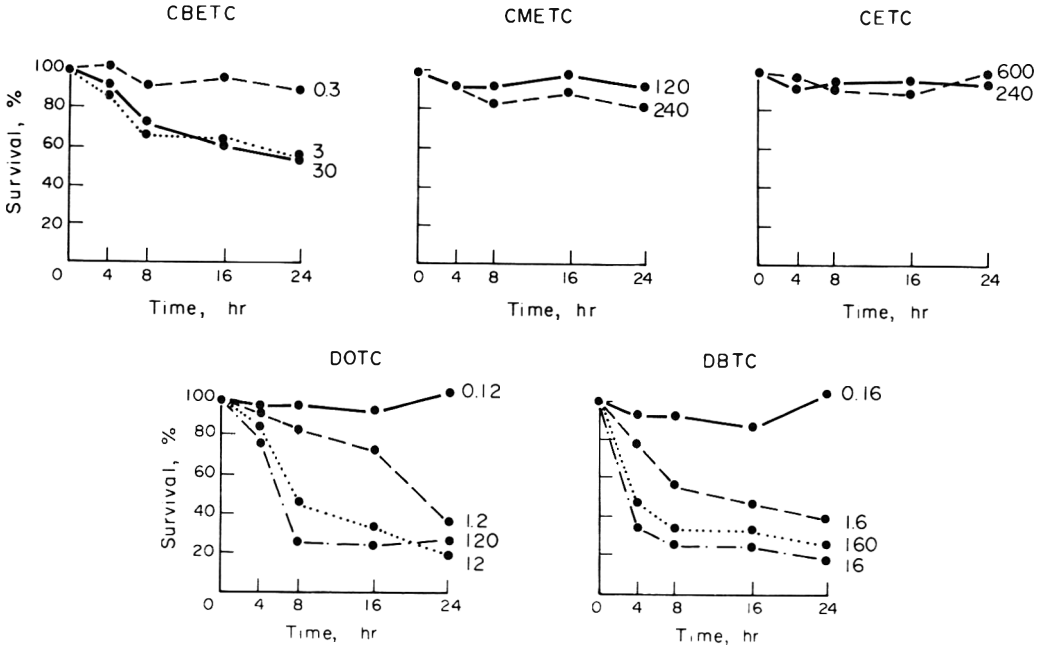

Fig. 1. Survival of rat thymocytes exposed to stated (μM) concentrations of the estertins. CBETC and CMETC, their hydrolysis product, CETC, or the dialkyltins, DOTC and DBTC for 0–24 hr. Results are expressed as percentages of the survival of the control cultures.

caused a progressive decrease in thymocyte survival until, after a 24-hr incubation with 50 μg DOTC or DBTC/ml, survival was only some 25–30% of the control values. With concentrations of 0·5 μg/ml (1·2 μM-DOTC and 1·6 μM-DBTC), thymocyte survival still showed a considerable decrease. Of the estertin compounds, only CBETC decreased thymocyte survival in a dose-related and time-dependent fashion. The survival was 56% of the control value after a 24-hr incubation with 30 μM-CBETC, the maximum concentration that could be achieved in the cell culture medium. The more water-soluble CMETC decreased thymocyte survival slightly, to 81% of the control value at the relatively high concentration of 240 μM. CETC had no effect on the viability and number of the cultured thymocytes even in concentrations up to 600 μM.

Lymphocyte transformation

Table 3 illustrates the extent of [^{3}H]TdR incorporation in two typical experiments in which thymic and splenic lymphocytes were cultured with various mitogens or in their absence. The figures demonstrated the variability between separate experiments, but within each experiment the variation was relatively small. Because of this variability between experiments, the values showing the effects of the various organotin compounds on [^{3}H]TdR incorporation (Fig. 2) are expressed as percentages of the controls, to facilitate the comparison of results obtained in separate experiments.

The blast transformation of thymocytes was inhibited in a dose-related manner in the presence of graded concentrations of DOTC, DBTC or CBETC. In concentrations of 0·1 μg DBTC/ml of medium, 0·5 μg DOTC/ml and 2·5 μg CBETC/ml, [^{3}H]TdR incorporation was completely inhibited. Concentrations five times lower still caused marked reductions, but with a further fivefold reduction in organotin concentration, [^{3}H]TdR incorporation approached control values. In contrast, CMETC and CETC did not affect blast transformation up to concentrations of 12·5 μg/ml medium. The relative inhibition of [^{3}H]TdR incorporation by the various organotin compounds tested showed essentially the same pattern in PHA- and Con A-stimulated cultures and in non-stimulated cultures (not presented in Fig. 2).

DOTC, DBTC and CBETC were shown to have similar effects on the transformation of lymphocytes from the spleen (Fig. 2) as on those from the thymus, and again the effects were similar in PHA- and Con A-stimulated cultures and also in splenocyte cultures incubated with LPS.

Table 3. [^{3}H]*Thymidine incorporation in thymic and splenic lymphocytes cultured in the presence or absence of various mitogens (results of typical experiments)*

Mitogen	[^{3}H]TdR incorporation (counts/min) Experiment 1	Experiment 2
	Splenocytes	
—	236 ± 117	326 ± 86
PHA	1017 ± 194	2951 ± 508
Con A	3285 ± 320	5773 ± 975
LPS	712 ± 168	1152 ± 94
	Thymocytes	
—	518 ± 64	228 ± 33
PHA	3974 ± 747	1227 ± 167
Con A	8086 ± 972	4082 ± 796

PHA = Phytohaemagglutinin Con A = Concanavalin A
LPS = *Escherichia coli* lipopolysaccharide

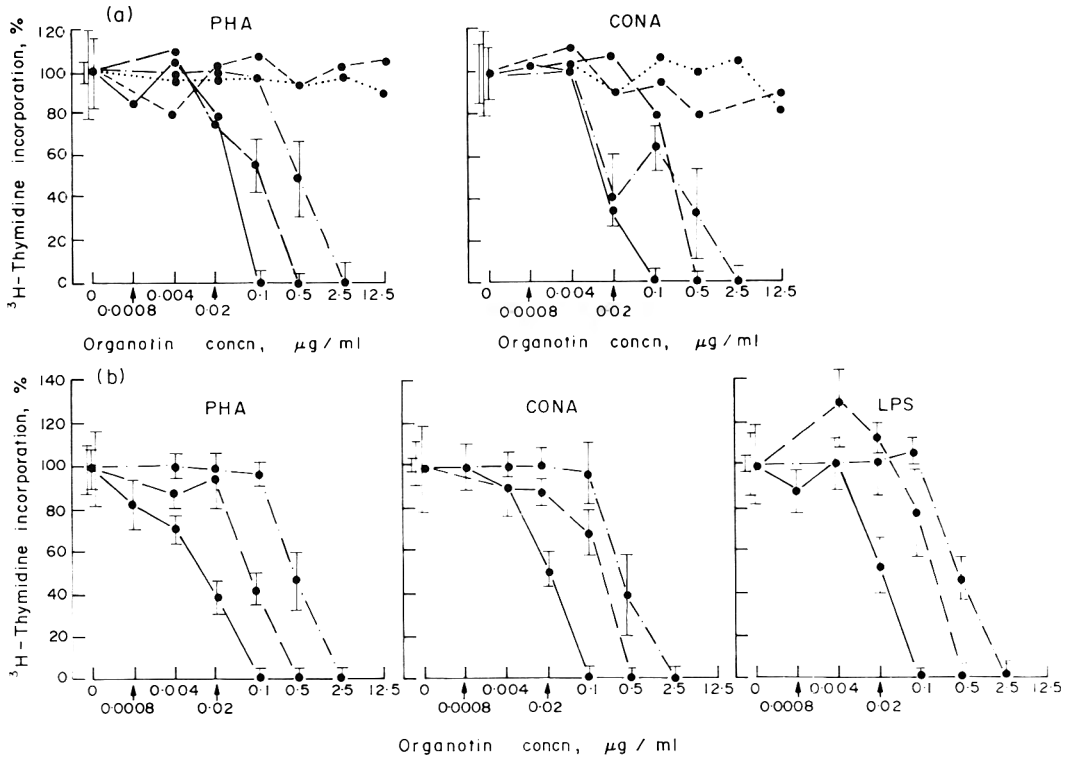

Fig. 2. Effects of various concentrations of CBETC (—·—), CMETC (·····), CETC(— —), DOTC (——) or DBTC (— —) *in vitro* on [^{3}H]thymidine incorporation in rat thymus and spleen cells in response to phytohaemagglutinin (PHA), concanavalin A (Con A) and *Escherichia coli* lipopolysaccharide (LPS). Results are expressed as percentages of [^{3}H]TdR incorporation in the control cultures and vertical bars indicate standard deviations for triplicate cultures.

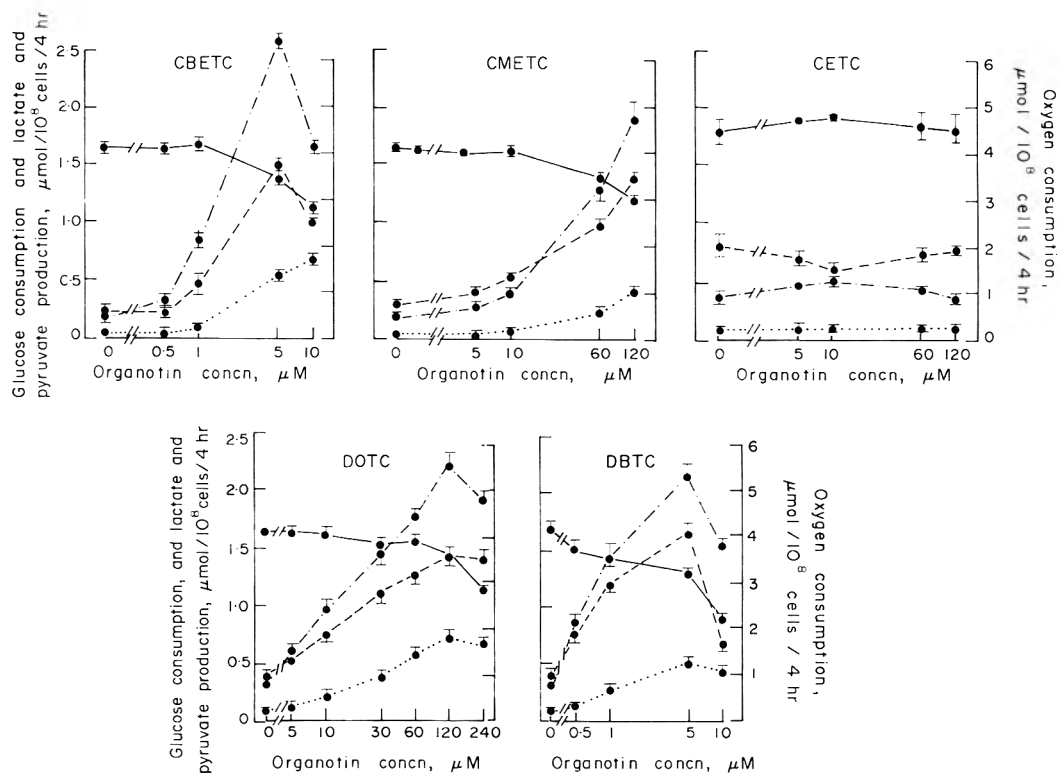

Fig. 3. Consumption of glucose (——) and of oxygen (— —) and production of lactate (—·—) and of pyruvate (·····) by isolated rat thymocytes incubated with various concentrations of CBETC, CMETC, DOTC or DBTC. Results are means ± SEM for at least three incubations each performed in triplicate.

Glucose metabolism studies

The effects of the various dialkyltin- and estertin compounds on glucose consumption, lactate and pyruvate production and the consumption of oxygen in thymocyte cultures are presented in Fig. 3. These graphs indicate an increase in substrate consumption by the thymocytes in the presence of DOTC, DBTC, CBETC and CMETC, the increases being directly related to the dose of organotin. Glucose uptake showed some increase with DOTC, DBTC, CBETC and CMETC concentrations of only 5, 0·5, 1 and 5 μM, respectively. Maximal stimulation of glucose consumption occurred with concentrations of 5 μM DBTC or CBETC and at the much higher concentration of 120 μM for DOTC and CMETC; further increases in the exposure levels progressively decreased glucose consumption. In contrast, CETC in concentrations up to 600 μM, the highest tested, did not interfere with glucose metabolism.

The increased amount of glucose consumed by thymocytes incubated with the various tin compounds was not completely metabolized but was largely converted to lactate and pyruvate (Fig. 3). Thus conversion is mainly by the glycolytic pathway, with little oxidation by the TCA cycle. Lactate and pyruvate production was stimulated maximally at the concentrations of DOTC, DBTC, CBETC and CMETC that also induced maximum glucose consumption.

The oxygen consumption of the thymocytes showed a slight, but dose-related, decrease in incubations both with the dialkyltins and with the estertin compounds, but not with the dicarboxylic acid CETC. At maximum stimulation of the substrate uptake, oxygen consumption was never reduced by more than 30% and at lower organotin concentrations it was only slightly diminished. This is notable, since the accumulation of lactate and pyruvate showed that the increased amount of glucose consumed undergoes little oxidative metabolism.

At organotin levels up to those inducing maximum stimulation of the substrate consumption, the number and viability of rat thymocytes was not affected during the 4-hr incubation period. Cell survival declined at higher exposure levels but after a 4-hr incubation it was never reduced by more than 10% when scored with nigrosin exclusion.

DISCUSSION

In the comparative 2-wk feeding studies in rats, the estertin compounds, CMETC and CBETC, appeared to be much less toxic than the dialkyltin compounds, DBTC and DOTC, while the dicarboxyltin compound, CETC, showed no toxic effects at dietary levels up to 1350 ppm. At the relatively high dietary level of 1350 ppm, the estertins induced a slight but statistically significant growth retardation, associated with a significant decrease in the relative weight of the liver and, in the case of CBETC, also of the thymus and spleen. Histologically, a diminished amount of glycogen was demonstrated in these livers but no other treatment-related organ changes were observed. At dietary levels of 450 ppm the estertins did not alter any of the parameters tested, whereas administration of the dialkyltins at this level causes severe growth retardation and is rapidly fatal (authors' unpublished data, 1981). In the group fed 150 ppm DBTC, two animals died with signs of jaundice. Relative liver weights of this group were increased and proliferation of bile-duct epithelial cells and bile ductules, as originally described by Barnes & Magee (1958), were observed. The most prominent feature of DBTC and DOTC toxicity was their effect on lymphoid tissue. The relative weights of the lymphoid organs showed a dose-related decrease following the feeding of 50 or 150 ppm of either dialkyltin in the diet. Most pronounced was the effect on thymus weight, which was decreased by more than 70% after consumption of 150 ppm DBTC or DOTC for 2 wk. Lymphocyte depletion was observed in all dialkyltin-exposed animals, especially in the thymic cortex. CETC, the hydrolysis product of the estertins, had no effect up to a dietary level of 1350 ppm, on any of the parameters tested.

DBTC also proved the most toxic compound on parenteral administration, causing death in rats given an iv or ip injection of 10 mg/kg. Body-weight reduction and severe lymphoid atrophy were seen even with a dose of 2·5 mg DBTC/kg, whereas parenteral application of 10 mg CBETC or CETC did not affect body weight or the weight of any organ, including the thymus.

From the various *in vitro* studies, it appears that the estertin and dialkyltin compounds tested all affect survival, blast transformation and the glucose metabolism of rat thymocytes, whereas CETC is completely inactive in these test systems.

In suspension cultures of rat thymocytes the viability of the cells decreased as the DBTC, DOTC or CBETC concentration in the nutrient medium increased. The more water-soluble estertin, CMETC, was much less active, decreasing thymocyte survival only at the relatively high concentration of 240 μM. Comparable differences in cytotoxicity were noted between the lipophylic dialkyltin homologues DBTC and DOTC and the more hydrophylic dimethyltin dichloride (Seinen *et al.* 1977b). The latter slightly decreased thymocyte survival at a concentration of 120 μM (26 μg/ml), whereas the lipophylic estertin and dialkyltin compounds showed cytotoxicity at a concentration of 0·5 μg/ml medium.

Blast transformation of rat lymphocytes, measured by [^{3}H]TdR incorporation, was completely inhibited at concentrations as low as 0·1, 0·5 and 2·5 μg/ml for the dialkyltins DBTC and DOTC and the estertin CBETC, respectively, whereas CMETC and CETC up to a level of 12·5 μg/ml did not affect DNA-replication of rat lymphocytes.

The estertins and dialkyltins were similar in their impairment of glucose metabolism in rat thymocytes *in vitro*. DBTC and CBETC were the most active, inducing maximum stimulation of glucose consumption at a level of 5 μM. At higher levels of exposure to these organotins a marked inhibition of thymocyte metabolism was observed. A sharp fall in glucose consumption was accompanied by marked inhibition of thymocyte oxygen consumption and by a decrease in cell viability. The effects of DOTC and CMETC were essentially the same as those of DBTC and CBETC but occurred at much higher concentrations, thymocyte glucose consumption being stimulated maximally

by these compounds at a level of 120 μM. In contrast, CETC, the hydrolysis product of the estertin compounds, did not interfere with glucose metabolism in the thymocytes or with any other parameter tested *in vitro*.

Penninks & Seinen (1980) have indicated that the inhibition of energy metabolism in rat thymocytes *in vitro* is related to the thymolytic effects of these organotins *in vivo*. Therefore, it is surprising that the estertins, which display *in vitro* lymphocytotoxic effects comparable to those of the dialkyltins, do not induce lymphoid atrophy when administered *in vivo* to rats. Probably, *in vivo*, the estertins are detoxified effectively. The simplest mechanism for such a detoxification would be a hydrolysis reaction, a possibility suggested by the finding that CETC, the dicarboxylic acid formed by the hydrolysis of estertins, was found to be inactive in both the *in vivo* and *in vitro* test systems. This hypothesis is supported by preliminary metabolism studies with CBETC. Thin-layer chromatography of the urine of CBETC-exposed rats showed CETC to be the major metabolite.

From this study we conclude that the bis-(β-carbomethoxyethyl)- and bis-(β-carbobutoxyethyl)tin stabilizers are less toxic than the di-*n*-butyl- and di-*n*-octyltin stabilizers. Although there are many similarities between the effects of these compounds *in vitro*, only the estertins are effectively detoxified *in vivo*.

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ENZYME-MEDIATED MUTAGENICITY IN *SALMONELLA TYPHIMURIUM* OF CONTAMINANTS OF SYNTHETIC INDIGO PRODUCTS

W. M. F. JONGEN and G. M. ALINK

Department of Toxicology, Agricultural University, De Dreijen 12, 6703 BC Wageningen, The Netherlands

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Abstract—The mutagenic potential of two natural and seven synthetic, commercial indigo dye products was investigated. The natural products showed no mutagenicity in *Salmonella typhimurium* strains TA98 and TA100. In the presence of rat-liver homogenate from Aroclor 1254 pretreated rats all of the synthetic products were mutagenic towards strain TA98 but not towards strain TA100. The mutagenic effect produced was highly dependent on the amount of rat-liver homogenate added. Because of its high mutagenic potential, one product was further investigated. In the presence of rat-liver homogenate this product was weakly mutagenic towards strain TA1537 and strongly mutagenic towards strain TA1538. No mutagenicity was observed in strain TA1535. Experiments with purified synthetic indigo and natural indigo revealed that the mutagenic activity of the synthetic commercial products can be ascribed to one or more contaminants.

INTRODUCTION

Indigo [($\Delta^{2,2'}$-biindoline)3,3'-dione] is one of the earliest known naturally occurring dyes. Originally it was obtained from the plants *Indigofera tinctoria* and *Isatis tinctoria* where it occurs as a glycoside of indoxyl. At the beginning of this century the extraction of indigo from plants was superseded by chemical synthesis. Nowadays this compound is mainly used in clothing industries. Because of its strong colouring ability and the striking changes of colour it shows in different solvents the chemistry of indigo has been well studied (Schweizer, 1964).

As far as we know, no data have yet been published concerning the possible genotoxic effects of indigo. Contradictory data have been published concerning the chemically related dye indigo carmine. Hansen, Fitzhugh, Nelson & Davies (1966) found that it was tumorigenic in rats following sc injection, but did not produce tumours in feeding experiments. Price, Suk, Freeman *et al.* (1978) showed that indigo carmine induced malignant cell transformation in Fischer rat embryo cells. The same authors (*loc. cit.*) reported an increase in tumour incidence in Graffi hamsters after sc injection. However Brown, Roehm & Brown (1977) and Auletta, Kuzava & Parmar (1977) observed a lack of mutagenicity in the Salmonella/mammalian-microsome assay. The present study concerns the mutagenic potential of indigo (indigotin: C.I. no. 73000) using the Salmonella/mammalian-microsome assay. Several batches of both natural and synthetic indigo were investigated both in the presence and absence of rat-liver homogenate.

EXPERIMENTAL

Chemicals. The different batches of synthetic indigo were purchased from: Riedel de Haen AG, Hannover, FRG (A); BDH Chemicals Ltd, Poole, England (B); Fluka AG, Bucks SG, Switzerland (C); Pfaltz & Bauer Inc., Stamford, CT, USA (D); Kodak, Rochester, NY, USA (E); ACF, Maarsen, (F); Serva, Heidelberg, FRG (G). Purified synthetic indigo was kindly provided by ACF, and natural indigo was obtained from Pfaltz & Bauer (I) and ACF (II). DMSO used as a solvent for indigo was obtained from E. Merck AG, Darmstadt, FRG. The NADPH-generating system contained $MgCl_2$ (Merck) and the cofactors NADP and glucose-6-phosphate from Boehringer Mannheim GmbH (Mannheim, FRG). Ethidium bromide (used as a positive control) was obtained from Sigma (St. Louis, MO, USA), 4-nitroquinoline-*N*-oxide (4-NQO) from Fluka AG, nutrient broth from Oxoid (Basingstoke, England) and Aroclor 1254 from Monsanto (St. Louis, MO, USA). All other compounds were of analytical grade.

Preparation of S-9 mix. Three-month-old male Wistar rats were pretreated with Aroclor 1254, to induce their drug-metabolizing enzymes, according to Ames, McCann & Yamasaki (1975). The rats were killed by decapitation and their livers were removed aseptically, washed with sterile ice-cold 0·15 M-KCl solution and homogenized in three volumes of KCl (3 ml/g rat liver). The homogenates were pooled and centrifuged for 15 min at 9000 g. The supernatant (S-9) was collected, divided into 3-ml aliquots and stored in sterile polypropylene vials in liquid nitrogen.

Mutagenicity assay. The experiments were performed using the standard plate assay of Ames *et al.* (1975). Bacteria were grown in an Erlenmeyer flask containing 10 ml nutrient broth (Oxoid No. 2, fortified with 10 μg L-histidine.HCl/ml) in a shaking incubator at 37°C until the suspension had reached a density of about 2×10^9 bacteria/ml (measured as optical density at 700 nm). From this suspension 0·1 ml was added to the molten agar overlayer together with appropriate amounts of the other components and poured onto minimal agar petri dishes. In all experi-

Table 1. *The mutagenicity in* Salmonella typhimurium *strains TA98 and TA100 of seven batches of commercial synthetic indigo in the presence of varying amounts of S-9 in the mix*

		Mutagenicity in strain ... (no. of revertants/plate)*					
		TA98			TA100		
Test substance	S-9 (μl/plate) ...	0	30	150	0	30	150
Control		21	18	23	113	143	138
Indigo†	A	16	1041	225	111	198	198
	B	20	91	53	108	138	127
	C	16	113	46	82	126	133
	D	22	133	71	113	124	126
	E	21	74	39	98	132	138
	F	12	924	220	103	190	176
	G	20	127	69	85	133	117
4-NQO‡		612	—	—	> 2000	—	—
Ethidium bromide§		—	261	—	—	—	—

4-NQO = 4-Nitroquinoline-*N*-oxide

*Values are means for three plates.
†To each plate was added 3·8 μmol indigo.
‡0·5 μg/plate.
§5 μg/plate.

ments where the NADPH-generating system (S-9 mix) was used the method described by Ames *et al.* (1975) was followed. All experiments were performed in triplicate and each experiment was repeated at least once.

RESULTS

Table 1 shows the results of experiments with several batches of synthetic indigo. None of the compounds showed mutagenicity in the absence of S-9 mix. On addition of S-9 mix, all products showed a mutagenic effect in strain TA98 varying from a fourfold to a 60-fold increase in the number of revertants compared with the control but did not show mutagenicity in strain TA100. The results of experiments with natural products and purified synthetic indigo are presented in Table 2. Using concentrations of up to 19·1 μmol/plate, with and without the addition of S-9 mix no mutations were generated either with the natural or the purified synthetic product. These data suggest that the mutagenic factors originate from one or more contaminants, present in the synthetic products.

On the basis of its high mutagenic potential in strain TA98 (Table 3) one batch of synthetic indigo (F) was chosen for further research. Table 4 shows the influence of different amounts of S-9 mix on the mutagenicity of batch F strain TA98. The amount of S-9 appeared to be critical for all of the synthetic products (Table 1). Table 5 shows the results obtained with the other strains used. On addition of S-9 mix a weakly positive response was found in TA1537 which was clearly dose related. A strong positive effect was found in TA1538. The decrease in the number of revertants in both of these strains at the highest dose level may indicate a cytotoxic effect. No mutagenic response was observed in strain TA1535 with or without metabolic activation.

Table 2. *The mutagenicity in* Salmonella typhimurium *strain TA98 of some natural and synthetic indigo products in the presence and absence of S-9 mix*

Test substance	Purity (%)	Amount of product added (μmol/plate)	Mutagenicity in strain TA98 (no. of revertants plate)*	
			− S-9	+ S-9†
Control	—		42	47
Natural indigo I	Unknown	19·1	42	53
Natural indigo II	12%	19·1	35	26
Synthetic indigo (F)	92%	1·9	44	713
Synthetic indigo (pure)	99·9%	19·1	29	35
4-NQO‡	—	—	746	—
Ethidium bromide§	—	—	—	295

4-NQO = 4-Nitroquinoline-*N*-oxide

*Values are means for three plates.
†To each plate was added 0·3 ml S-9 mix.
‡0·5 μg/plate.
§5 μg/plate.

Table 3. *The mutagenicity of a synthetic indigo product (batch F) in* Salmonella typhimurium *strain TA98 in the presence and absence of S-9 mix*

Test material	Concentration (μmol/plate)	Mutagenicity in strain TA98 (no. of revertants/plate)* +S-9†	−S-9
Control	0	40	36
Indigo (batch F)	0·4	147	32
	1·0	480	27
	2·5	842	34
	4·0	1352	29
	10·0	471	21
4-NQO‡		—	801
Ethidium bromide§		317	—
DMSO‖		39	42

DMSO = Dimethylsulphoxide
4-NQO = 4-Nitroquinoline-*N*-oxide

*Values are means of three plates.
†To each plate was added 0·3 ml S-9 mix.
‡0·5 μg/plate.
§5 μg/plate.
‖0·25 ml/plate.

Table 4. *The effect of varying the amount of S-9 in the mix added on the mutagenicity in* Salmonella typhimurium *strain TA98 of a synthetic indigo product (batch F)*

Amount of S-9 added (μl/plate)	Mutagenicity of synthetic indigo (batch F) in strain TA98 (no. of revertants/plate)*
0	40
6	100
12	292
24	945
30	1190
60	904
120	437
240	227

To each plate was added 3·8 μmol synthetic indigo (batch F). Values are means of three plates.

DISCUSSION

Both purified indigo and natural indigo products appeared to be non-mutagenic in the *S. typhimurium* strains used. However all of the synthetic indigo products contained impurities which showed enzyme-mediated mutagenicity, but only in the strain detecting frameshift mutagens. The amount of S-9 in the mix added was critical for all of the products. These data suggest the presence of one or more structurally related contaminants in commercial indigo products.

The finding that the compound itself is not responsible for the mutagenic activity may also be relevant to the studies on indigo carmine. Price *et al.* (1978) used technical grade products and the discrepancy between their positive findings and the lack of mutagenicity observed by Brown *et al.* (1977) and Auletta *et al.* (1977) may be explained by the presence of one or more contaminant(s).

This study indicates that pure indigo is not mutagenic in the Salmonella/mammalian-microsome assay. Although differences in synthesis procedures may explain the variations in mutagenicity between different products, all of the products investigated showed considerable mutagenic activity. Indigo from plant origin might provide a non-mutagenic alternative.

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Table 5. *The mutagenicity of a synthetic indigo product (batch F) in* Salmonella typhimurium *strains TA1535, TA1537 and TA1538 in the presence and absence of S-9 mix*

Test material	Concentration (μmol/plate)	Mutagenicity in strain ... (no. of revertants/plate)* TA1535 +S-9	TA1535 −S-9	TA1537 +S-9	TA1537 −S-9	TA1538 +S-9	TA1538 −S-9
Untreated	0	20	14	15	22	13	13
Indigo (batch F)	0·4	20	17	34	23	118	16
	1·0	29	22	48	31	314	15
	3·8	21	21	75	31	959	22
	19·1	18	17	52	16	462	22
4-NQO‡		—	100	—	82	—	360
DMSO§		17	11	13	20	14	10

*Values are means of three plates.
†To each plate was added 0·3 ml S-9 mix.
‡0·5 μg/plate.
§0·5 ml/plate.

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PERCUTANEOUS ABSORPTION OF 2-AMINO-4-NITROPHENOL IN THE RAT

H. HOFER, G. W. SCHWACH and CH. FENZL

Institut für Biologie, Österreichisches Forschungszentrum Seibersdorf, A-2444 Seibersdorf, Austria

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Abstract—The absorption of ^{14}C-labelled 2-amino-4-nitrophenol (ANP) in two hair dyeing formulations was investigated after application to the skin of rats. After 1 and 5 days, 0·21 and 0·36%, respectively, of the administered radioactivity was absorbed from formulation 1 which contained carboxymethylcellulose as a thickening agent. Absorption was greater (1·12% after 1 day, 1·67% after 5 days) from formulation 2 which contained oleic acid and isopropanol. Complementary studies of absorption after administration of [^{14}C]ANP by sc injection or oral gavage were also carried out. The radioactivity was rapidly excreted, predominantly in the urine, in both cases. Biliary excretion was also detected in an oral study.

INTRODUCTION

2-Amino-4-nitrophenol (ANP) is a constituent of hair dyeing formulations. The studies reported here were designed mainly to obtain data on the percutaneous absorption of ANP from two formulations under approximately realistic conditions. Complementary studies were carried out with subcutaneous and oral administration. ^{14}C-labelled ANP was used in order to simplify the methodology.

EXPERIMENTAL

Materials

Using [^{14}C]benzene as precursor, ANP, labelled in the ring, was synthesized at the Institute of Chemistry, Österreichisches Forschungszentrum Seibersdorf.

Formulation 1 was prepared and had the following composition: [^{14}C]ANP, 1·54%; Relatin-soaking (Relatin 25,000, Henkel AG, Düsseldorf, FRG, 4% in water), 50%; 25% ammonia solution (E. Merck AG, Darmstadt, FRG), 4·5%; distilled water, to 100%. ANP was suspended in water plus ammonia solution, in which it partially dissolved, and then mixed into the Relatin-soaking (carboxymethylcellulose as a thickening agent).

Formulation 2 was prepared and had the following composition: [^{14}C]ANP, 0·77%; 1,4-phenylenediammonium dichloride (E. Merck AG), 2·52%; 2,4-diaminoanisole sulphate (Kuhlmann, FRG), 1·7%; resorcinol (E. Merck AG), 0·85%; oleic acid (E. Merck AG), 2·5%; isopropanol (E. Merck AG), 1·5%; sodium sulphite (E. Merck AG), 0·10%; 25% ammonia solution (E. Merck AG), 6%; distilled water, to 50%; 6% hydrogen peroxide solution (diluted 30% Perhydrol, E. Merck AG), 50%. ANP, oleic acid and isopropanol were mixed at room temperature. The other dyes were mixed with water at 60°C, 25% ammonia solution was added and the mixture was stirred into the first solution. Sodium sulphite was added as a 3% aqueous solution. Immediately before application to the skin of the rats, individual portions for each animal were mixed with equal amounts of hydrogen peroxide solution.

Animals

Male and female Sprague–Dawley rats (Him: OFA(SD)SPF, 5 wk old) were used. They were kept at 24°C in metabolism cages and had free access to water and feed (Altromin GmbH, Lippe, FRG).

Experimental design

Cutaneous application, 24 hr examination. This investigation was carried out to investigate rapid percutaneous absorption and the ^{14}C content of the expired air, which was determined only in this experiment. Each of the formulations was applied to five male and five female rats.

One day before application of the hair dye the animals were anaesthetized with ether and their dorsal hair was clipped. A 30 × 30-mm area was marked. Before treatment the animals were anaesthetized with urethane and were kept in this state until the end of the experiment. An aliquot (0·5 g corresponding to about 0·2 mCi ^{14}C) of one of the hair dyeing formulations was applied to the marked area and spread with a spatula. It was left for 30 min and then was rinsed off with 100 ml of a 3% solution of hair shampoo and 100–200 ml of distilled water. Rinsings were collected. The skin was dabbed dry with absorbent tissue and then covered with gauze to avoid contamination of the cages. Air-tight all-glass metabolism cages were used to collect urine and faeces from individual rats. Air (20 litres/hr) was circulated through each of the ten cages and then through gas-washing bottles filled with ethanolamine to trap CO_2. The air was then passed through a further washing bottle that was common to all ten air lines. After 24 hr the animals were killed, the treated area of skin was removed and the remaining carcass was homogenized.

Cutaneous application, 5 days examination. The treatment was as described above except for a shorter

Abbreviations: ANP = 2-Amino-4-nitrophenol.

duration of anaesthesia. Each of the formulations was applied to ten females. The treated area was covered with an elastic-type bandage and a frill made of cardboard was fitted to the animals to prevent their licking the treated area and to avoid contamination of urine and faeces by skin scabs. Urine and faeces were collected daily from the metabolism cages.

Subcutaneous administration, 5 days examination. Two millilitres of a 0·2% solution of [^{14}C]ANP in neutral, physiological saline was administered sc in the dorsal region of nine male and nine female rats. The radioactivity administered was about 0·01 mCi per animal. The animals were housed three to a metabolism cage and samples from the three animals in each cage were pooled. The tissue around the injection site was removed at the end of the study and analysed for radioactivity separately from the remainder of the carcass.

Oral administration, 5 days examination. The experimental design was much the same as for the subcutaneous study. The ANP solution (0·2% in physiological saline) was intubated by gavage. At the end of the experiment the gastro-intestinal tract was removed and its radioactivity was determined separately from the remainder of the carcass.

Oral administration, elimination in bile. Three male and three female rats were used. A tracheal tube and a gavage tube were fixed to the anaesthetized animals. A polyvinyl chloride tube was inserted into the bile duct, the abdominal cavity was closed by two ligatures and 30 min afterwards 2 ml of a 0·2% solution [^{14}C]ANP in physiological saline was administered by gavage. Bile was collected for 3 hr.

^{14}C determination

For determination of expired ^{14}C in expired air 2 ml of ethanolamine was mixed directly with a scintillator containing 10 ml methanol, 7 ml toluene, 7 mg *p*-bis-(*o*-methylstyryl)benzene and 105 mg 2,5-diphenyloxazole. All other samples were homogenized and combusted in a Packard Sample Oxidizer. A liquid-scintillation counter (Packard model 3375) with automatic external standardization was used for determination of radioactivity in the samples, which was calculated as percentage of the administered ^{14}C activity. Detection limits (mean counts/min + 2SD) were taken from control experiments.

RESULTS AND DISCUSSION

Cutaneous application

The treated skin contained about double the amount of ^{14}C after dyeing with formulation 2 than with formulation 1 (Table 1). This was true for both the 1-day and the 5-day experiment. Absorption of ^{14}C from formulation 2 was significantly greater than from formulation 1. [This absorption was calculated as the combined radioactivity of the urine, faeces, expired air (where analysed) and carcass without the treated area of skin.] As expected significantly greater absorption was observed after 5 days than after 1 day, but the amount of ^{14}C absorbed was small in each case. Therefore it seems that ANP in the Relatin-containing formulation 1 can be more easily washed off or can penetrate the skin less easily than that in formulation 2.

Table 1. *Distribution of ^{14}C activity 24 hr and 5 days after cutaneous application to rats of two hair dyeing formulations containing ^{14}C-labelled 2-amino-4-nitrophenol*

Hair dyeing formulation	^{14}C activity (% of administered dose) in						Absorbed ^{14}C activity*
	Rinsing water	Skin, area of application	Remaining carcass	Expired air	Urine	Faeces	
After 24 hr							
1	81·2 ± 3·7	7·7 ± 2·2	0·044 ± 0·017	UD	0·152 ± 0·046	0·0153 ± 0·0085	0·211 ± 0·046
2	87·4 ± 8·1	17·8 ± 5·1	0·148 ± 0·034	0·00111 ± 0·00042	0·92 ± 0·26	0·049 ± 0·042	1·12 ± 0·25
After 5 days							
1	85·5 ± 8·5	10·2 ± 4·2	0·032 ± 0·012	ND	0·283 ± 0·097	0·044 ± 0·019	0·36 ± 0·10
2	70·2 ± 8·4	19·3 ± 3·7	0·085 ± 0·024	ND	1·32 ± 0·50	0·269 ± 0·087	1·67 ± 0·54

UD = Not detected (detection limit 0·0007%) ND = Not done

*Absorbed ^{14}C was calculated as the sum of the radioactivity detected in the carcass (without the treated area of skin), expired air (24-hr experiment only), urine and faeces.

Values are means ± 1 SD for groups of ten rats.

Table 2. *^{14}C activity in urine and faeces after cutaneous application to rats of two hair dyeing formulations containing ^{14}C-labelled 2-amino-4-nitrophenol*

Period after administration (days)	Formulation ...	^{14}C activity (% of administered dose) in Urine		Faeces	
		1	2	1	2
0–1		0·237	1·21	0·0127	0·090
1–2		0·026	0·057	0·010	0·091
2–3		0·0099	0·025	0·0069	0·039
3–4		0·0057	0·0136	0·0039	0·0214
4–5		0·0048	0·0106	0·0066	0·026

Values are means for groups of ten rats.

Table 3. *Distribution of ^{14}C activity 5 days after administration of ^{14}C-labelled 2-amino-4-nitrophenol to rats by sc injection or oral gavage*

Route of administration	^{14}C activity (% of administered dose) in Site of injection	Gastro-intestinal tract	Remaining carcass	Urine	Faeces
Sc injection	0·37 ± 0·17	—	0·80 ± 0·18	58 ± 22	42 ± 25
Oral gavage	—	0·39 ± 0·41	0·59 ± 0·34	68·3 ± 9·4	25·4 ± 6·9

Each value is the mean ± 1 SD for six groups of three rats. (The rats were housed three to a cage and the results for the rats in each cage were pooled.)

Very low amounts of ^{14}C in the expired air, which were close to the detection limit, were observed after cutaneous application of formulation 2. This could be due to volatile contaminants or to metabolic degradation to CO_2.

Excretion of absorbed ^{14}C was mainly and rapidly in the urine and to a lesser extent and more slowly in the faeces (Table 2).

Subcutaneous and oral administration

[^{14}C]ANP was almost entirely absorbed from the site of sc injection. Excretion of ^{14}C was predominantly in the urine (Table 3). After 1 day 89% of the administered ^{14}C had already been eliminated.

At least 68·9% of orally administered [^{14}C]ANP is absorbed within 5 days (Table 3); this minimum absorption is calculated from the amount of ^{14}C detected in the urine and in the carcass without the gastro-intestinal tract. Radioactivity in the faeces could also possibly originate from absorbed ^{14}C since within 3 hr of administration 4·4 ± 2·2% (mean ± 1 SD, n = 6) of the administered ^{14}C was eliminated in the bile. As with sc administration, excretion occurred predominantly in the urine and mostly during the first day after administration.

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REVERSIBILITY OF NEPHROTOXICITY INDUCED IN RATS BY NITRILOTRIACETATE IN SUBCHRONIC FEEDING STUDIES

M. C. MYERS, R. L. KANERVA, C. L. ALDEN and R. L. ANDERSON
The Procter & Gamble Company, Miami Valley Laboratories, P.O. Box 39175, Cincinnati, OH 45247, USA

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Abstract—The reversibility of nitrilotriacetate (NTA)-associated nephrotoxicity was investigated by comparing renal tissues from rats fed nephrotoxic levels of NTA for 7 wk with those from rats allowed 5 wk of recovery after the 7-wk exposure. In addition the toxicity of 2% $Na_3NTA.H_2O$ in the diet (73 μmol/g diet) was compared with that of 1·5% H_3NTA (79 μmol/g diet). The two forms of NTA induced comparable renal tubular cell toxicity which was characterized by proximal convoluted cell vacuolation and hyperplasia. These effects were noted in all of the exposed animals although the extent of damage varied. This specific renal tubular cell toxicity was completely reversed during the 5-wk recovery period. Renal pelvic transitional cell toxicity was induced primarily by $Na_3NTA.H_2O$. Renal pelvic toxicity was characterized by hydronephrosis, and erosion, ulceration and hyperplasia of the transitional epithelium. All forms of renal toxicity except that accompanying hydronephrosis were reversed when $Na_3NTA.H_2O$ feeding was discontinued.

INTRODUCTION

Nitrilotriacetate (NTA) is an amino-carboxylate ligand that can be used as a phosphate replacement in laundry detergents. In rodents ingestion of NTA has been shown to be associated with nephrotoxicity after a single oral dose given by gavage and in subchronic and chronic feeding studies (for review see Anderson, Alden & Merski, 1982). In the National Cancer Institute (NCI) chronic feeding study (NCI, 1977) NTA ingestion was also associated with renal tubular cell and renal pelvic transitional cell tumours at high dietary levels. Renal tubular cell carcinogenicity has also been reported by Goyer, Falk, Hogan *et al.* (1981).

Alden & Kanerva (1982) and Squire (1981) have proposed that renal tubular cell tumorigenesis associated with exposure to high doses of NTA is the end result of a defined sequence of toxic events in the renal tubular cells that are dependent on both NTA dose and time. The proposed toxic sequence leading to tubular cell tumours has both an NTA-specific component that is characterized by a progressive hyperplastic response and a nonspecific component that is manifested as an exacerbation of the glomerular nephrosis that occurs in ageing rats. The NTA-specific toxic sequence has been divided into four stages: (1) tubular cell cytoplasmic vacuolation, (2) simple hyperplasia comprised of cells with cytoplasmic vacuoles, (3) nodular hyperplasia comprised of cells with cytoplasmic vacuoles and (4) adenomatous hyperplasia. The hallmark of the nonspecific toxicity is tubular hyperplasia comprised of cells with cytoplasmic basophilia but not vacuoles. Similarly, tumorigenic levels of NTA have been shown to induce toxic effects in the renal pelvis that are characterized by hydronephrosis and urothelial erosion, ulceration and hyperplasia. The urothelial toxic response has also been causally associated with the pelvic urothelial tumorigenic response by Alden, Kanerva, Anderson & Adkins (1981).

The present report describes the reversibility of renal tubular cell lesions that had progressed to nodular hyperplasia after 7 wk of feeding diets containing tumorigenic levels of NTA to weanling rats. Administration of an NTA-free diet for 5 wk resulted in the complete reversal of all of the lesions induced by NTA except in severe cases of hydronephrosis.

EXPERIMENTAL

Male Charles River (Sprague–Dawley derived) rats were obtained as weanlings from Charles River Breeding Laboratories, Inc., Wilmington, MA and were individually housed in stainless steel metabolism cages in a temperature- and humidity-controlled room with a 12-hr light/dark cycle. Feed and distilled water were provided *ad lib.* The control animals were given a diet of ground Purina laboratory chow (Ralston Purina Co., St Louis, MO), while test animals were given Purina laboratory chow containing 1·5% nitrilotriacetic acid (H_3NTA; 79 μmol/g diet) or 2% trisodium nitrilotriacetate monohydrate ($Na_3NTA.H_2O$; 73 μmol/g diet). [$Na_3NTA.H_2O$ was obtained from Monsanto, St Louis, MO and H_3NTA was prepared by HCl treatment of $Na_3NTA.H_2O$.] The animals were divided into recovery and non-recovery groups. Animals in the non-recovery groups were killed after 49 days of treatment. After 49 days of NTA exposure the recovery group animals were given the control diet for a further 35 days and then killed.

Throughout the study weekly weight gains and feed consumption were measured. At the end of the NTA-

Abbreviations: H & E = Haematoxylin and eosin; NTA = nitrilotriacetate; PAS = periodic acid–Schiff; PCT = proximal convoluted tubule; TE = transitional epithelium.

exposure or recovery periods the rats were anaesthetized with sodium pentobarbital (100 mg/kg body weight) and the left kidney was fixed by retrograde vascular perfusion through the descending aorta. When perfusion was unsuccessful, fixation was completed by immersion. A 1% glutaraldehyde 4% formaldehyde mixture in 0·1 M-phosphate buffer was used for fixation (McDowell & Trump, 1976). The perfused kidney was excised and processed by routine histological methods for paraffin embedding. Longitudinal midsagittal sections were stained with haematoxylin and eosin (H & E) and a representative section from each kidney was randomly selected and the number and grade of pathological alterations in each section were recorded. Sections from all well-perfused kidneys from each treatment group were selected and stained with periodic acid–Schiff (PAS) reagent.

RESULTS

The amounts of NTA consumed by the two treatment groups were approximately equivalent (Table 1). The two forms of NTA resulted in comparable decreases in growth rate during the exposure phase of the study. During the recovery phase the animals that had been exposed to NTA gained more weight than the controls, primarily as a result of increased feed consumption.

In the renal cortex the predominant responses to NTA treatment were vacuolar and hyperplastic alterations of the epithelium of the proximal convoluted tubules (PCTs). The development of tubular epithelial cell vacuoles occurred in response to both 1·5% H_3NTA and 2% $Na_3NTA.H_2O$ treatments. This effect was characterized by the occurrence of well-delineated clear round spaces in the epithelial cell cytoplasm (Fig. 1). The degree of vacuolation within a tubule cross-section was variable, ranging from a few of the cells being affected to generalized involvement. The number of convoluted tubules affected in an individual animal varied from the usual case of an occasional focus to the diffuse involvement of the majority of tubules. PAS staining of well-perfused kidneys from animals treated with NTA showed a PAS-positive brush border in vacuolated tubules which suggested the primary involvement in the PCTs. Normal PCT epithelium showed many PAS-positive intracytoplasmic granules, presumably lysosomes, which were not evident in vacuolated PCTs.

Tubules with NTA-induced vacuolation were frequently hyperplastic. Cross-sections of the hyperplastic tubules were characterized by increased numbers of vacuolated cells arranged in one or two cell layers. Generally the affected tubules were of slightly larger diameter than normal tubules. Karyomegaly was observed frequently, but mitotic figures were uncommon. Sometimes groups of affected tubules were arrayed linearly, suggesting the involvement of an extended portion of a single nephron. Tubules more severely affected by hyperplasia had significantly increased diameters compared with normal tubules. They were variable in appearance, some having greatly dilated lumina and others with the lumen absent or completely filled with vacuolated cells. Tubules that had a diameter more than twice that of a normal PCT were classed as tubular hyperplastic nodules of the vacuolated cell type (Figs 2 & 3).

Ingestion of H_3NTA resulted in a slightly greater incidence of vacuolated cell nodular hyperplasia (Table 2). The increased incidence of PCT-cell cytoplasmic vacuolation associated with ingestion of $Na_3NTA.H_2O$ was primarily observed in four animals with hydronephrosis (Table 1). Kidneys from every animal fed NTA for 7 wk contained one or more tubules with vacuolar alterations whereas none were observed in the kidneys of control animals. The kidneys of rats allowed 5 wk recovery after NTA treatment showed no vacuolar alterations in the PCTs, indicating that these specific manifestations of NTA-induced renal toxicity are completely reversible even when they have progressed to the state of nodular hyperplasia (Table 2).

After the 49-day treatment period foci of tubular hyperplasia with cellular cytoplasmic basophilia were observed in control animals, and there was an increased incidence of such lesions in animals given either form of NTA (Table 2). Therefore the effect of NTA was an exacerbation of lesions commonly associated with age-related nephrosis, and is interpretable as a nonspecific effect associated with NTA ingestion (Gray, 1977). The affected tubules were lined by one or two layers of nonvacuolated epithelium with cytoplasmic basophilia and had obliterated lumina. Cell nuclei were normal in size or enlarged, and mitotic figures were rarely observed. Tubules that had a diameter more than twice that of a normal tubule were regarded as hyperplastic nodules of the basophilic cell type (Fig. 4).

The total incidence of basophilic cell hyperplasia was greater in the animals ingesting $Na_3NTA.H_2O$ than in those given H_3NTA. However, the incidence of nodular lesions was similar in both NTA groups.

Table 1. *Overall weight gain and incidence of hydronephrosis in male rats given nitrilotriacetate in the diet for 49 days with or without a subsequent 35-day recovery period*

Dietary level of NTA	NTA feeding period (49 days)			Recovery period (35 days)	
	Weight gain (g)*	NTA ingested (mmol 49 days)	Hydronephrosis†	Weight gain (g)*	Hydronephrosis†
0 (control)	274 ± 17 (6)	0	0/6	113 ± 15 (6)	0/6
1·5% H_3NTA	197 ± 21 (8)	54	0/8	130 ± 23 (8)	0/6
2% $Na_3NTA.H_2O$	193 ± 32 (7)	49	4/7	164 ± 22 (8)	3/8

NTA = Nitrilotriacetate

*Mean ± SEM for the number of rats indicated in brackets.

†No. of animals affected no. examined.

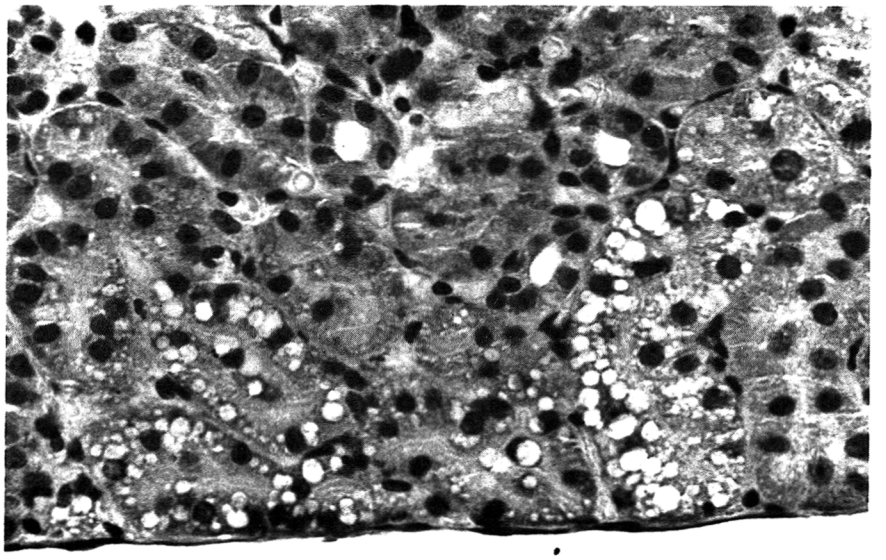

Fig. 1. Vacuolization in the subcapsular proximal convoluted tubules of a male rat fed 2% $Na_3NTA.H_2O$ in the diet for 49 days. Immersion fixed and H & E stained. × 400.

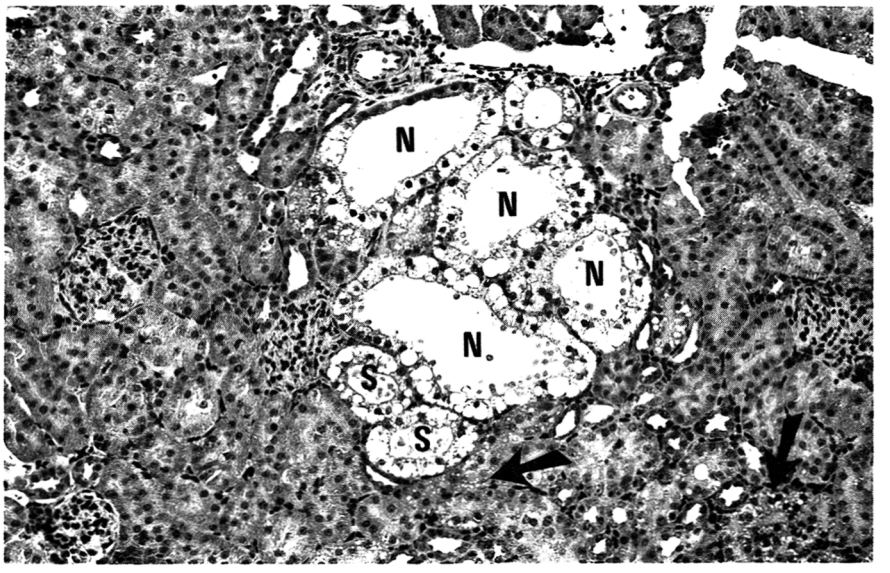

Fig. 2. A focus of hyperplastic convoluted tubules surrounded by apparently normal renal parenchyma. The transition from simple (S) to nodular (N) hyperplasia is apparent within this focus. A few adjacent tubules (arrowed) are slightly vacuolated. From a male rat given 2% $Na_3NTA.H_2O$ in the diet for 49 days. Immersion fixed and H & E stained. × 140.

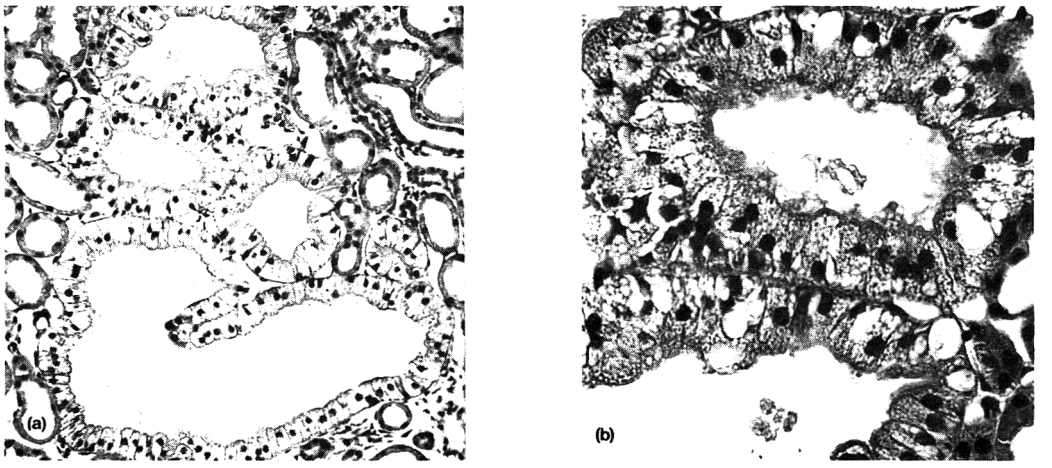

Fig. 3. (a) A focus of vacuolated cell nodular hyperplasia; the adjacent cortical parenchyma appears normal (× 140). (b) At a higher magnification (× 300) the clear reticulated appearance of severely vacuolated and hyperplastic tubular epithelium can be observed. From a male rat given 1·5% H_3NTA in the diet for 49 days. Perfusion fixed and H & E stained.

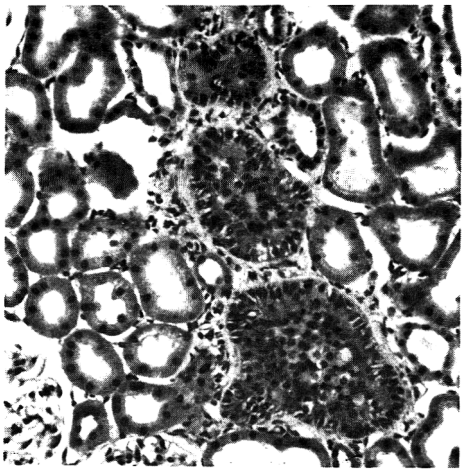

Fig. 4. A focus of nodular hyperplasia with basophilic cells. From a male rat given 1·5% H_3NTA in the diet for 49 days. Perfusion fixed and H & E stained. × 200.

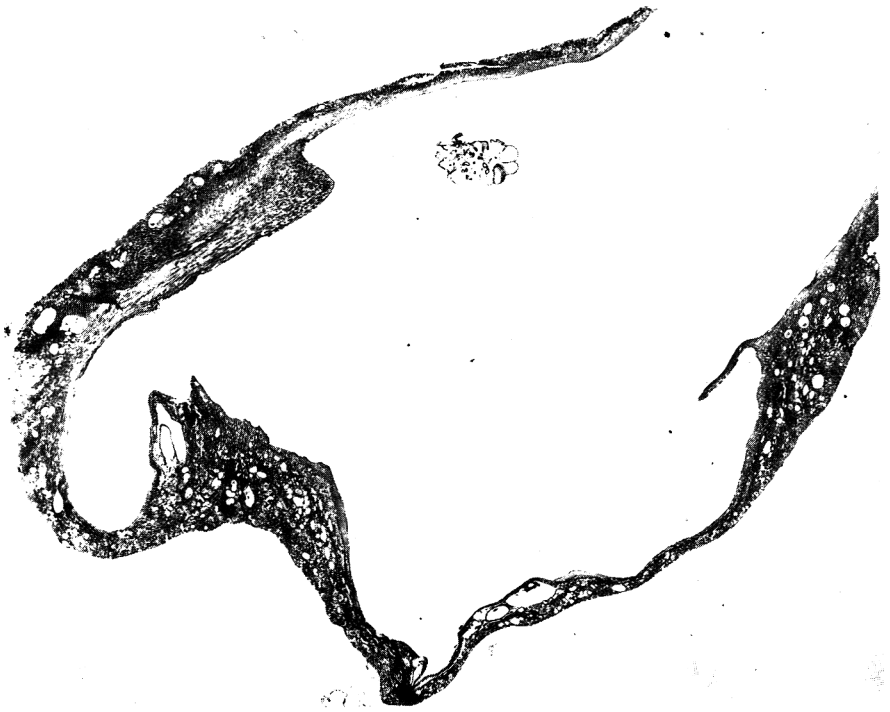

Fig. 5. Severe hydronephrosis of a kidney from a male rat given 2% $Na_3NTA.H_2O$ in the diet for 49 days. × 5.

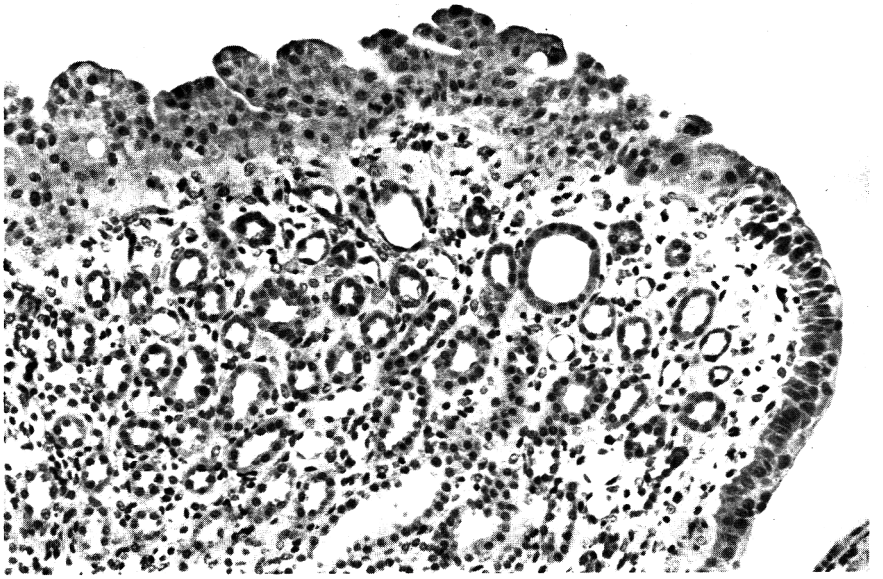

Fig. 6. Hyperplasia of the transitional epithelium is shown in the renal pelvis of a male rat given 2% $Na_3NTA.H_2O$ in the diet for 49 days. Perfusion fixed and H & E stained. × 140.

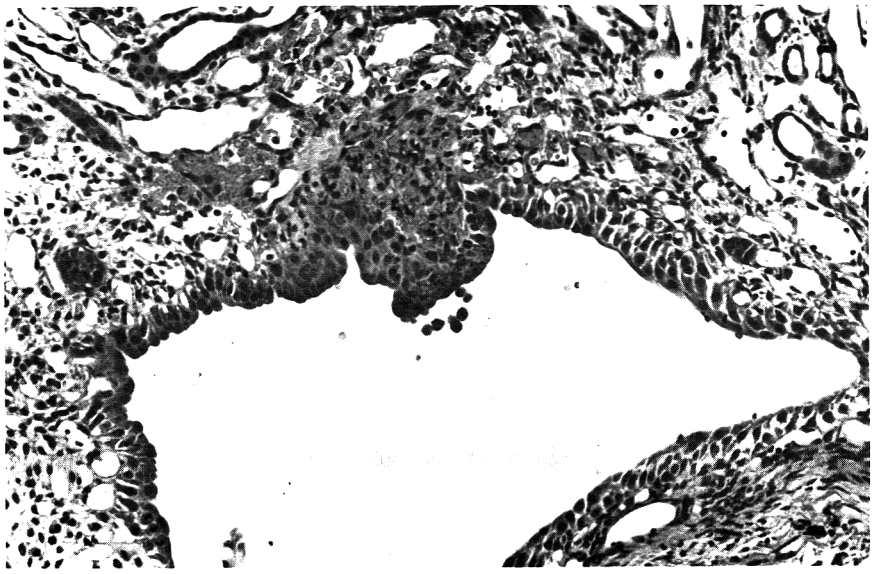

Fig. 7. Hyperplastic transitional epithelium with a local ulceration characterized by haemorrhage and polymorphonucleocyte invasion in the renal pelvis of a male rat given 2% $Na_3NTA.H_2O$ in the diet for 49 days. Perfusion fixed and H & E stained. × 140.

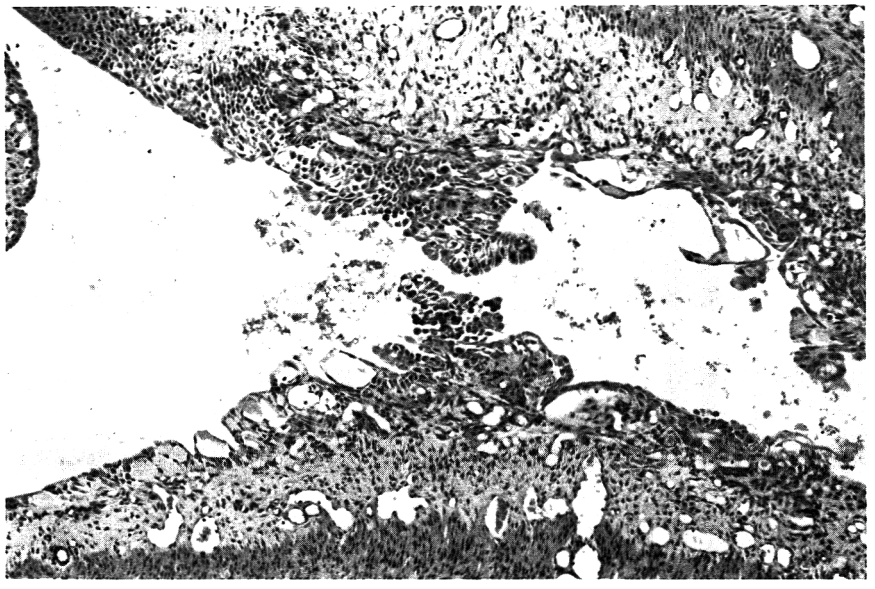

Fig. 8. Hyperplasia, erosion and haemorrhage are shown at the ureteral–pelvic junction of an animal given 2% $Na_3NTA.H_2O$ in the diet for 49 days. Perfusion fixed and H & E stained. × 60.

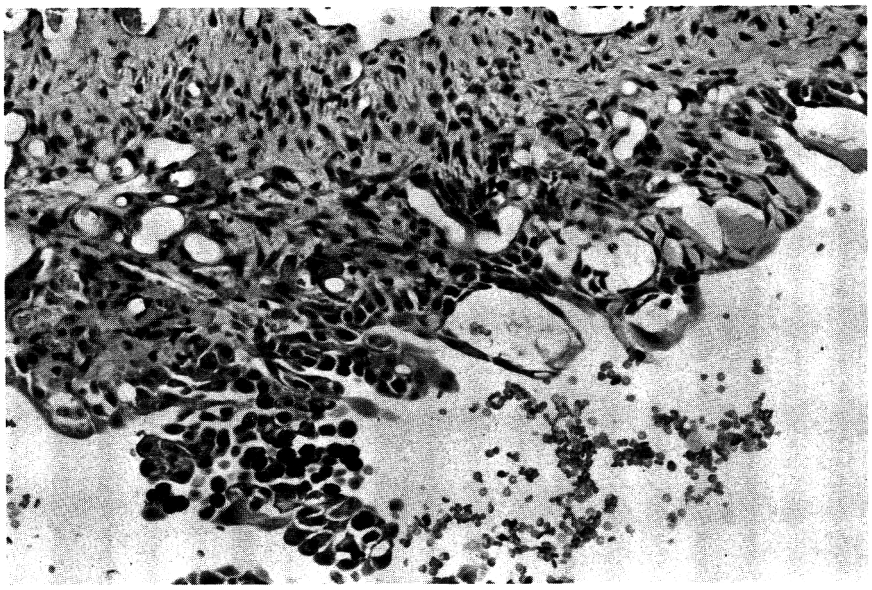

Fig. 9. A higher magnification view of Fig. 8 shows dysplastic cells in a focus of exophytic transitional cell hyperplasia. × 160.

Table 2. *Incidence of convoluted tubular cell lesions in kidneys from male rats fed nitrilotriacetate for 49 days with or without a subsequent 35-day recovery period*

Dietary level of NTA	No. of rats	Incidence* of vacuolated alterations			Incidence* of nonvacuolated alterations		
		All lesion types	Vacuolated cell hyperplasia†	Vacuolated cell nodular hyperplasia†	All lesion types	Basophilic cell hyperplasia†	Basophilic cell nodular hyperplasia†
After 49 days NTA treatment							
0 (control)	6	0	0	0	3	3 (Md)	0
1·5% H_3NTA	8	8	6 (M–S)	6 (M–S)	7	6 (M–Md)	4 (M)
2% $Na_3NTA.H_2O$	7	7	5 (M–S)	4 (M–Md)	7	5 (M–S)‡	3 (M)
After 49 days NTA treatment and 35 days recovery							
0 (control)	6	0	0	0	2	2 (Md)	0
1·5% H_3NTA	8	0	0	0	2	2 (M)	0
2% $Na_3NTA.H_2O$	8	0	0	0	5	5 (M–S)§	0

NTA = Nitrilotriacetate

*No. of kidney cross-sections showing lesion. One section per rat was examined for the number of rats indicated.

†The grade of severity of the lesion is given in brackets: M = minimal (1 lesion focus in a kidney cross-section); Md = moderate (2–4 lesion foci); S = severe (>5 lesion foci).

‡Four of the animals had hydronephrosis.

§Three of the animals had severe hydronephrosis.

In the treated animals given an NTA-free diet for 5 wk no sites of basophilic nodular hyperplasia were detected and the incidence of simple hyperplasia of the basophilic cell type in the animals without hydronephrosis was similar to that in the controls. A high incidence of simple basophilic hyperplasia was still present in the three animals that had been fed $Na_3NTA.H_2O$ and that displayed hydronephrosis even after the 5-wk recovery period (Table 1). Thus the nonspecific renal toxicity associated with the ingestion of high doses of NTA is reversible except when it is accompanied by irreversible hydronephrosis (Fig. 5).

Pelvic epithelial and subepithelial inflammation and fibrosis were observed in the groups given either form of NTA for 49 days. Mild to severe hyperplasia of the transitional epithelium (TE) in the renal pelvis occurred in four out of seven animals given diets containing 2% $Na_3NTA.H_2O$ (Fig. 6) and moderate TE hyperplasia was observed in one out of eight rats given 1·5% H_3NTA in the diet. Animals with TE hyperplasia also exhibited pelvic dilation and TE erosion, ulceration and haemorrhage (Fig. 7). TE dysplasia, intracytoplasmic globules, and mitotic figures were also noted. One animal given $Na_3NTA.H_2O$ exhibited dramatic proliferative alteration of the TE at the ureteral–pelvic junction (Figs 8 & 9). This alteration was characterized by exo- and endophytic nodular hyperplasia, intra-epithelial polymorphonuclear infiltration, and haemorrhage. Mitotic figures and dysplasia were observed in the cells comprising these hyperplastic nodules.

Tissues from animals in the recovery groups showed no abnormal cellular morphology in the renal pelvic urothelium in the presence of persisting hydronephrosis.

DISCUSSION

Feeding high doses of NTA for 7 wk induced renal tubular cell toxicity in all 15 animals examined. The NTA-specific toxicity characterized by the presence of vacuoles in the PCT-cell cytoplasm progressed to nodular hyperplasia (Table 2) in ten of the rats. The complete absence of any PCT-cell vacuolation in the animals given an NTA-free diet for 5 wk after NTA exposure demonstrates that the maintenance of degenerative or proliferative vacuolar lesions is dependent upon continued NTA insult.

The basophilic-type tubular cell hyperplasia as well as other lesions commonly associated with age-related nephrosis were present in all of the groups, including controls. Basophilic tubular cell hyperplasia is commonly observed in rat kidneys as a sequel to age-related nephrosis and is indicative of a cellular regenerative response (Gray, 1977). Consequently, these responses are considered to be nonspecific. They were clearly exacerbated by 7 wk of ingestion of high doses of NTA, as indicated by the occurrence of nodular basophilic cell hyperplasia in seven out of 15 animals given NTA but in none in the controls (Table 2). The reversibility of this type of tubular cell lesion upon cessation of NTA ingestion is illustrated by the absence of nodular basophilic cell lesions in both NTA-treated recovery groups and the reduced incidence (to control levels) of simple basophilic cell hy-

perplasia in the H_3NTA-treated recovery group. An increased incidence of simple basophilic hyperplasia occurred in animals of the $Na_3NTA.H_2O$-treated recovery group that had developed severe hydronephrosis during $Na_3NTA.H_2O$ feeding.

TE hyperplasia and hydronephrosis occurred mainly in rats fed $Na_3NTA.H_2O$ and was associated with urothelial erosion and ulceration. The renal pelvic toxicity in $Na_3NTA.H_2O$-fed animals is paralleled by alterations in urinary characteristics: $Na_3NTA.H_2O$ ingestion is associated with an increase in urine volume, a marked alkaline shift in urine pH, and haematuria (Anderson, 1979). Comparable urinary alterations do not occur with H_3NTA ingestion. The urothelial proliferative and toxic response in the renal pelvis, like that in the tubules, was reversed during the 5-wk recovery phase of the study. The hydronephrotic response in the $Na_3NTA.H_2O$-treated group was clearly sufficiently severe to preclude the restoration of normal renal structure in a few rats.

In summary, this work shows that both the induction and maintenance of renal toxic responses are dependent upon continuous exposure to high doses of NTA. The findings in this study are compatible with the proposed pathogenesis for renal tubular cell tumorigenicity associated with NTA and demonstrate that, once proliferative lesions are formed, their maintenance is dependent upon continued NTA exposure even after they attain a state of nodular hyperplasia. The difference in renal pelvic response to the two forms of NTA studied parallels the pelvic tumorigenic responses observed in the 2-yr chronic feeding study (NCI, 1977), and appears to be a unique response of male rats to diets containing 2% $Na_3NTA.H_2O$ (Anderson, 1979).

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REVERSIBILITY OF RENAL CORTICAL LESIONS INDUCED IN RATS BY HIGH DOSES OF NITRILOTRIACETATE IN CHRONIC FEEDING STUDIES

C. L. ALDEN and R. L. KANERVA

The Procter & Gamble Company, Miami Valley Laboratories, P.O. Box 39175, Cincinnati, OH 45247, USA

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Abstract—The effects in the renal cortex of the male rat of continuous administration of a high dose of nitrilotriacetate (NTA) for 24 months were compared with those of the administration of a similar dose for 18 months followed by a 6-month recovery period on the control diet. The results suggest that discontinuation of treatment interrupts the sequence of events leading to tumour formation. This study indicates that all stages in the proposed pathogenesis of renal tubular tumour formation by NTA, up to the occurrence of adenomatous hyperplasia and neoplasia, are reversible. Apparently, tumour development is dependent on the continuous administration of high doses of NTA in the presence of toxic injury to the majority of nephrons.

INTRODUCTION

Nitrilotriacetate (NTA), an amino-acid type chelator, is an efficient detergent builder. NTA has been widely used for 10 years in detergents in Canada and is a candidate for similar usage in the USA. Because of the potential for widespread high-volume use in the USA, the National Cancer Institute (NCI) selected NTA for testing in its bioassay program. The NCI has shown that long-term administration of high doses of NTA to rodents is associated with tumorigenicity in, and restricted to, urinary tracts (NCI, 1977).

We have previously reported on the pathogenesis of renal cortical tumours in NTA-treated rats and the role of nephron toxicity as a prerequisite for NTA tumorigenicity (Alden & Kanerva, 1982; Alden, Kanerva, Anderson & Adkins, 1981). The next major facet of our pathogenetic studies investigates whether an inexorable sequence of events is initiated when nephron toxicity is induced with NTA, or whether, and at what step, reversal of the tumorigenic process can occur in the sequence if the toxic insult is discontinued. To this end we have demonstrated that lesions characteristic of nephron toxicity and proliferative sequelae induced by short-term exposure to NTA are reversible after a 5-week recovery period (Myers, Kanerva, Alden & Anderson, 1982). The reversibility after a 6-month recovery period of lesions induced by chronic (18-month) administration of NTA will be addressed in this communication.

EXPERIMENTAL

Slides from the NCI bioassay of NTA archived by Tracor Jitco, Inc. provide the case material for this report. Renal cortices from Fisher 344 rats fed nitrilotriacetic acid (H_3NTA) at a concentration of 1·5% in the diet, a carcinogenic level, for 18 months followed by a 6-month recovery period (recovery group) were examined microscopically. This dose level, 79 μmol/g diet, involves an exposure of the urinary tract to NTA that is equivalent to the high dose of trisodium nitrilotriacetate monohydrate ($Na_3NTA.H_2O$; 2% in the diet, 73 μmol/g diet) which was fed to male Fischer 344 rats for 24 months (continuous treatment group), also in the NCI bioassay. Renal cortices from this latter group have been previously evaluated and results have been reported in establishing the pathological sequence of events leading to tumour formation (Alden & Kanerva, 1982).

Results of microscopic evaluation of renal cortices from the above two groups would be expected to differ primarily because of the 6-month recovery period in the H_3NTA-treated group. However, additional potential variables include the following: (1) the two groups were treated at different laboratories: the continuous treatment group at Stanford Research Institute (SRI) and the recovery group at Litton Bionetics (LB); (2) the two groups were fed different forms of NTA: $Na_3NTA.H_2O$ at SRI and H_3NTA at LB; (3) short-term studies have shown that the two forms have different effects on urinary parameters (Anderson & Kanerva 1978a,b): $Na_3NTA.H_2O$ induces polyuria, increases urinary pH and increases urinary sodium excretion whereas H_3NTA decreases urinary pH and does not induce polyuria.

The experimental design for evaluating the effect of a 6-month recovery period from chronic NTA administration is shown in Table 1.

RESULTS

Our criteria for categorization of renal cortical lesions have been previously described (Alden &

Abbreviations: LB = Litton Bionetics; NCI = National Cancer Institute; NTA = nitrilotriacetate; SRI = Stanford Research Institute.

Table 1. *Groups from which slides were evaluated in order to determine the effects of a long-term recovery period on renal cortical histopathological alterations induced by chronic administration of NTA*

NCI Bioassay group	Number of male rats evaluated	Treatment	Laboratory
Control	14	Control diet for 24 months	Litton Bionetics
Control	10	Control diet for 24 months	Stanford Research Institute
Continuous treatment	24	2% $Na_3NTA.H_2O$ in control diet (73 μmol NTA/g diet) for 24 months	Stanford Research Institute
Recovery	15	1·5% H_3NTA in control diet (79 μmol NTA/g diet) for 18 months followed by control diet for 6 months	Litton Bionetics

Kanerva, 1982; Alden *et al.* 1981). Age-related nephrosis was graded on the basis of the amount of parenchyma affected by the lesion: 1 = <1%, 2 = 1–10%, 3 = 11–25% and 4 = >25%. The results of our microscopic evaluation of renal cortices and the incidence of renal tubular neoplasms as reported by the NCI (1977) are shown in Fig. 1. We are using our own evaluation of the non-neoplastic parameters since the NCI report primarily concerned itself with the tumorigenic response, thus incompletely addressing the toxic injury (Alden & Kanerva, 1982).

All renal cortical lesions in the controls are compatible with those known to be associated with spontaneous age-related nephrosis in the rat. The severity of age-related nephrosis in LB controls (mean grade = 2·1) was slightly increased compared to SRI controls (mean grade = 1·2). The severity of age-related nephrosis was greater in the continuous treatment group (mean grade = 3·9) than in the recovery group (mean grade = 3·1), and both were significantly increased over the corresponding control groups.

Comparison of parameters of tubular epithelial proliferation also reveals a significant increase in continuous treatment and recovery groups compared to control groups. Additionally, all categories of tubular hyperplasia as well as tubular neoplasms were increased in the continuous treatment group compared with the recovery group. Tubular hyperplastic lesions characterized by vacuolated epithelial cells did not occur in the recovery group but were frequently observed in the continuous treatment group. Two-year studies on NTA carried out at the Procter & Gamble Co. laboratories with an interim kill at 19 months (Nixon, Buehler & Niewenhuis, 1972) revealed that renal lesions at 18 months are qualitatively similar to, but less frequent than, those observed at 2 yr.

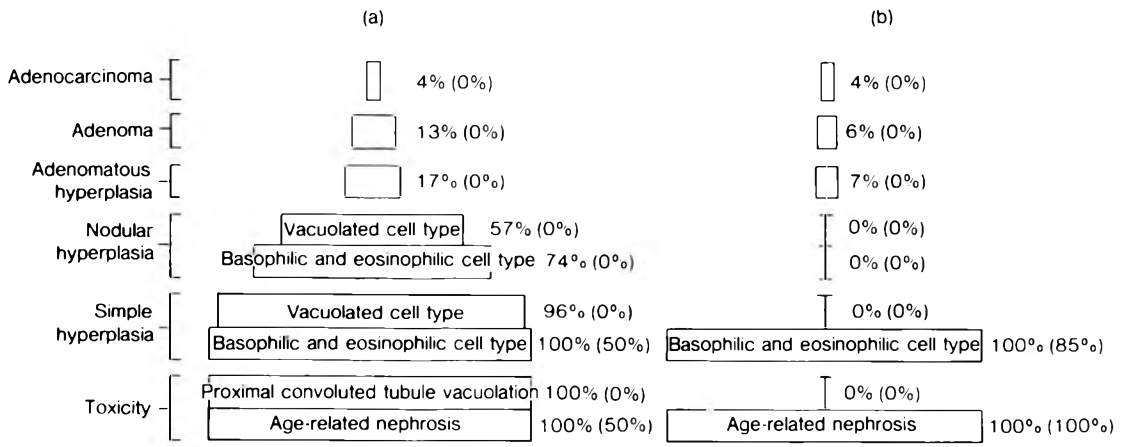

Fig. 1. The incidence of lesions in the renal cortex of Fischer 344 male rats (a) given a carcinogenic dose of nitrilotriacetate (as $NA_3NTA.H_2O$) in the diet for 24 months (continuous treatment group) or (b) given an equivalent dietary dose (as H_3NTA) for 18 months followed by 6 months on the control diet (recovery group). The values in brackets are for the corresponding control groups. The lesions are listed in ascending order on the basis of frequency and time course sequence in rats given high doses of NTA (Merski, 1981; Nixon, Buehler & Niewenhuis, 1972). A previously described (Alden & Kanerva, 1982) pathological sequence of events is apparent in the continuous treatment group. A recovery period has interrupted the sequence.

DISCUSSION

A morphological sequence of events initiated by nephron toxicity leading to renal tubular tumour formation has been previously described in rats fed high dietary levels of NTA for 2 yr (Alden & Kanerva, 1982; Alden *et al.* 1981). Cortical effects associated with short-term exposure to NTA are rapidly and completely reversible (Myers *et al.* 1982). Such reversibility does not necessarily demonstrate reversal of the tumorigenic process. Hence, this report addresses the issue of reversibility of chronic effects leading ultimately to tumorigenesis. Results of our comparison of the effects of high doses of NTA administered continuously for 24 months with the effects of 18-months' administration followed by a 6-month recovery period demonstrate that discontinuation of treatment interrupts the described sequence as illustrated in Fig. 1 and summarized as follows.

> The evidence of the specific toxic effect of NTA on proximal convoluted tubules, i.e. vacuolated epithelium in both degenerative and proliferative modes (previously described by Alden *et al.* 1981), is completely reversed by discontinuing NTA treatment after 18 months. Evidence of nonspecific NTA toxicity in the nephron manifested as exacerbation of age-related nephrosis (previously described by Alden *et al.* 1981 and Alden & Kanerva, 1982) is reduced by discontinuing treatment after 18 months although not to control levels. Also, discontinuation of the administration of high doses of NTA after 18 months results in decreased incidences of tumours and of the exaggerated tubular hyperplastic lesions (designated hyperplastic nodules and adenomatous foci) compared with those in rats fed NTA continuously for 24 months.

Our interpretation of these data correlate well with our view that NTA exerts its effect through epigenetic means. This concept has been developed on the basis of experimental evidence that nephron toxicity is a prerequisite for NTA tumorigenesis and that the nephron toxicity is the result of cation perturbation at the cellular level (Anderson, Alden & Merski, 1982). Also, NTA is nonmutagenic and nonmetabolized in the mammalian system. In addition, these data suggest that the toxic injury does not inexorably proceed to tumour formation and that, on discontinuation of the toxic insult, all stages in the pathogenesis of NTA-induced tumour formation are reversible up to the occurrence of adenomatous hyperplasia and neoplasia. These data also suggest that tumour development depends on the continuous administration of high doses of NTA in the presence of pathological evidence of injury to the majority of nephrons. Hence, the effects of short-term administration of NTA should be completely reversible not only with respect to the return of the kidney to normal histological appearance (as previously demonstrated) but possibly also in the reversibility of the tumorigenic stimulus(i) once the toxic insult is discontinued.

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ESTIMATION OF VOLATILE *N*-NITROSAMINES IN RUBBER NIPPLES FOR BABIES' BOTTLES

D. C. HAVERY and T. FAZIO

Division of Chemistry and Physics, Food and Drug Administration, Washington, DC 20204, USA

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Abstract—A method is described for the estimation of volatile *N*-nitrosamines in the rubber nipples of babies' bottles. In a study of rubber nipples from one manufacturer, *N*-nitrosodimethylamine, *N*-nitrosodiethylamine and *N*-nitrosopiperidine were determined by gas chromatography, using a thermal energy analyser, and their presence was confirmed by mass spectrometry with average levels of individual nitrosamines ranging from 22 to 281 ppb. When the nipples were sterilized in a conventional sterilizer together with milk or infant formula the three nitrosamines migrated into the milk or formula. Storing a bottle of milk with a rubber nipple inverted in it for 2 hr at room temperature or overnight in a refrigerator after sterilization resulted in an 8–13% average increase in the nitrosamine levels migrating into the milk. On repeated sterilization of a single nipple, the quantities of nitrosamines migrating into milk from rubber nipples declined steadily, but after seven sterilizations, nitrosamines were still readily detectable in the milk. Nitrosamine levels were higher in rubber nipples after sterilization, indicating the presence of nitrosamine precursors in the nipples. No nitrosamines were found in raw, uncured rubber. Chemical accelerators and stabilizers added during the vulcanization process are the source of the amine precursors in rubber nipples.

INTRODUCTION

Several epidemiological studies in years past have reported a higher than average incidence of cancer in rubber industry workers (Monson & Fine, 1978; Monson & Nakano, 1976). Recent studies have reported finding volatile nitrosamines, a class of potent chemical carcinogens, in rubber factory atmospheres (Fajen, Carson, Rounbehler *et al.* 1979; McGlothlin, Wilcox, Fajen & Edwards, 1981). The origin of nitrosamines in the factory air has been traced to accelerators and stabilizers used in the vulcanization process (Ireland, Hytrek & Lasoski, 1980; Yeager, Van Gulick & Lasoski, 1980). Preliminary surveys of rubber materials and manufactured rubber products have indicated the presence of volatile nitrosamines (Ireland *et al.* 1980). Of particular concern was the recent finding by Preussmann, Spiegelhalder & Eisenbrand (1981) of volatile nitrosamines in rubber nipples for babies' bottles. The aqueous extraction technique described by Preussmann *et al.* (1981) suggests that these toxic compounds could be ingested when an infant sucks on a rubber nipple while feeding.

The potential exposure of infants to these toxic compounds prompted the US Food and Drug Administration to look further into the problem. We describe here a method for the extraction of volatile nitrosamines from rubber nipples. We also report the results of tests to determine the quantity of volatile *N*-nitroso compounds migrating into milk and infant formula during a typical sterilization, the effect of storage on the migration of nitrosamines from a nipple into milk or infant formula and the effects of repeated sterilizations on the levels of nitrosamines in rubber nipples and on their migration.

Abbreviations: GC–TEA = Gas chromatography-thermal energy analysis; NDEA = *N*-nitrosodiethylamine; NDMA = *N*-nitrosodimethylamine; NDPA = *N*-nitrosodipropylamine; NDPhA = *N*-nitrosodiphenylamine; NPIP = *N*-nitrosopiperidine.

EXPERIMENTAL

Rubber nipple samples were obtained by a Food and Drug Administration Field Inspector from one manufacturing plant. Volatile nitrosamines were extracted from the rubber nipples with methylene chloride. Each nipple was cut into pieces (approx. 5 mm^2) and soaked overnight (16–21 hr) in a 250-ml round-bottomed flask with 100 ml methylene chloride. The methylene chloride extract and rubber pieces were filtered through a coarse sintered-glass Soxhlet extraction thimble suspended in a Soxhlet extractor. The extraction flask was rinsed with 25 ml methylene chloride, and the rinse was added to the Soxhlet extractor. The nipple pieces were extracted in the Soxhlet extractor for 1 hr. After cooling the methylene chloride extract, 100 ml 5 *N*-sodium hydroxide and boiling chips were added. The flask was connected to an atmospheric distillation apparatus, and the methylene chloride was distilled and discarded. The aqueous phase was distilled and 70 ml was collected in a 250-ml separatory funnel. To the distillate was added 300 mg anhydrous sodium carbonate, and the distillate was extracted three times with 50-ml portions of methylene chloride. The pooled extracts were dried by passing through 30 g anhydrous sodium sulphate, held in a coarse sintered-glass filtering funnel, into a 250-ml Kuderna-Danish evaporator concentrator. The sodium sulphate was rinsed with 15 ml methylene chloride and collected in the concentrator. Carborundum grains were added, a Snyder column was attached, and the methylene chloride extract was concentrated to 4 ml at a rate of 1 ml/min in a 60°C water bath, and further concentrated to 1 ml at room temperature under a gentle stream of nitrogen.

The nitrosamines were detected and quantitated by injecting 8-μl aliquots of the concentrated extract and appropriate nitrosamine standards into a Hewlett-Packard 5710A gas chromatograph. The coiled glass column (2·7 m × 4 mm ID) was packed with 10% Carbowax 1540 plus 5% potassium hydroxide on a 100–120 mesh Chromosorb W-HP. The argon carrier gas flow rate was 40 ml/min and the column was temperature programmed from 100 to 180 C at 4 /min. The injection port was maintained at 200 C. The gas chromatograph was interfaced to a Model 502L thermal energy analyser (Thermo Electron Corp., Waltham, MA) operated under the following conditions: furnace, 450°C; pressure, 1 mm; liquid nitrogen cold trap.

In an attempt to reproduce the sterilization process used in a typical home, nipples (i.e. two halves) were inverted on glass bottles containing milk or infant formula and loosely capped. The bottles were placed in a boiling water sterilizer for 25 min. The nipples were removed and analysed for nitrosamines, as was the milk or infant formula, after cooling to room temperature.

Milk and infant formula samples were prepared by vacuum distillation and analysed (Havery, Fazio & Howard, 1978). The sample (50 g) was vacuum distilled with mineral oil. The aqueous distillate was partitioned with three 25-ml portions of methylene chloride and concentrated, and the nitrosamines were determined using a gas chromatograph coupled to a thermal energy analyser (GC–TEA).

Apple juice samples were analysed for volatile nitrosamines by a column elution method similar to that previously described for beer (Hotchkiss, Havery & Fazio, 1981). Apple juice (50 g) was mixed with 35 g Celite in a beaker. The mixture was packed in a chromatographic column, and the nitrosamines were eluted with 150 ml methylene chloride. The eluate was concentrated, and the nitrosamines were quantitated by GC–TEA.

The extracts of rubber nipples, milk, infant formula and water containing nitrosamines were further cleaned up on acid-Celite (Howard, Fazio & Watts, 1970) and silica gel (Fazio, Howard & White, 1972) chromatographic columns before confirmation by gas chromatography–mass spectrometry (GS–MS). A Finnigan Model 3300F quadrupole mass spectrometer with a Finnigan 9500 gas chromatograph and Finnigan Incos 2300 data system was used for the nitrosamine confirmation. The injection port was fitted with an SGE splitless injection system. The GC column was a 50 m × 0·5 mm ID Carbowax 20 M fused silica capillary column and was interfaced directly into the mass spectrometer. The temperatures used were: injection port, 180°C; transfer line, 170°C; column oven, programmed as follows: after injection of the sample the temperature was held at 30°C for 90 sec, rapidly elevated to 80°C (30 sec), and subsequently programmed more slowly from 80 to 180°C at 4°/min. Full mass scans were acquired under computer control at a rate of 1·0 sec/scan for the mass range m/z 20 to 200.

RESULTS

Initially, an aqueous extraction method as described by Ireland *et al.* (1980) was evaluated. However, it was found that nitrosamines were artefactually formed when the nipple was extracted with hot water. A method involving vacuum distillation of a cut-up nipple was also ineffective in extracting the nitrosamines from the rubber. By soaking pieces of rubber nipple in methylene chloride at room temperature, the nitrosamines could be extracted at an efficiency of more than 90%. Recovery of an internal standard, *N*-nitrosodipropylamine (NDPA), added at the 10 ppb level to the methylene chloride extract of a rubber nipple before distillation, averaged 96%.

Application of the methylene chloride extraction method to several nipples from the same manufactured lot showed that nitrosamine levels varied considerably from nipple to nipple, some differing by as much as 140%. However, if a single nipple was cut in half, the nitrosamine levels in each half agreed with average coefficients of variation of 12·1 and 7·0 for *N*-nitrosodimethylamine (NDMA) and *N*-nitrosodiethylamine (NDEA), respectively. Therefore, for the remainder of the study, to have some basis against which to compare nitrosamine levels in rubber nipples before and after sterilization, two nipples from the same lot were cut in half, one half of each was exchanged, and the two new sets of nipples were used for experimentation.

It was evident from our work with a hot aqueous extraction of rubber nipples that nitrosamine precursors were present in these products. We therefore examined the methylene chloride extraction method described here for the potential to form nitrosamine artefacts. Because of the physical nature of rubber, artefactual formation of nitrosamines during the initial methylene chloride extraction was difficult, if not impossible, to evaluate. However, since the nitrosamine precursors added to rubber during vulcanization are often present at levels in excess of 1·5%, as discussed later, one would expect nitrosamine levels in rubber nipples far in excess of the levels found if they were artefactually formed. Further tests were performed to check the distillation step for the potential for artefact formation. When nitrite and morpholine were added to methylene chloride extracts of rubber nipples, neither an enhancement of the nitrosamine levels previously found in the nipple nor *N*-nitrosomorpholine were observed, providing further evidence that the nitrosamines found in rubber nipples were not artefactually formed during analysis.

Our primary concern as a regulatory agency was whether the nitrosamines would migrate from the nipple into milk or infant formula during sterilization. Recoveries from milk after sterilization of the three nitrosamines found in rubber nipples, namely, NDMA, NDEA and nitrosopiperidine (NPIP), averaged 85, 95 and 96%, respectively. The results of the analysis of five nipples from the same lot before and after sterilization are shown in Table 1. The data show that the levels of NDMA and NDEA found in the nipples vary considerably from nipple to nipple within the same lot. Average levels of 54 ng NDMA and 27 ng NDEA migrated into the milk during the 25-min sterilization, equivalent to averages of 17 and 20% migration, respectively, of the nitrosamine found in the unsterilized nipple. These nanogram values would produce NDMA and NDEA levels of approxi-

Table 1. *The effects of sterilization on the levels of* N-*nitrosodimethylamine*, N-*nitrosodiethylamine and* N-*nitrosopiperidine in rubber nipples, milk and infant formula*

Sample no.	Nitrosamine concn in rubber nipples (ppb)						Nitrosamine concn in milk or formula								
	Unsterilized			Sterilized			ng			ppb*			Migration (%)		
	NDMA	NDEA	NPIP	NDMA	NDEA	NPIP	NDMA	NDEA	NPIP	NDMA	NDEA	NPIP	NDMA	NDEA	NPIP
Nipples of type 1 sterilized with milk															
1	60	28	—	32	35	—	49	24	—	0·4	0·2	—	16	17	—
2	76	32	—	24	29	—	48	26	—	0·4	0·2	—	13	17	—
3	90	42	—	70	73	—	87	44	—	0·7	0·4	—	18	20	—
4	37	14	—	25	25	—	51	23	—	0·4	0·2	—	26	32	—
5	59	24	—	25	27	—	33	19	—	0·3	0·2	—	11	15	—
Mean	64	28	—	35	38	—	54	27	—	0·4	0·2	—	17	20	—
Nipples of type 2 sterilized with milk															
6	—	66	283	—	103	331	—	56	110	—	0·4	0·9	—	16	7
7	—	50	296	—	56	273	—	38	76	—	0·3	0·6	—	15	5
8	—	57	333	—	103	430	—	77	152	—	0·6	1·2	—	26	9
9	—	50	219	—	75	255	—	68	115	—	0·5	0·9	—	27	10
10	—	61	275	—	90	317	—	57	102	—	0·5	0·8	—	18	7
Mean	—	57	281	—	85	321	—	59	111	—	0·5	0·9	—	20	8
Nipples sterilized with infant formula															
11	54	20	—	23	26	—	45	23	—	0·4	0·2	—	16	21	—
12	71	30	—	50	53	—	100	46	—	0·8	0·4	—	27	30	—
13	52	18	—	21	19	—	48	28	—	0·4	0·2	—	18	30	—
Mean	59	23	—	31	33	—	64	32	—	0·5	0·3	—	20	27	—

NDEA = *N*-Nitrosodiethylamine NDMA = *N*-Nitrosodimethylamine NPIP = *N*-Nitrosopiperidine

*Calculated on the basis of the weight of 120 ml of milk or infant formula.

mately 0·45 and 0·2 ppb, respectively, or a total of almost 0·7 ppb, in 120 ml milk. No nitrosamines were found in unsterilized milk. NDEA in these nipples increased by an average of 10 ppb after sterilization, indicating the presence of nitrosamine precursors in the rubber nipples.

Results of the analysis of five nipples of a different type, from the same lot, are also shown in Table 1. Two nitrosamines, NDEA and NPIP, were found in the nipples. NDEA and NPIP were also found in the milk following sterilization at average levels of 59 and 111 ng, respectively, equivalent to 0·5 and 0·9 ppb, or a total of 1·4 ppb, in 120 ml milk. The average percentage migration figures for NDEA and NPIP into milk were 20 and 8, respectively. Both NDEA and NPIP levels were higher in rubber nipples after sterilization, again indicating the presence of nitrosamine precursors.

Three nipples from a single lot were also sterilized with infant formula as previously described to determine whether infant formula would affect the quantity of nitrosamine migrating from the nipple. The results are shown in Table 1. The average amounts of NDMA and NDEA that migrated into the formula were 20 and 27% of the amounts found in the nipples before sterilization, figures somewhat higher than those for milk.

To confirm the presence of the observed nitrosamines in rubber nipples and milk by GC–MS, sample extracts from several analyses were pooled. The presence of NDMA, NDEA and NPIP in rubber nipples was confirmed by GC–MS. NDMA and NPIP were confirmed by GC–MS in milk that had been sterilized along with a rubber nipple. NDEA could not be confirmed in sterilized milk due to interferences. When water was substituted for milk in the sterilization process, followed by extraction, concentration and analysis of the sterilized water, the presence of NDMA, NDEA and NPIP in the water was confirmed.

A common procedure in the home is to sterilize a number of bottles at one time and store for later use. The effect of storage on the quantity of nitrosamines migrating into milk was examined. Nipples and bottles containing milk were sterilized together as previously described. The bottles were removed from the sterilizer, the caps tightened, and the bottles with nipples were stored, either for 2 hr at room temperature or in the refrigerator overnight. The results are shown in Table 2. No significant difference in the nitrosamine migration rate was observed between the 2-hr room temperature storage and overnight refrigerated storage. However, both storage conditions resulted in average migration rates of 26 to 32% for NDMA and NDEA, respectively, an increase of from 9 to 12% above the rate obtained for milk that had not been stored following sterilization.

Since rubber nipples are reusable items, and since earlier experiments had shown an increase in nitrosamine levels in nipples following sterilization, an experiment was conducted to determine the effect of multiple sterilizations on the nitrosamine levels migrating from a single nipple into milk. Two whole nipples were placed on bottles of milk and sterilized as previously described. Following sterilization, the milk samples were cooled and analysed for volatile nitrosamines. The same nipples were placed on two fresh bottles of milk and sterilized, and the milk was analysed as before. The procedure was repeated a total of six times for both nipples. The results are shown in Fig. 1. The data show that the levels of nitrosamines migrating into milk continued to decline as the rubber nipples were repeatedly sterilized. Portions of the curves in Fig. 1 approach a zero slope, demonstrating that nearly as much nitrosamine is being formed as is migrating into the milk.

Table 2. *Effect of storage on migration (%) of N-nitrosamines into milk*

	Percentage migration after storage for ...			
	2 hr at 21 C		19 hr at 4 C	
Sample	NDMA	NDEA	NDMA	NDEA
1	38	34	22	34
2	21	29	15	20
3	19	33	27	27
4	27	30	39	43
5	31	38	21	31
6	–	–	26	36
	27	33	25	32

NDMA = *N*-Nitrosodimethylamine
NDEA = *N*-Nitrosodiethylamine

Another method of sterilizing rubber nipples commonly used in the home is by direct immersion in boiling water. To determine the effects of such treatment on nitrosamine levels in rubber nipples, a nipple (i.e. two matched halves) was immersed for 5 min in boiling water in a round-bottomed flask fitted with a water-cooled condenser. The sterilized nipple and its unsterilized matching set were analysed for nitrosamines, as was the water after cooling to room temperature. The levels of NDMA in the unsterilized and sterilized nipples were 43 and 69 ppb respectively and the levels of NDEA in the unsterilized and sterilized nipples were 23 and 64 ppb respectively. The levels of NDMA and NDEA in the sterilized water were 34 and 21 ppb. There was a considerable increase in the levels of NDMA and NDEA found in the rubber nipples after sterilization, again showing the presence of nitrosamine precursors. The nitrosamine levels would be expected to decline as the nitrosamine precursors are used up. The results also suggest that if the nipple is subjected to elevated temperatures during nitrosamine analysis, such as the boiling water extraction described by Ireland *et al.* (1980), the nitrosamine data obtained may be in considerable error.

Fruit juices are frequently consumed by older infants through rubber nipples, and it is common practice for a parent to carry a bottle of juice, complete with a rubber nipple, for several hours before feeding. Since the pH of fruit juice is between 3 and 4, the optimum pH for nitrosamine formation, an experiment was conducted to determine the effect of fruit juice on the migration of nitrosamines from a rubber nipple. A nipple was allowed to remain in contact with apple juice for 4 hr, after which the nipple and the apple juice were analysed for volatile nitrosamines. Average levels of 25 ng NDMA and NDEA migrated into the apple juice during the 4-hr contact.

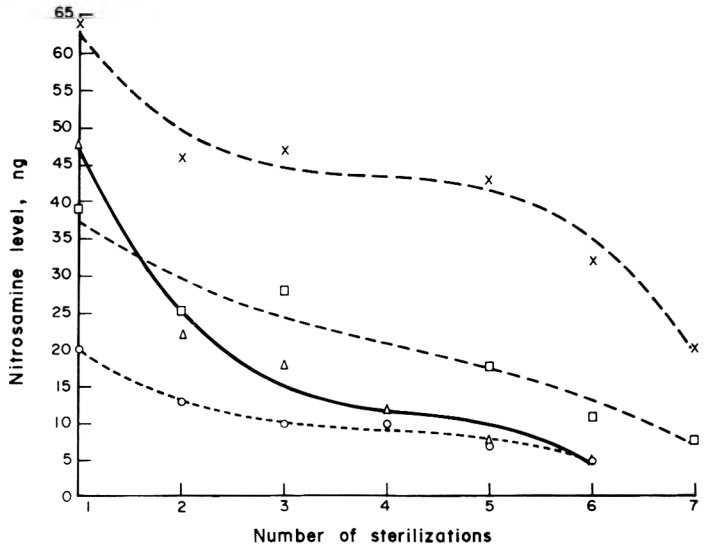

Fig. 1. Nitrosamines migrating from a nipple to milk upon repetitive sterilization: NPIP (--- x ---), NDEA (—— △ ——), NDMA (---□---), NDEA(---○---).

These levels are a little more than half those found in milk sterilized with a rubber nipple.

A sample of bulk, raw (uncured) rubber was obtained from a rubber manufacturer. A portion of the raw rubber was analysed for volatile nitrosamines to determine if any of these compounds were present prior to processing. The sample was extracted using the hot water technique of Ireland *et al.* (1980), since the methylene chloride extraction technique was not suitable. No volatile nitrosamines were found in the sample. To determine if nitrosatable amines were present in the raw rubber, the same aqueous extract was acidified to pH 3, and nitrite was added. The water was extracted with methylene chloride and analysed for volatile nitrosamines by GC–TEA. Both NDMA and NDEA were found at the 10–20 ppb level, indicating the presence of nitrosatable amine precursors in the raw rubber.

DISCUSSION

Both nitrosamine precursors, namely, amines and a nitrosating agent, must be present for a nitrosamine to form. The chemical accelerators and stabilizers added during the vulcanization process are the source of the amines in rubber nipples. Depending on formulation, these compounds, often added at levels in excess of 1·5%, contain amine functional groups which are designed to break off when heated during vulcanization to release sulphur. Typical examples of accelerators used are dialkyl dithiccarbamates and dialkyl thiuram mono- and polysulphides (Fig. 2). Methyl and ethyl accelerator substituents are commonly used in the rubber nipple industry as evidenced by the presence of their corresponding nitrosamines. Studies have shown that when these compounds are not present or are present at reduced levels, the nitrosamine levels in the resulting rubber and in the surrounding air are significantly reduced (McGlothlin *et al.* 1981; Preussmann *et al.* 1981).

It is not certain at this time what the specific nitrosating agent(s) are, although several possibilities have been suggested for the rubber tyre industry. McGlothlin *et al.* (1981) have shown that nitrosodiphenylamine (NDPhA) was the nitrosating agent at a rubber tyre manufacturing plant. NDPhA, a commonly used vulcanization retardant, will transnitrosate other amines under the proper conditions (Buglass, Challis & Osborne, 1974). Nitrogen oxides in contaminated air in rubber manufacturing plants may also act as nitrosating agents.

Although the nitrosation of amines during the vulcanization process appears to be the primary source of nitrosamines, the accelerators themselves are contaminated with nitrosamines (Ireland *et al.* 1980; Spiegelhalder & Preussmann, 1982).

Recently, considerable interest has focused on the endogenous formation of nitrosamines from ingested amines (Tannenbaum, 1979). Nitrites, present in human saliva, may react with amines ingested by an infant while sucking on a rubber nipple. Recently, German scientists described a method for estimating quantities of nitrosamines ingested by feeding infants by using simulated saliva containing nitrite to extract rubber nipples (Preussmann *et al.* 1981; Spiegelhalder & Preussmann, 1982). Both volatile and nonvolatile nitrosamines were extracted from rubber nipples at levels up to 230 ppb, while nitrosatable amine levels in the nipples were as high as 22,000 ppb. German law

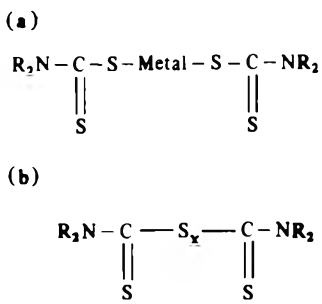

Fig. 2. Types of rubber accelerators: (a) dithiocarbamates; (b) thiuram mono- and polysulphides.

presently allows a maximum of 10 ppb volatile nitrosamines and 200 ppb nitrosatable compounds in rubber nipples as determined by this method of extraction (Spiegelhalder & Preussmann, 1982).

Two approaches to reducing the nitrosamine hazard in rubber products have been suggested. Either the accelerators used in the vulcanization process must be replaced by compounds that will not nitrosate, or they must be replaced by accelerators that, when nitrosated, form nitrosamines that are not carcinogenic. A number of compounds that meet these criteria are available. Alternative vulcanization inhibitors have also been suggested (Spiegelhalder & Preussmann, 1982).

Our work in this area is continuing. Future studies will include a survey of rubber nipples from the US retail market. Also, the presence of nonvolatile nitrosamines in these products will be studied.

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SHORT PAPERS

THE MOBILIZATION OF ALUMINIUM FROM THREE BRANDS OF CHEWING GUM

A. LIONE
Associated Pharmacologists & Toxicologists, 5510-16th Street, NW, Washington, DC 20011

and

J. C. SMITH
Institute of Environmental Health, University of Cincinnati Medical Center, Cincinnati, OH 45267, USA

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Abstract—The aluminium content of three chewing gums was measured before and after chewing. Analysis by atomic absorption spectrometry demonstrated that a single stick of chewing gum may contain as much as 4 mg aluminium. Analysis of samples after chewing revealed that between 2 and 21% of the aluminium in some chewing gums is mobilized during chewing. These data suggest that a stick of chewing gum can yield aluminium levels that correspond to 0·05–2·22% of the typical daily intake of aluminium from all dietary sources. Therefore, although the aluminium content of some chewing gums is relatively large, only a small proportion of the aluminium is mobilized by chewing. These gums are unlikely to contribute significantly to the daily intake of dietary aluminium.

Introduction

Kupchella & Syty (1980) recently reported the aluminium content of some chewing gums to be 600–900 μg/g. For a typical 5 g stick of chewing gum, this corresponds to 3·0–4·5 mg aluminium per stick. Thus, a single stick of gum may contain between 15 and 22% of the typical daily aluminium intake of 20 mg (Federation of American Societies for Experimental Biology, 1975; Underwood, 1977). In the light of the increasing concern regarding the toxicity of aluminium to the brain (Crapper McLachlin & DeBoni, 1980), the parathyroid (Cann, Prussin & Gordon, 1979) and bone (Spencer & Lender, 1979), we have investigated the mobilization of aluminium from chewing gum to estimate its importance as a source of dietary aluminium.

Experimental

The following chewing gums were obtained from retail suppliers and analysed: 'Wrigley's Spearmint Gum', Wrigley Company, Chicago, IL; 'Bubble Yum', Life Savers, Inc., New York, NY; 'Trident Sugarless Gum', Warner-Lambert Company, Morris Plains, NJ.

All of the samples were chewed by one subject (A. L.). Because many toothpastes contain aluminium oxide and other aluminium salts (Penna, 1979), no toothpaste was used during the sample collection period. A sodium chloride/sodium bicarbonate mixture was used for teeth cleaning, which was completed at least 1 hr before a gum sample was chewed. Saliva pH was measured immediately before and after each gum sample was collected. All pH measurements were 6·0. Gum was chewed for 20 min, then placed on a clean glass plate and covered loosely to dry for 12 hr. The chewed gum was then transferred to a clean glass vial. The interval between each sample collection was at least 24 hr.

A wet ashing procedure was used to prepare samples for analysis. Approximately 0·5 g of each chewing-gum sample was digested with 20 ml concn. HNO_3 at 150°C for 10 hr in a covered beaker. Ten millilitres of an acid mixture (HNO_3–$HClO_4$–H_2SO_4, 2:2:1, by vol.) was then added, and digestion at 150°C was continued for a further 5 hr. Next the beaker was uncovered and the sample was taken to near dryness. The residue was dissolved using 10% HNO_3 to a final volume of 5·0 ml. Aluminium analysis by atomic absorption spectrometry was performed as described previously (Fetterolf & Syty, 1979) using a Perkin Elmer Model 403 atomic absorption spectrometer.

Aluminium recovery by the method of standard additions averaged 105%.

Results

The aluminium contents of the gums before chewing were as follows: Trident Sugarless Gum, 132 μg/g total weight (ppm); Wrigley's Spearmint Gum, 176 ppm; Bubble Yum, 515 ppm. In the process of chewing, sugar, flavourings and other components of the gums are dissolved and removed, while some moisture is added to the remaining gum base. For this reason, comparison of the aluminium contents of the gum samples before and after chewing is reported in terms of the aluminium content of the whole sample

Table 1. *Aluminium content of chewing gums*

Product*	Total weight (g)	Al concn (ppm)	Al content (mg/stick)	Al extracted by chewing (mg)	% of total Al extracted
I: unchewed	7·724	515	3·978	—	—
chewed	2·348	–	3·533	0·445	11·2
II: unchewed	1·590	132	0·210	—	—
chewed	0·574		0·166	0·044	21·0
III: unchewed	3·169	176	0·558	—	—
chewed	0·877	–	0·547	0·011	2·0

*The products analysed were as follows: I –Bubble Yum; II– Trident Sugarless Gum; III– Wrigley's Spearmint Gum.
Values are means of duplicate analyses.

(Table 1). Between 2·0 and 21% of the aluminium content of the gums was mobilized by chewing. Amounts of aluminium extracted by chewing were: 0·445 mg from Bubble Yum, 0·11 mg from Wrigley's Spearmint Gum and 0·44 mg from Trident Sugarless Gum.

Discussion

The aluminium content of chewing gums reported here (132–515 ppm) is consistent with the values (610–900 ppm) reported previously by Kupchella & Syty (1980). Thus, some chewing gums contain quantities of aluminium in a single stick that equal 20% of the normal daily intake of aluminium. However, the total amount of aluminium extracted from the gums by chewing is small, ranging between 0·011 and 0·445 mg. These quantities correspond to 0·05–2·22% of the estimated daily intake of aluminium from all sources (Federation of American Societies for Experimental Biology, 1975; Underwood, 1977). Therefore, moderate use of the chewing gums analysed in this report would not contribute substantially to daily aluminium intake. Heavy use of certain gums could represent a significant dietary source of aluminium. The bioavailability of the aluminium in chewing gums has not been investigated.

The percentages of aluminium extracted from the various gums differed by more than tenfold (2·0–21%). Thus, the data suggest that more than one form of aluminium may be present in the gums analysed. In future work it may be useful to determine at what point in the processing or manufacture of chewing gums the aluminium content of these products is elevated, in order to identify the sources of soluble aluminium that may be inadvertently added to chewing gums (Junière & Sigwalt, 1964).

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MUTAGENIC EFFECTS OF IRRADIATED GLUCOSE IN *DROSOPHILA MELANOGASTER*

M. B. VARMA, K. P. RAO*, S. D. NANDAN and M. S. RAO
Department of Genetics, Osmania University, Hyderabad-500007, India

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Abstract—The mutagenic effects of irradiated glucose were studied using the sex-linked recessive lethal test in *Drosophila melanogaster*. Oregon K males of *D. melanogaster* reared on a medium containing 20 or 40% glucose irradiated with a dose of 0·02, 0·10, 0·20, 2 or 5 Mrad γ-rays were scored for the induction of sex-linked recessive lethals. The results showed no significant increase in the frequency of X-lethals in Drosophila at any of the dose levels.

Introduction

The use of ionizing radiation in food preservation has increased considerably during the last few years. Earlier the genetic hazards known to be associated with ionizing radiation had led to extensive studies on the possible cytotoxic and mutagenic effects of irradiated food using *in vitro* and *in vivo* test systems.

Ehrenberg (1960) reported that irradiated glucose induced a high frequency of chlorophyll mutations in the M_2 generation of barley seeds. Subsequent studies revealed that irradiated sugar solutions produced variations in chromosomal structure and function in cells both from plant sources and from mammalian organisms (Kesavan & Swaminathan, 1971). Irradiated sugar solutions that exerted mutagenic effects in *Salmonella typhimurium in vitro* failed to do so in an *in vivo* host-mediated assay using the mouse as the host, a difference that was attributed to the metabolism that ocurred *in vivo* (Aiyar & Subba Rao, 1977).

An attempt was made in the present study to evaluate the mutagenic response of irradiated glucose in *Drosophila melanogaster*, using the sex-linked recessive lethal test, which is one of the sensitive prescreening methods for detecting mutagenic substances (Sobels & Vogel, 1976).

Experimental

Test material. D-Glucose (anhydrous), obtained from M/s Sarabhai Chemicals, Baroda, was used. The powder was γ-irradiated at dose levels of 0·02, 0·10, 0·20, 2 and 5 Mrad, using a cobalt-60 source. The irradiation was carried out in polythene bags in the presence of air and at room temperature (25 ± 1°C).

Experimental design. Two series of experiments were conducted differing only in the level of glucose (20 or 40%) incorporated in the medium. Each series involved seven groups of *D. melanogaster* reared, respectively, on a standard corn-meal medium (control 1), on corn-meal medium containing 20 or 40% unirradiated glucose (control 2) or on corn-meal medium containing 20 or 40% glucose freshly irradiated with 0·02, 0·10, 0·20, 2 or 5 Mrad. The standard corn-meal food medium contained 3·14% agar, 3·14% dried yeast powder, 21·43% corn meal, 18·00% molasses, 0·7% propionic acid, and 54% water. The molasses was omitted when glucose was added to this medium.

Procedure. Male and female *D. melanogaster* (Oregon-K strain) were allowed to feed on the standard corn-meal medium for 4 days and were then discarded. The larvae that hatched were divided into seven groups for each series of experiments and allowed to feed on one of the test or control media and to emerge as adult flies. The males from each test or control group were then collected and mated with homozygous females of the *y* sc^{s1} *In 49* sc^{s8}*; bw, st* stock. The flies emerging from the vials were randomly selected for F_1 matings. The F_1 females were mated individually with *y* sc^{s1} *In 49* sc^{s8}*; bw, st* males and the F_2 generation was screened for induced frequency of sex-linked recessive lethal mutations. The data were subjected to the chi-square test of 2 × 2 contingency.

Results and Discussion

The data obtained on the frequency of sex-linked recessive lethals in *D. melanogaster* reared on medium containing 20 or 40% irradiated glucose are presented in Table 1. Statistical analysis of the data showed no significant differences between the control and treated groups at any level of glucose irradiation.

The reports already available on the mutagenicity of irradiated food media in *D. melanogaster* are conflicting. Munoz & Barnett (1968) reported a high incidence of recessive lethal mutations in the spermatogonia of Drosophila injected with irradiated solutions of fructose and glucose. However, we observed no effect with the feeding of irradiated glucose, while Rinehart & Ratty (1967) failed to show any increase in the frequency of sex-linked recessive lethal mutations in Drosophila reared on media containing irradiated sucrose. The differences in mutagenic activity observed

*To whom correspondence should be addressed.

Table 1. *The frequency of sex-linked recessive lethals in* Drosophila melanogaster *reared on media containing 20 or 40% glucose irradiated in powder form with various doses of γ-rays*

Test group*	Total no. of X-chromosomes	Incidence of lethals: No.	Incidence of lethals: % of total
Tests on 20% glucose			
Control 1	4251	13	0·30
Control 2	3384	11	0·33
0·02 Mrad	3528	11	0·31
0·10	3016	10	0·33
0·20	3195	10	0·31
2·00	2778	10	0·35
5·00	3255	12	0·36
Tests on 40% glucose			
Control 1	5000	14	0·28
Control 2	3435	11	0·32
0·02 Mrad	3310	10	0·30
0·10	3034	10	0·32
0·20	3580	12	0·33
2·00	2842	12	0·42
5·00	2754	11	0·39

*Control 1—Drosophila reared on standard corn-meal medium; control 2—F_0 larvae reared on corn-meal medium with molasses replaced by 20 or 40% unirradiated glucose; 0·02–5·00 Mrad—F_0 larvae reared on corn-meal medium with molasses replaced by 20 or 40% glucose previously irradiated (as powder, with a γ-ray dose between 0·02 and 5·00 Mrad as indicated).

For all the test values, $P > 0·05$ by chi-square analysis.

in these studies may be attributable to the different modes of administration of the compounds.

An increase in the frequency of dominant and sex-linked recessive lethals and also of visible mutations in Drosophila fed an irradiated medium has been reported by Rinehart & Ratty (1965 & 1967) and by Swaminathan, Nirula, Natarajan & Sharma (1963). Other studies have shown a high frequency of sex-linked and autosomal recessive lethals and dominant lethal mutations in Drosophila cultured on medium supplemented with irradiated DNA (Parkash, 1965) or on irradiated basic medium (Kesavan & Swaminathan, 1969). The absence of evidence of genetic damage by irradiated glucose in our study supports the lack of any mutagenic response in Drosophila cultured on irradiated media in earlier studies reported by Chopra (1965), Holsten, Sugii & Steward (1965), Hossain, Mollah & Malik (1967) and Reddy, Rao *et al.* (1965) and on medium supplemented with irradiated potatoes, soya flour or irradiated DNA (Chopra, 1965; Khan & Alderson, 1965; Rinehart & Ratty, 1967; Scarascia-Mugnozza, Natarajan & Ehrenberg 1965).

These variations in the mutagenic response of *D. melanogaster* to irradiated media and irradiated food components have been attributed to differences in the catalase content of the flies (Kesavan & Swaminathan, 1969). Sobels (1956) pointed out that catalase in the body cavity of flies nullifies the mutagenic activity of organic peroxides. It is possible that catalase may have been a factor in the absence of mutagenic effect in the study described here.

On the other hand, it is now known that radiation-induced hydrogen peroxide plays a precursor role in the ultimate toxicity of irradiated carbohydrates. Irradiation of water or of aqueous solutions of carbohydrates produces hydroperoxide radicals ($HO_2^{\cdot}$) and hydrogen peroxide (H_2O_2), which can react with carbohydrates to produce other toxic substances like glyoxal (Berry, Hills & Trillwood, 1965). If solid carbohydrate is irradiated and then dissolved in water, production of $HO_2^{\cdot}$ and H_2O_2 and hence of other toxic substances is not likely. In this study the experiments were carried out using glucose irradiated in powder form and no toxicity was observed. This suggests that $HO_2^{\cdot}$ and H_2O_2 and toxic substances produced as a result of the reaction of $HO_2^{\cdot}$ and H_2O_2 with organic substances may have been responsible for the toxicity that was observed in earlier studies.

Since carbohydrates form one of the main components of food materials, the genotoxicity of these substances is of major importance in the overall toxicological assessment of irradiated food. The results reported here clearly demonstrate the inability of irradiated glucose to induce genetic damage and thus contribute to the evidence for the non-mutagenic activity of irradiated foods in general reported by earlier workers.

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THE EFFECT OF DOSE AND VEHICLE ON EARLY TISSUE DAMAGE AND REGENERATIVE ACTIVITY AFTER CHLOROFORM ADMINISTRATION TO MICE

D. H. MOORE, L. F. CHASSEAUD, S. K. MAJEED, D. E. PRENTICE and F. J. C. ROE

Huntingdon Research Centre, Huntingdon, Cambs.

and

N. J. VAN ABBÉ

Beecham Products Research Department, Randalls Road, Leatherhead, Surrey, England

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Abstract—The relationship between the acute toxicity of orally-administered chloroform and its long-term tumorigenic potential was studied in male mice of the CFLP outbred Swiss albino mouse strain. A single dose of approximately 18 mg $CHCl_3$/kg had no detectable acute toxic effect on the liver or kidneys and did not stimulate regenerative activity, whereas both toxicity and subsequent tissue regeneration were observed with single doses of about 60 mg/kg or higher. The severity of the toxic effects and regenerative changes was greater when corn oil was used as a vehicle for chloroform than when the vehicle was a toothpaste base. In earlier long-term studies in mice of the same strain, kidney tumours occurred in males given 60 mg/kg/day throughout life but not in mice given 17 mg/kg/day. The tumour response was greater when the 60-mg/kg/day dose was given in an oily vehicle than when it was given in toothpaste. The findings are consistent with the hypothesis that early acute toxic change and subsequent repair are a *sine qua non* for tumorigenesis in the kidney and liver in response to chloroform.

Introduction

For many decades, chloroform has been widely used as a preservative and flavouring agent in medicines and toothpaste, but the safety of these uses was questioned when, in some experiments (National Cancer Institute, 1976; Roe, Palmer, Worden & Van Abbé, 1979), the long-term administration of chloroform in high dosage led to enhanced incidences of liver or kidney tumours in laboratory rats and mice. From earlier work (Ilett, Reid, Sipes & Krishna, 1973; Klaassen & Plaa, 1967; Waters, 1951) it was known that the same tissues are the principal targets for the acute toxicity of chloroform. It seemed possible therefore that the later development of tumours was dependent on the much earlier production of tissue damage and/or on regenerative activity following such damage. The present study was carried out to establish the threshold dose for acute toxicity and repair, to determine whether this was influenced by the nature of the vehicle in which chloroform was administered and to test the hypothesis that kidney tumour development in CFLP mice exposed to chloroform depends on prior chloroform-induced damage and repair in the kidney.

Experimental

Dosage forms. Two chloroform formulations, one with corn oil as the vehicle and the other in a toothpaste base (see Roe *et al.* 1979), were prepared for administration to the mice. The chloroform was of pharmaceutical grade and the corn oil of food grade. The corn oil solutions contained 0, 0·45, 1·8 and 7·2% (w/w) chloroform. The toothpaste formulations contained 0, 0·325, 0·94 and 3·59% (w/w) chloroform, as shown by gas-chromatographic analysis, when administered.

Animals and treatment. Male 6-wk-old CFLP outbred Swiss albino mice (from Hacking and Churchill Ltd, Wyton, Huntingdon) were allocated to treatment groups at random. Groups, each consisting of at least three animals, were given single doses of one of the test materials by intragastric intubation, the dose levels of chloroform being as close as possible to a planned schedule of 0, 15, 60 and 240 mg/kg body weight. After 3 days, each mouse received an intraperitoneal injection of [6-^{3}H]thymidine (supplied by The Radiochemical Centre, Amersham; specific activity 83 mCi/mg and radiochemical purity 98%) to provide a 25·7-μCi dose. After a further 24 hr, the mice were anaesthetized with halothane, and blood samples were taken by cardiac puncture into heparinized tubes and centrifuged to separate the plasma. The mice were then killed and the liver and kidneys were dissected out and weighed.

Sample preparation and measurements. Plasma glutamic–pyruvic transaminase (GPT) and glutamic–oxalacetic transaminase (GOT) activities were measured on a Union Carbide Centrifichem analyser using the appropriate Roche diagnostic test kits at a working temperature of 30°C. Plasma urea was measured using the Smith Kline SK1 test kit. Liver and kidney samples were preserved in 10% buffered

Table 1. *Liver and kidney weights and [^{3}H]thymidine uptake in male CFLP mice given single intragastric doses of chloroform in corn oil or toothpaste*

Group	$CHCl_3$ dose (mg/kg body weight)	Liver weight (g)	Kidney weight (g)	Radioactivity uptake from [^{3}H]thymidine (% of dose in tissue)	
				Liver	Kidney
Vehicle: Corn oil					
Control	0	2·41 (0·37)	0·56 (0·05)	2·46 (0·49)	0·61 (0·17)
Low	17·3 (0·9)	2·44 (0·10)	0·58 (0·02)	2·87 (0·19)	0·70 (0·01)
Intermediate	65·6 (9·4)	2·57 (0·37)	0·70 (0·11)	3·87 (1·03)	3·02 (0·30)**
High†	273 (30·6)	2·61 (0·25)	0·87 (0·08)***	6·24 (3·43)*	2·53 (1·21)**
Vehicle: Toothpaste					
Control	0	2·38 (0·37)	0·58 (0·14)	2·66 (0·18)	0·65 (0·11)
Low	18·2 (0·6)	2·48 (0·15)	0·58 (0·04)	2·70 (0·43)	0·63 (0·08)
Intermediate	59·2 (2·1)	2·16 (0·09)	0·53 (0·04)	2·44 (0·60)	0·85 (0·37)
High‡	199 (6·5)	2·40 (0·38)	0·89 (0·18)*	3·87 (1·18)	2·88 (0·85)**

†Group of five mice.
‡Group of four mice.
Values are means for groups of three mice (except where indicated otherwise) with standard deviations in parentheses, and those marked with asterisks differ significantly (by Student's *t* test) from the corresponding control value: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

formalin and embedded in paraffin wax; sections 5 μm thick were stained with haematoxylin and eosin. For radioactivity measurements, further samples of liver and kidney (about 0·1–0·2 g) were burned in oxygen using a Model 306 automatic sample oxidizer (Packard Instrument Co. Ltd. Reading) and the combustion products were absorbed in Monophase-40 scintillator solution. The efficiency of the oxidizer was checked by combustion of tritium standards (obtained from the Radiochemical Centre). The dose, in terms of radioactivity, given to the mice was checked by mixing aliquots of the [^{3}H]thymidine dosing solution with a toluene–Triton X-100 (2:1, v/v) scintillator solution (10 ml) containing 0·39% (w/v) 2,5-diphenyloxazole and 0·01% (w/v) *p*-bis-(*o*-methylstyryl)benzene. Radioactivity was measured using a Philips automatic liquid scintillation analyser (Philips NV, Eindhoven, The Netherlands). Counting efficiencies were approximately 25–30% for all samples. Samples were counted for 4 min, thereby accumulating at least 10^4 counts, with a counting error $< 1\%$ of the mean gross count. Recovery of radioactivity from combusted standards was 99·1 ± 3·0%.

Results

No signs of toxic effects on the liver or kidneys were detected in mice given chloroform at the low dose level (means of 17·3 mg/kg in corn oil solution, 18·2 mg/kg in toothpaste). Liver and kidney weights, [^{3}H]thymidine uptake and values for plasma urea, GPT and GOT all closely approximated to those for the control mice (Tables 1 and 2) and histological examination revealed no abnormalities (Table 3).

With the intermediate dose of chloroform (mean 65·6 mg/kg) in corn oil, there was little sign of toxicity in the liver, no increase in liver weight, a minimal and non-significant increase in [^{3}H]thymidine uptake by the hepatocytes, no significant change in GPT or GOT and no histological abnormality. However, an increase in kidney weight approached significance at the 5% level and this was accompanied by a marked increase in [^{3}H]thymidine uptake in the kidneys, conspicuous tubular necrosis and areas of tubular basophilia indicative of tubular regeneration, but there was no rise in plasma-urea concentrations. With the intermediate dose of chloroform (59·2 mg/kg) in toothpaste, however, there was little sign of either liver or kidney toxicity and [^{3}H]thymidine uptake in the kidney was not significantly increased.

In mice given the high chloroform dose in either vehicle (means of 273 mg/kg in corn oil, 199 mg/kg in toothpaste), there was an increased uptake of [^{3}H]thymidine in the kidneys, plasma urea was elevated and kidney necrosis was seen in all animals. All

Table 2. *Biochemical indices of tissue damage in male CFLP mice given single intragastric doses of chloroform in corn oil or toothpaste*

Group	Mean plasma concentrations		
	Urea (mg/100 ml)	GPT (mU/ml)	GOT (mU/ml)
Vehicle: Corn oil			
Control	48	32	78
Low	49	31	80
Intermediate	47	35	64
High†	378***	71*	66
Vehicle: Toothpaste			
Control	68	25	60
Low	54	44	56
Intermediate	63	34	62
High‡	348‡	55	64

†Group of five mice.
‡Group of four mice. Individual urea values were 119, 145, 361 and 768; the difference from the control group was not statistically significant.
Values are means for groups of three mice (except where indicated otherwise) and those marked with asterisks differ significantly (Student's *t* test) from the corresponding control values: *$P < 0.05$; ***$P < 0.001$.

Table 3. *Histological findings in the liver and kidneys of male CFLP mice given single intragastric doses of chloroform in corn oil or toothpaste*

Group	No. of mice examined	Liver findings*		Kidney findings*			
		Minimal centrilobular enlargement	Areas of degenerate or necrotic cells	Tubular basophilia		Tubular necrosis	
				Occasional areas	Widespread	Occasional areas	Widespread
Vehicle: Corn oil							
Control	3	0	0	0	0	0	0
Low	3	0	0	0	0	0	0
Intermediate	3	0	0	1	2	2	1
High	5	4	1	0	3	0	5
Vehicle: Toothpaste							
Control	3	0	0	0	0	0	0
Low	3	0	0	0	0	0	0
Intermediate	3	0	0	1	0	0	0
High	4	2	0	0	4	0	4

*No. of mice affected.

the kidneys were enlarged and pale compared with those of the control mice. Plasma GPT and the uptake of [^{3}H]thymidine in the liver were both increased, particularly in the mice given chloroform in corn oil.

Discussion

Reitz, Quast, Watanabe & Gehring (1979) contrasted the negative findings for chloroform in bacterial mutagenicity studies with the positive findings recorded in some tumorigenicity bioassays. This group (Reitz, Quast, Stott *et al.* 1980) also reported their own investigations in which they gave single doses of chloroform dissolved in corn oil to male B6C3F1 mice and male Osborne–Mendel rats, in order to determine a no-effect level for liver and kidney damage and for tissue repair. They reported a no-effect level of 15 mg/kg. We have described here the outcome of similar experiments in which we examined the responses to single doses of chloroform given to males of the CFLP outbred Swiss albino mouse strain previously used in our own tumorigenicity bioassays. We had earlier reported (Roe *et al.* 1979) the development of an excess of kidney tumours in male CFLP mice given chloroform at 60 mg/kg/day for 80 wk though not in those given 17 mg/kg/day. The response had been numerically greater when chloroform was given in oil rather than in a toothpaste vehicle. The work described here offers a comparison of the effects produced by single doses of chloroform in these vehicles.

The intended dose levels of chloroform in this study were 15, 60 and 240 mg/kg. Although actual dose levels differed slightly in practice, we do not attach any importance to the minor discrepancies involved (e.g. between a nominal dose level of 60 mg/kg and actual levels of 59 mg/kg in toothpaste or 66 mg/kg in corn oil).

With both vehicles, we found that effects were seen in the kidney at a lower dose level than was needed to produce changes in the liver. These effects were more noticeable with corn oil as the vehicle than with toothpaste, a water-miscible vehicle. No detectable changes in either organ were recorded with single doses of 17 mg $CHCl_3$/kg in corn oil or 18 mg $CHCl_3$/kg in toothpaste.

We could not detect any kidney necrosis after giving the intermediate dose level (approximating to 60 mg $CHCl_3$/kg) in toothpaste but the appearance of tubular basophilia, indicative of regenerative activity, in one animal suggested that this dose may have reached a threshold at which regeneration following toxic action was beginning to appear. At the higher dose (approximating to 240 mg $CHCl_3$/kg), both tubular necrosis and basophilia were pronounced in all the mice.

With chloroform dissolved in corn oil, tubular necrosis and basophilia were readily seen at the intermediate dose level in all the mice and these changes became more marked with the high dose.

Corresponding data for [^{3}H]thymidine uptake in the kidneys (indicative of regenerative activity) corroborated the histological findings, showing a significant increase with the intermediate chloroform dose in corn oil.

With respect to the liver, histological evidence for toxic changes after a single chloroform dose was seen only at the high dose level. It was more noticeable with the corn-oil vehicle than with toothpaste, in terms of both centrilobular enlargement and [^{3}H]thymidine uptake, and was probably also reflected in the elevation of plasma GPT.

Taken as a whole, our findings agree with those of Reitz *et al.* (1980), confirming that toxic changes due to single doses of chloroform appear at lower dose levels in the kidney than in the liver. However, we have also shown that the changes are accentuated when chloroform is given in corn oil rather than in toothpaste.

Thus no toxic or regenerative changes were detectable following single-dose chloroform administration at approximately the dose level (17 mg/kg) at which no excess of tumours was found after long-term daily administration. However, toxic and/or regenerative changes began to appear when single doses of chloroform were given at the dose level (approximating to 60 mg/kg) at which long-term daily administration led

to the development of excess kidney tumours in elderly male mice of the strain used. A higher proportion of these mice (12/48 compared with 5/47) developed kidney tumours when the chloroform was given to them in an oily vehicle (arachis oil) than when the same chloroform dose was given in toothpaste. This appears to parallel our observation of a greater toxic effect and regenerative activity after the giving of a single dose of chloroform in corn-oil rather than in toothpaste.

Chloroform dose levels in our long-term studies with male CFLP mice and in the NCI study (National Cancer Institute, 1976) with Osborne–Mendel rats did not differ greatly, and excess liver tumours were not seen under these conditions. The present work and that of Reitz *et al.* (1980) point to the fact that little or no hepatotoxic effect would occur at these dose levels. However, at the much higher chloroform dose levels given to B6C3F1 mice (National Cancer Institute, 1976) hepatotoxic changes and regenerative activity would be expected, especially as a corn-oil vehicle was used, and these mice had a high incidence of liver tumours.

Numerous short-term studies using a wide variety of experimental techniques have shown that chloroform has little or no genotoxic activity (de Serres & Ashby, 1980; Diaz Gomez & Castro, 1980; Kirkland, Smith & Van Abbé, 1981; Perocco & Prodi, 1981; Petzold & Swenberg, 1978; Simmon, Kauhanen & Tardiff, 1977; Sturrock, 1977; Styles, 1979; Uehleke, Werner, Greim & Krämer, 1977; Van Abbé, Green, Jones *et al.* 1982). In looking for a non-genotoxic mechanism to explain the excess tumours observed when chloroform is given repeatedly to laboratory rodents, it is interesting to note the apparent relationship between chloroform dose level, choice of vehicle, onset of toxic changes in the organs concerned and early signs of regenerative activity even with single-dose administration. This suggests the possibility that a relatively modest toxic insult frequently repeated may suffice to increase opportunities for "spontaneous" development of neoplasms in those tissues where regeneration is repeatedly provoked. On the other hand, if dosage is insufficient to provoke regenerative activity, the risk of developing excess tumours may be negligible with a compound having little or no genotoxic potential. The limited evidence from the work reported here appears to be consistent with such a hypothesis.

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Review Section

DRAFT EEC METHOD FOR THE DETERMINATION OF THE GLOBAL MIGRATION OF PLASTICS CONSTITUENTS INTO FATTY-FOOD SIMULANTS: APPLICABILITY TO LACQUERS, PLASTICS AND LAMINATES*

D. VAN BATTUM, M. A. H. RIJK and R. VERSPOOR

Central Institute for Nutrition and Food Research, TNO, Utrechtseweg 48, Zeist, The Netherlands

and

L. ROSSI†

Commission of the European Communities (DGIII), 200 rue de la Loi, 1049 Brussels, Belgium

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Summary—An experimental study was carried out to establish whether the draft EEC method for the determination of the global migration of constituents from plastics packaging materials into fatty food simulants could be applied to all plastics, including lacquers and laminates. Some difficulties were encountered in the use of the EEC method for melamine, for hotmelt-coated packaging materials and for laminates containing one or more layers of materials sensitive to moisture, such as paper, cardboard or regenerated cellulose film.

INTRODUCTION

An earlier publication (Rossi, 1977a) discussed the principal rules and standards that, according to the Commission of the European Communities, should form the basis of Community standards on plastics materials and articles intended to come into contact with foodstuffs.

One of the standards that has come under most criticism (Figge, Cmelka & Koch, 1977) lays down a limit for the total quantity of constituents that can migrate from the material into the food or food simulant in contact with it ('global migration limit'). There were doubts as to whether—from the point of view of hygiene and health—there was any real need to establish such a limit, and objections were raised with regard to the practical difficulties involved in checking it. This was particularly the case with regard to migration into liquids simulating fatty foodstuffs (the fat test), because of the analytical problems involved in applying the proposed testing methods, which were all complicated to perform, were sometimes not very accurate and were reproducible only to a very limited extent.

In order to remedy the various difficulties, the Commission of the European Communities, with the active collaboration of governments and industrial organizations, organized four cycles of round-robin analyses between 1972 and 1978 (i) to try out the numerous methods proposed for the fat test (Figge, 1973; Pallière, 1972; Rossi, Sampaolo & Gramiccioni, 1972; van Battum & Rijk, 1972), (ii) to select the most reliable ones, (iii) to standardize procedures as much as possible, (iv) to establish their repeatability and reproducibility and (v) to ascertain whether or not they could be applied to the principal materials on the market. The results of this research have already been published (Rossi, 1977b; Rossi, 1981).

The conclusions may be briefly summarized as follows: although the various methods developed for this purpose (Community method, modified Figge's method, modified van Battum's method) are difficult to perform and are reproducible only to a limited extent—which means that the tests have to be repeated to obtain a sufficiently precise value—they are considered to be acceptable from the point of view of accuracy, at least as far as the materials that were actually tested are concerned. In particular, they are regarded as acceptable in comparison with previously proposed methods. However, the materials tested were limited to a small number of homogeneous materials that are easily conditioned (polyethylene with various melt-flow indices, polypropylene, various types of polystyrene, rigid polyvinyl chloride, copolymers of acrylonitrile–butadiene–styrene and a polyester).

Therefore the Commission of the European Communities decided in 1979 to ask the Central Institute for Nutrition and Food Research (CIVO-TNO, Zeist) to extend the experiments to other materials in order to ascertain whether or not the Community method could be applied generally and, if so, to establish the extent of its validity and any modifications required. For this purpose, an *ad hoc* group of experts, including representatives of the industrial organizations concerned, selected the materials to be tested from

*Study carried out by the CIVO-TNO Institute under EEC contract.

†Istituto Superiore di Sanità, Rome, Italy.

among those that had already posed analytical problems at national level (melamine, polyamide and various types of laminate that included a hygroscopic layer such as board, etc.) or for which there was a lack of data at Community level (lacquers). This paper presents the results obtained.

EXPERIMENTAL

Test samples. Table 1 lists the 27 samples analysed, giving for each a description of the type of plastics material and the abbreviation used. Samples 19, 20 and 21 were lacquered (one side only) tin plate.

Test method and conditions. The method used was the Community method described in full in Document III/1521/78 of the Commission of the European Communities and referred to in another publication (Rossi, 1981). This method consists basically of weighing a conditioned sample before and after the period of contact with the simulant. The difference in mass is then adjusted for the quantity of simulant absorbed by the sample, as measured by a gas-chromatographic method after extraction with a suitable solvent. All tests involved contact for 10 days at 40 C, using refined olive oil except where a different liquid simulant is specifically indicated. All tests were carried out in triplicate and, where appropriate, only the side

Table 1. *Identification of the samples examined*

Reference no.	Description	Abbreviation
1	Lacquer code 10 – white pigmented internal acrylic lacquer; applied to cans 211 × 400 size (*c.* 68 mm diam. × 100 mm); film weight *c.* 14 g/m^2	Pigm. acrylic lacquer
2	Lacquer code 16 – alum-pigmented internal epoxyphenolic lacquer containing neat release agent (Acrawax C); applied to cans 300 × 207 size (*c.* 75 mm diam. × 62 mm); film weight approximately 5·5 g/m^2	Pigm. epoxyphenol lacquer
3	Lacquer code 20 – clear gold internal epoxyphenolic lacquer; applied to cans 300 × 401 size (*c.* 75 mm diam. × 103 mm); film weight *c.* 5 g/m^2	Clear epoxyphenol lacquer
4	Paper–Alufoil–hotmelt	PAPER–ALUFOIL–*HOTMELT*
5	Paper–polyethylene (low density)–polyvinylidene chloride, thickness 0·07 mm	PAPER–LDPE–*PVDC*
5A	As 5; different manufacturer	PAPER–LDPE–*PVDC*
6	MXDT/A–polyethylene (low density), the polyethylene being applied as extrusion coating; thickness 0·08 mm	MXDT/A–*LDPE*
6A	MXDT/A–polyurethane adhesive–LDPE; thickness 0·07 mm	MXDT/A–PU adh–*LDPE*
7	Paper–polyvinylidene chloride–polyethylene (low density); thickness 0·10 mm	PAPER–PVDC–*LDPE*
7A	As 7; different manufacturer	PAPER–PVDC–*LDPE*
8	Nitrocellulose–regenerated cellulose film–polyethylene (low density); thickness 0·02 mm	NITROCEL–RCF–*LDPE*
9	Paper–Alufoil–Surlyn; thickness 0·10 mm	PAPER–ALUFOIL–*SURLYN*
10	Polyethylene (high density)–EVA (coextrusion); thickness 0·05 mm	HDPE–*EVA coextr.*
11	Polyamide–Surlyn; thickness 0·10 mm	PA–*SURLYN*
12	Polypropylene–high slip EVA; thickness 0·06 mm	PP–*EVA high slip*
13	Nylon–adhesive–polyethylene (medium density); thickness 0·06 mm	PA–ADH–*MDPE*
14	Polyvinyl chloride plasticized (575 g/m^2)–polyamide–polyvinyl chloride plasticized (575 g/m^2)–polyurethane (70 g/m^2); thickness 0·85 mm	PVC pl–PA–PVC pl–*PU*
15	Polyamide 6 film; thickness 0·095 mm	PA-6
16	Melamine resin 152·7; thickness 2·00 mm	MELAMINE
17	Polyvinyl alcohol film, waterproof; thickness 0·04 mm	PVA
18	Polyvinylidene chloride (14 g/m^2)–cardboard (210 g/m^2)–polyvinylidene chloride (9 g/m^2); thickness 2·50 mm	PVDC–CARDB–*PVDC*
19	White pigmented epoxy-melamine-phenolic lacquer, applied to tinplate on one side only	Epox. melam. phenol lacquer
20	Alum-pigmented vinyl organosol lacquer, applied to tinplate on one side only	Vinyl organosol lacquer
21	Clear gold epoxy phenolic lacquer, applied to tinplate on one side only	Clear epoxyphenol lacquer

For laminates, the total thickness is given and the polymer italicized in the abbreviation (column 3) is the layer exposed to the food simulant.

Table 2. *Global migration from various materials into the fatty-food simulant*

Test sample		Global migration (mg/dm²)*		
No.	Identity	1	2	3
1	Pigm. acrylic lacquer	−1·9	−1·5	−1·2
2	Pigm. epoxyphenol lacquer	−0·8	−0·4	−1·5
3	Clear epoxyphenol lacquer	−2·2	−1·6	−2·8
4	PAPER–ALUFOIL–HOTMELT	ND†	ND†	ND†
5	PAPER–LDPE–PVDC‡	2·2	−3·5	−6·0
		(2·9)	(−2·9)	(−5·6)
5A	PAPER–LDPE–PVDC	3·6	−0·5	−2·2
6	MXDT/A–LDPE	−0·4	0·6	4·1
6A	MXDT/A–PU adh–LDPE	4·1	4·1	0·7
7	PAPER–PVDC LDPE	1·1	0·2	0·1
7A	PAPER–PVDC–LDPE	−3·4	−1·2	−1·9
8	NITROCEL–RCF–LDPE	12·1	8·3	9·0
9	PAPER–ALUFOIL–SURLYN	−1·5	−2·2	−1·1
10	HDPE–EVA coextr.	0·4	−0·1	−0·4
11	PA–SURLYN	3·0	3·1	3·5
12	PP–EVA high slip	0·6	1·2	0·1
13	PA–ADH MDPE‡	−3·4	−3·7	−4·1
		(−0·5)	(−1·1)	(−2·1)
14	PVC pl–PA PVC pl–PU	600†	630†	860†
15	PA-6	−0·3	−0·4	0·2
16	MELAMINE	ND	ND	ND
17	PVA	9·6	6·8	9·3
18	PVDC–CARDB–PVDC‡	−195	−176	−160
		(−7·4)	(6·0)	(−3·9)
19	Epox. melam. phenol lacquer	−0·9	−0·4	−0·3
20	Vinyl organosol lacquer	−0·7	−0·7	−0·6
21	Clear epoxyphenol lacquer	−1·2	−1·7	−1·6

ND = Not determinable

*Results of triplicate tests.
†The fat simulant HB 307 was used instead of olive oil.
‡The values in brackets were obtained after a second extraction of the sample (no. 5) or after the sample had been dissolved and reprecipitated (nos 13 and 18; see text).
For full identification of the test samples, see Table 1.

intended to come into contact with the foodstuff was exposed to the simulant.

RESULTS AND DISCUSSION

The results of the tests are summarized in Table 2. These results may appear satisfactory, given the difficulties inherent in this type of analysis, but application of the method has revealed a number of problems. These problems relate to the conditioning of the samples, the extraction of the oil absorbed by the sample, the determination of the quantity of oil extracted, the presence in the material examined of substances that may interfere with the monitoring of the oil, and various technical difficulties.

Problems connected with the conditioning stage

Figure 1 gives the humidity absorption curves for a number of samples during conditioning, which was carried out at 22°C and 50% relative humidity. It can be seen that in the case of sample 16—melamine—it is not possible to establish equilibrium sufficiently (a difference between two successive weighings $\leqslant 1\ mg/dm^2$) for the Community method to be applied. In the other examples given, analysis is possible provided conditioning continues for the time necessary to achieve equilibrium. The length of conditioning time varied according to the sample, ranging

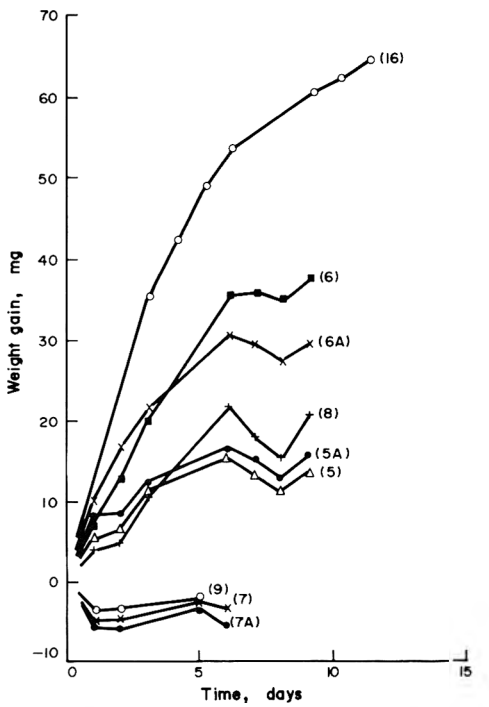

Fig. 1. Moisture uptake by the test samples during conditioning at 22°C and 50% relative humidity.

from 1 to 8 days. Only samples 1, 2, 3, 10, 12, 19, 20 and 21 required no conditioning at all. The fact that with some samples successive weighings did not reach the specified degree of agreement, although a state of equilibrium appeared to have been reached, obviously stems from an analytical error inherent in the method itself and is one possible explanation for the negative global-migration values obtained.

Problems at the sample-extraction stage

As stated in the method, the analyst must take special care to ensure that the oil absorbed is fully recovered. If it is not, the migration values obtained will be lower than the true value and will sometimes even be negative. This happened with samples 13 and 18. In these cases, re-extraction with trichlorotrifluoroethane (sample 13) or with trichlorotrifluoroethane and diethyl ether (sample 18) was not enough to extract all the oil, but it was largely (though not entirely) recovered by dissolving the MDPE layer of sample 13 in toluene and the layers of PVDC of sample 18 in tetrahydrofuran. In the case of sample 5, however, re-extraction with diethyl ether did not affect the result significantly. In this case, therefore, the negative values obtained must have resulted from other factors.

Problems at the oil determination stage

According to the Commission's proposals, this method allows for an absolute deviation of 3 mg/dm^2 to take some account of the various possible analytical errors inherent in the method. If this deviation is accounted for entirely at the oil monitoring stage, the maximum theoretically determinable quantity of oil absorbed must not exceed 150 mg if the sample has a surface area of 2 dm^2 and if the normal error in the quantitative determination of oil by the gas-chromatographic process is around 4%. In practice the limit for the quantity of oil that can be determined is around 50 mg/dm^2. This means that it is not possible to apply the method correctly when the oil absorption of the sample is higher than this value, or rather, it suggests that the tolerable deviation should be increased. In the samples tested, oil absorption was greater than the maximum permissible on the above basis in the following cases: samples 5 and 5A (*c.* 175 mg/dm^2), samples 7 and 7A (*c.* 150 mg/dm^2) and 18 (*c.* 400 mg/dm^2).

Problems of interference

Problems arise in determining global migration when packaging materials contain constituents that interfere with the gas-chromatographic fat determination. These difficulties can sometimes be bypassed by using another fatty food simulant, such as sunflower oil or the synthetic triglyceride mixture HB 307 instead of olive oil. In this study olive oil could not be used in the determination of global migration from samples 4 and 14. However, the fat simulant HB 307 proved to be suitable.

Technical problems

Samples 4 and 14 also gave rise to technical problems. Sample 4 was a hotmelt-coated laminate. When the Maturi cell was dismantled after the 10-day contact period, it appeared that part of the hotmelt coating remained stuck to the polytetrafluoroethylene U-frame of the cell. It was not possible therefore to determine the true weight of the sample after contact with the fat simulant or, consequently, to determine the global migration.

Another type of technical problem arose in determining global migration from sample 14. It is not possible to extract the fat absorbed by the food-contact side of a laminate while the sample is still part of the Maturi cell; the sample must first be removed from the cell. As, subsequently, the extraction of the sample with trichlorotrifluoroethane involves both sides of the sample, constituents of the layers of the laminate that do not come into contact with food can interfere with the fat determination either because of their chemical or physical properties or just by quantity. In the case of sample 14 the latter cause was predominant; the migration values obtained were, therefore, not reliable and in any case they were too high. It is not yet clear why the migration values obtained in testing the lacquers with the fatty-food simulant were consistently negative.

Conclusions

Because of the limited number of samples tested, it is not possible to make any conclusive judgements about the applicability of the Community method of determining global migration to fatty foodstuffs. Nevertheless, the tests carried out do reveal the main difficulties that may be encountered in applying the method to commercially used samples. Provision is made in the method itself for meeting some of these difficulties: e.g. the need to condition the sample, to check that the oil is fully extracted and to replace the simulant where the material contains interfering substances. The method includes a number of ways of dealing with these problems, or at least of ascertaining whether or not they exist. On the other hand, there are other difficulties, such as the technical problems mentioned above and the problem with melamine, that have not so far been tackled at European level, although they have already been noted at the national level.

However, despite the fact that the number of materials tested imposes certain limitations, as explained above, the following points can be drawn from this study:

(a) the draft fat test can most probably also be applied to lacquers;
(b) it is also very probable that the fat test can be applied to laminates consisting of two or more layers of plastic, provided none of the plastics used is sensitive to moisture, can absorb an appreciable quantity (> 50 mg/dm^2) of fatty-food simulant, or contains substances that because of their quantity or some chemical or physical property interfere with the determination of the amount of fatty-food simulant absorbed by the sample;
(c) the fat test is virtually not applicable to laminates containing one or more layers of materials, such as paper, cardboard or regenerated cellulose film, that are sensitive to moisture;
(d) the fat test is not likely to be applicable to melamine-based packaging materials;

(e) the fat test is not likely to be applicable to hotmelt-coated packaging materials.

In view of these conclusions, and until future progress resolves outstanding problems in the application of the fat test, it seems worthwhile to reconsider the use of a volatile food simulant. The experience gained in the studies on the determination of global migration using such fat simulants as olive oil and HB 307 shows that most of the difficulties encountered result from the fact that the food simulant cannot be evaporated. The use of a volatile food simulant would save much trouble and would allow the determinations to be performed more quickly. In the past, several volatile solvents have been used as food simulants, including diethyl ether, hexane and heptane. None of them proved to be satisfactory for all types of packaging material. Recently, however, in some preliminary migration tests carried out under conventional contact conditions, it was found that the results obtained with isooctane appeared to be in fairly good agreement with those obtained in tests with olive oil as the fatty-food simulant. In fact they were so satisfactory that it was considered worthwhile to start a series of experiments to explore the use of isooctane. The results of these experiments will be reported later.

Acknowledgements – The authors would like to thank the following producers for supplying samples for this research: Metal Box Ltd, Wantage, UK; UK Flexible Packaging Association, c/o Metal Box Ltd, Wantage; Mardon Flexible Packaging Ltd, Bath, UK; Association Européenne des Enducteurs Calandreurs et Fabricants de Revêtements de Sols Plastiques et Synthétiques, Brussels, Belgium; 4 P Verpackungen GmbH, Kempten, FRG; Thomassen & Drijver-Verblifa NV, Deventer, The Netherlands.

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REVIEWS OF RECENT PUBLICATIONS

Human Nutrition: Current Issues and Controversies. Edited by A. Neuberger & T. H. Jukes. MTP Press Ltd, Lancaster, 1982. pp. x + 249. £16.95.

As our knowledge of biology extends and yet our technology outstrips our understanding, there is greater scope for the charlatan and fraud. Pseudoscience easily outpaces science, giving much greater scope for the quick-witted quack to bamboozle the gullible. Nowhere is this better seen than in the field of human nutrition among affluent nations, where fads abound, special diets are the stuff of fantasy and there are endless arguments about minutiae which are beyond the comprehension of the hungry.

This book deals authoritatively and in detail with a few of the problems that confront the serious nutritional toxicologist. It is not possible to escape the contemporary absurdities but these are dealt with in a sensible and forthright manner. The book covers such wide-ranging topics as energy balance, fluoride, nitrite and nitrosamines, vitamins A and D, and laetrile.

It is to be regarded as a ready source of sound factual sense and as such is highly recommended.

Lipids in Human Nutrition: An Appraisal of Some Dietary Concepts. By G. J. Brisson. MTP Press Ltd, Lancaster, 1982. pp. xiv + 175. £14.95.

This is very good book. It deals with a complex subject in a manner that seems intended for the intelligent layman and as such is a model for its kind. Although the professional scientist will find many of the illustrations irksome or facile they are ideally suited to the presumed reader and almost all are immediately comprehensible.

All of this is good. But what makes the book outstanding is its iconoclasm. None of the current hypotheses concerning the role of lipids in cardiovascular disease escapes unscathed. For each, the evidence is sifted, the essentials exposed and the shortcomings displayed. None is discarded, but for each the further evidence needed is identified. All of this comes as a breath of fresh air to a topic which has been dominated by personal fads and charlatanry. The book is highly recommended to all moderate thinkers.

Flavouring Substances and Natural Sources of Flavourings. 3rd Edition. Partial Agreement in the Social and Public Health Field. Council of Europe, Strasbourg. Maisonneuve S.A., Moulins-lès-Metz, 1981, pp. 376. 270 F.Fr.

This new edition of the Council of Europe 'blue book' has been considerably revised since the last version was published (*Cited in F.C.T.* 1975, **13**, 459). It is now considered that effective legal control must be based on positive lists, and the 'negative' list of flavouring substances, for which the available data indicate definite toxicity, has consequently been omitted. The old distinction between natural and artificial flavouring substances has disappeared, and a 'flavouring substance' is now stated to be a chemically defined compound that has flavouring properties, obtained either by isolation from a natural source or by synthesis. Such substances are distinguished from natural sources of flavourings—i.e. products of plant or animal origin from which flavourings may be obtained exclusively through the appropriate physical processes or by biological processes which occur spontaneously (e.g. fermentation)—and from natural flavourings, i.e. complex mixtures derived from natural sources which have flavouring properties.

Natural sources of flavourings are now subdivided as follows: fruits and vegetables or parts thereof consumed as food (N1); plants and parts thereof, including herbs, spices and seasonings commonly added to foodstuffs in small quantities, the use of which is considered acceptable with a possible limitation of an active principle in the final product (N2); plants or parts thereof for which potential long-term toxicity cannot be adequately assessed, but that in view of their long history of use without evidence of acute untoward effects are temporarily acceptable for continued use, in the traditionally accepted manner, in certain beverages and other foodstuffs (N3); plants and parts thereof that are used for flavouring purposes at present but that cannot be classified in any of the above categories (N4). The single list of natural sources of flavourings, comprising these four categories, contains an additional five substances that were not present in the second edition.

Examples of natural sources of flavourings of animal origin are given in an appendix. A further appendix gives examples of natural sources of flavourings recognized as toxicologically unacceptable (the old N(2) series), but it is firmly stated that this must be considered neither as a negative nor as a complete list. The list of active components that require limitation has been shortened from 21 to 14, with omission of caffeine, emetine, methyl nonyl ketone, ricin, solanine, sparteine, creosote and tar and the addition of aloin, and the provisional limits recommended for all other components have been revised (generally downwards). Analytical methods published to date for determining these restricted compounds are also specified for the first time.

The list of flavouring substances that may be added to foodstuffs without hazard to public health now contains 69 additional substances. A separate column draws attention to ADIs established by JECFA, and notes which additives may also be used for purposes other than flavouring. Technological upper limits are prescribed separately for food and beverages, with exceptions for specific uses where necessary. The list of flavouring substances that may be added temporarily to foodstuffs without hazard to public health also includes an additional 69 substances, again with upper limits for use in food and beverages and specific ex-

ceptions. In addition to noting the toxicological data required, as before, the list records for the first time the need for a specification of technological data. Substances in the old 4000+ series of artificial flavouring substances not fully evaluated have where possible been transferred to one of these two lists.

Remaining substances not fully evaluated have been placed on a waiting list, which also includes some 1700 substances submitted by industry for evaluation, but for which insufficient technological and/or toxicological data were available. This list of 1950 substances (numbered from 10.001 onwards) is published separately as *Flavouring Substances not Fully Evaluated* (P-SG (81) 26, Council of Europe, Strasbourg, 1981; pp. ii + 235). Copies are available from the Partial Agreement Division, Council of Europe, F-67006 Strasbourg Cedex.

Datensammlung zur Toxikologie der Herbizide: 3. Lieferung. Die "Arbeitsgruppe Toxikologie" der Kommission für Pflanzenschutz-, Pflanzenbehandlungs und Vorratsschutzmittel der Deutschen Forschungsgemeinschaft. Verlag Chemie GmbH, Weinheim, 1981. pp. 280. DM 196.00.

This is the third issue of monographs on the toxicology of herbicides compiled by the West German authorities. Since the second issue (*Cited in F.C.T.* 1977, **15**, 641) new monographs have been added on allyl alcohol, benzthiazuron, chloramben, chloridazon, chlorphenprop-methyl, isocarbamide, maleic hydrazide, methabenzthiazuron, metobromuron, metribuzin, picloram and terbutryne. In addition supplements have been prepared to the original monographs on barban, chlorbufam, chlorpropham, cycluron, dalapon, dicamba, dichlobenil, dichlorprop and its salts and esters, dinoseb, dinoseb acetate, DNOC, linuron, MCPA and its salts and esters, MCPB, mecoprop, monuron, sodium chlorate, prometryne, propazine, prophem, simazine and 2,4-DB and its salts and esters. The monographs on amitrole, atrazine, diallate, diuron, monolinuron, phenmedipham, triallate and 2,4-D and its salts and esters have been reissued in expanded form, and in most of these cases the number of references cited has more than doubled. Changes within the monographs include the subdivision of the section on special studies into carcinogenicity, reproductive toxicity, mutagenicity and other studies, and the inclusion at the end of each monograph of a list of other relevant references not cited in the text.

Each monograph constitutes a comprehensive survey of the data, and the publication, which continues in the loose-leaf form of the earlier issues, will be of great value to anyone concerned with the effects of exposure to these herbicides, either during initial application or from residues in food or the environment.

Environmental Health Chemistry. The Chemistry of Environmental Agents as Potential Human Hazards. Edited by J. D. McKinney. Ann Arbor Science Publishers Inc., Ann Arbor, MI/The Butterworth Group, Sevenoaks, Kent. 1981. pp. xiv + 656. £27.40.

Hazard Assessment of Chemicals. Current Developments. Vol. 1. Edited by J. Saxena & F. Fisher. Academic Press, London, 1981. pp. xii + 461. £32.40.

These two books discuss, with little overlap, the problems of monitoring, evaluating and predicting the physicochemical and biological effects of environmental chemicals.

J. D. McKinney has assembled 29 short papers (almost all derived from an American Chemical Society symposium) under four headings: environmental health chemistry perspectives, environmental analysis, structure–activity and toxicity prediction, and chemical aspects of toxicological testing. *Hazard Assessment of Chemicals*, which is intended to be an annual serial publication, presents ten reviews which cover topics such as models for predicting contamination of water systems by spillages of toxic chemicals, the *in vitro* metabolism and activation of chemical carcinogens, the estimation of exposure to hazardous chemicals, and the environmental and health effects of polycyclic azaheterocyclic compounds. Aspects of reproductive toxicology are dealt with in both books. The effect of contaminants on the reproduction of aquatic organisms is discussed in *Hazard Assessment of Chemicals*, and the use of human sperm density as an indicator of toxicity is described in *Environmental Health Chemistry*.

Reviews of some of the topics (for instance analytical techniques for concentrating and determining trace metals and chlorinated organic contaminants) can be found in other publications; however, the value of these books lies in the discussion of work in newer fields, for example the prediction of toxicity from the molecular structure and physicochemical features of a molecule. A. J. Hopfinger *et al.* (in *Environmental Health Chemistry*) describe the types of molecular descriptors that can be generated for use in quantitative structure–activity relationship (QSAR) studies of toxicity but are cautious about its use if metabolic activation is disregarded. P. N. Craig and K. Enslein (in *Hazard Assessment of Chemicals*) report on the use of QSAR methods, based mainly on substructural analysis, to predict the LD_{50}s of a range of chemicals in rats and to estimate LD_{50}s in the rat from LD_{50}s determined experimentally in the mouse. Eventually this work may extend to the estimation of safe doses of new drugs for testing in man.

Both of these books cover the literature up to 1979–1980. Their multidisciplinary approach to the problem of assessing health risks associated with chemicals in the environment should be of interest and value to chemists and toxicologists working in this field.

Fundamentals of Industrial Toxicology. By K. Anderson & R. Scott. Ann Arbor Science Publishers Inc., 1981. pp. viii + 120. £8.60.

This book is aimed at those who are completely unfamiliar with the basic concepts and jargon of industrial toxicology. It is intended to fulfil the increasing need of people working in industry to have some understanding of toxicology. Unfortunately it falls short of fulfilling that need.

The first three chapters introduce the subject of toxicology and subsequent chapters outline the "Physiology of toxicant-affected systems", the "Mode of action of toxicants", "Dose-response relationships" and the "Classification and types of exposure". Regrettably the text is badly written, at times irritatingly patronizing and contains a number of errors (perhaps reflecting less than careful proof-reading) and some misleading statements. Almost half of the text is taken up by a chapter with the somewhat misleading heading "Identification of contaminants". The various groups of contaminants are considered separately in this chapter and are classified in three different ways—"physical classification", "chemical classification" and "physiological classification". The logic of this procedure and of the sub-headings within each classification are beyond my understanding—for example, there are two main sub-headings in the physical classification system, "Molecules" and "Particulates or Aerosols". A section on the characteristics of carcinogens includes those well-known polynuclear aromatics, beryllium, chromates and arsenic—one of the more conspicuous errors that remain in the final text. A brief chapter on the agencies involved in the regulation of the research on toxic substances is confined to the USA, and is of limited interest to readers elsewhere. The final chapter is a list of information sources (mainly books) relevant to industrial toxicology.

The index appears to have suffered some major mishap during its production—I can only conclude that much of the book was repaginated after the indexing was done.

The authors have deliberately confined themselves to the bare essentials of industrial toxicology and aimed at the completely uninitiated. The product is a book of little substance.

Cosmetic Ingredients, their Safety Assessment. Edited by R. L. Elder, and supported by Cosmetic, Toiletry and Fragrance Association. Pathotox Publishers, Inc., IL, 1980. pp. 170 + 3. $27.88.

This book contains the first in a series of reports on the safety-in-use of cosmetics ingredients to be conducted by the Cosmetic Ingredient Review (CIR) Expert Panel, a largely independent body of scientists set up by the Cosmetic, Toiletry and Fragrance Association (CTFA) in the United States in 1976. The nine safety evaluations included in this publication cover a total of 19 ingredients commonly used in cosmetics: wheat-germ glycerides and wheat gluten; wheat flour and wheat starch; wheat-germ oil; 2-bromo-2-nitropropane-1,3-diol; acetylated lanolin alcohol, lanolin, lanolin oil, lanolin acid, lanolin alcohol, lanolin wax, acetylated lanolin, hydrogenated lanolin and hydroxylated lanolin; avocado oil; caprylic/capric triglyceride; isopropyl lanolate; imidazolidinyl urea. The complete text of this book (without an index) has also been published as *J. envir. Path. Toxicol.* 1980. **4** (4).

The terms of reference of, and the procedures followed by the CIR are described in detail in an addendum to this publication. It seems that a great effort has been made to ensure impartiality. This is also apparent in the preparation of the final reports. The 19 priority materials were originally selected from a list of 189 ingredients which occurred in over 25 cosmetic formulations but excluding materials such as fragrances, food additives and GRAS food ingredients that are subject to other existing safety reviews. A weighted formula was devised in order to obtain the priority list by taking into account the frequency with which the ingredient is used in different formulations, the concentration of use, area of normal use, frequency of application, use by sensitive population subgroups, suggestion of biological activity and consumer complaints about products containing the ingredient. Each report goes through several stages during which data and comments are invited from industry and the general public, and the discussions leading up to the issue of the first tentative report and to the conclusions by the Expert Panel are held in public.

The format of each review is similar. At the beginning is a brief summary followed by the conclusion that the particular ingredients are considered to be safe as currently used, to be unsafe, or that there is insufficient information available to make an evaluation. In all but one case the data supported the conclusion that the ingredients were safe at current use levels. The exception was the antibacterial agent 2-bromo-2-nitropropane-1,3-diol which was thought to be safe at concentrations up to 0·1% (except under circumstances where its action with amines or amides can result in the formation of nitrosamines or nitrosamides), and it was reported that at least 16 different products sold in the USA contained levels exceeding this figure. In each report there is a section discussing the purpose and extent of use in cosmetics. A section on the biological properties of the materials discusses animal toxicity studies which are not limited to dermal exposure, and also gives reports on clinical experiences. A more detailed summary, including in some cases suggestions for further work, and the conclusions complete each review. All the data are presented in a clear and concise fashion. In at least one instance, however, the short summary given at the beginning is too brief to reflect accurately the relevant data. The reviews are based both on the published literature—the most recent references being for 1979—and on previously unpublished data supplied by industry, details of which are now available from the CIR. The result is a worthy attempt to draw together all the relevant information on these materials and to present an unbiased opinion on their safety in cosmetics. This book will be valuable to those within the cosmetics industry and may be of interest to others considering the use of these materials. Reports on additional materials are in various stages of progress and will no doubt be eagerly awaited.

Foreign Compound Metabolism in Mammals. Vol. 6. Senior Reporter D. E. Hathway. The Royal Society of Chemistry, London, 1981. pp. xvi + 390. £64.00.

Research in xenobiotic metabolism has made great strides forward in recent years and Volume 6 in the series 'Foreign Compound Metabolism in Mammals' attempts to review the literature published in this field during 1978 and 1979. With the ever-increasing volume of literature available, some selection becomes

inevitable. Many of the authors stress this point and most lean on the side of detailed discussion of specific points rather than attempting to cover the whole field. The first five chapters cover most aspects of xenobiotic metabolism including drug kinetics, enzymic mechanisms of oxidation, conjugation and hydrolysis, species and sex differences in metabolism and mechanisms of chemical carcinogenesis. Later chapters are devoted to individual types of xenobiotics such as drugs affecting the central nervous and cardiovascular systems, industrial and agricultural chemicals, and food additives.

In general the chapters are well written and reasonably balanced and contain much useful information considering the limitations on space and the wealth of literature available for review. However, unlike previous volumes, this issue contains only an index of compounds and metabolites, and no author index. This book can be recommended to those interested in updating their knowledge in the general field of xenobiotic metabolism.

Cell Proliferation in the Gastrointestinal Tract. Edited by D. R. Appleton, J. P. Sunter & A. J. Watson. Pitman Medical Ltd, Tunbridge Wells, 1980. pp. xxvii + 428. £25.00.

This book presents the papers given at a conference held in Newcastle-upon-Tyne in September 1979. The papers are divided into six sections: cell proliferation in normal experimental animals, growth control in normal experimental animals, stem cells, responses to cytotoxic agents and other stimuli, experimental carcinogenesis and cancer, and normal and disease states in man. Virtually all of the articles deal with the intestine.

The editors should be congratulated on a number of points. They have attempted, and largely succeeded, in standardizing the nomenclature used by the various authors. This, together with the uniformity in the presentation of the results and the logical ordering of the sections and the papers within sections has resulted in a well thought out and structured book unlike many conference proceedings. None of the discussions that followed the presentations are included, but the authors have modified their articles to take into account points that arose. The first paper in most of the sections presents a useful review of the topic, while the following papers are more specialized. The references cited in each paper are listed at the end of the book. This list of more than 800 references should serve as an invaluable guide to the literature especially for those beginning research in this field. The book should prove valuable to all those with an interest in research into cell proliferation in the gastrointestinal tract.

The Biochemical Basis of Chemical Teratogenesis. Edited by M. R. Juchau. Elsevier/North-Holland Inc., New York, 1981. pp. viii + 272. $60.00.

There is little doubt that many teratogens exert their effect on the foetus indirectly by the modification of either maternal metabolism or the maternal to foetal transfer of critical substances. Much of the content of this book illustrates this fact and the lengthy first chapter is devoted to this subject. The chapter begins with a section on placental anatomy and its changes during pregnancy in different species which is followed by a discussion of how alterations in the availability, transfer and utilization of substances necessary for normal foetal growth and differentiation may ultimately provide the explanation for the action of most teratogens.

One of the most interesting chapters is that written by the editor and A. G. Fantel on the current theories of the biochemical mechanism of teratogenesis of a selection of therapeutic agents. The evidence for and against each theory is discussed and illustrates the many different approaches used in experimental research on teratogenic mechanisms. Again maternally mediated effects feature prominently. In contrast, another chapter deals with the direct action of alkylating agents in developmental toxicity.

There are, as the editor admits, few teratogens that have been subjected to in-depth investigations and often scant data are available on biochemical mechanisms. As a result, much of the text lacks continuity and reads as a list of effects which inevitably makes rather heavy reading particularly for those new to the subject. Nevertheless the book can be recommended to those already familiar with biochemistry and reproductive toxicology and should serve as a stimulus to their research.

Gene Function. *E. coli* and its Heritable Elements. By R. E. Glass. Croom Helm Ltd, London, 1982. pp. 487. £9.95.

For many years *Escherichia coli* has been the prototype organism for the study of DNA metabolism and gene expression, and it is probable that more is known about the molecular biology of this organism than about any other. Any text that aims to collate and explain this vast amount of knowledge should be welcomed. Unfortunately the author has not been entirely successful in the task. The book covers the molecular genetics of *E. coli* extensively but at a rather superficial level. There is also a paucity of references to original research; most of the references are to either review papers or book chapters. The author has divided the book into four parts, beginning with an introduction to bacterial systems and their genetic material, structure, composition and growth, which is followed by sections on gene expression, transfer and regulation. However this has resulted in a somewhat illogical arrangement of the chapters within the book. For instance, RNA synthesis is described in Chapter 2, but it is not until Chapters 8 and 9 that control of RNA synthesis and gene expression are dealt with. Similarly, chapters devoted to mutation and "reactions of DNA" are separated by chapters on plasmids and bacteriophages.

However, the comprehensive nature of the text must be emphasized. The book covers everything from a basic introduction to *E. coli* physiology to plasmids and bacteriophages and includes coverage of topics such as restriction enzymes and RNA polymerase recognition sites. Although this book cannot be recommended as a standard reference source, as an intermediary text-book it would make a worthwhile addition to an undergraduate's book-shelf.

Immunoassays for the 80's. Edited by A Voller, A. Bartlett & D. Bidwell. MTP Press Ltd, Lancaster, 1981. pp. xiii + 508. £24.95.

This book takes its title from a conference held at the Zoological Society of London in 1980, on which the volume is largely based. The aim of the conference was to take an 'overview' of the subject and the contributions are in the form of summary papers by leading workers in the field. By careful choice of contributors, or perhaps by vigorous editorial control, most chapters avoid lengthy descriptions of original data, concentrating instead on a perspective of the current state of the art. The information is presented in an orderly, explanatory style and decorated with a smattering of those bipedal soap bubbles that immunologists must see whenever they gaze at the sky. Technical details are insufficient to enable the reader to 'go away and do it', but most chapters contain 50 or more references which should be more than adequate to provide details of the practicalities for those who want them.

The book is divided into two parts, 'Immunoassay techniques' and 'Applications of immunoassays'. The former consists of 15 chapters which consider the wide range of markers currently used in immunoassays, from the commonly used radioisotopes, to fluorescent and chemiluminescent labels, red cells, polystyrene particles and enzymes linked with the antibody or antigen. This section includes a chapter on the use of monoclonal antibodies, and is concluded by one considering the standardization of immunological reagents and the preparation of reference materials.

The 18 chapters in the second part of the book reflect the wide range of disciplines in which these highly sensitive and specific assay methods are now used. Applications of the techniques in endocrinology, pharmacology, toxicology, haematology and tissue typing are considered, as well as their use in the investigation of autoimmune disease and allergy, and in the diagnosis of viral, bacterial and fungal diseases in man, livestock and plants. A chapter is devoted to the antenatal diagnosis of neural tube defects by the assay of α-foetoprotein in maternal serum.

The book is a useful introduction to immunoassays and will be a particularly helpful source of reference to those who, although unfamiliar with the techniques, are wondering whether immunoassays might be applicable to their own research. If the predictions of many of the contributors are realized, during the 1980s there will be considerable further developments in the techniques and uses of immunoassays; perhaps the editors could look now to the task of assembling material for the book's sequel for the next decade.

Biological/Biomedical Application of Liquid Chromatography III. Edited by G. L. Hawk. Marcel Dekker Inc., New York, 1981. pp. xiv + 420. Sw. fr. 148.00.

This publication comprises a collection of 22 papers, selected from those presented at the Third International Symposium on the Biological/Biomedical Applications of Liquid Chromatography (LC), held in Boston, MA in October 1979. Like its predecessor in the series, taken from the 1978 Symposium (*Cited in F.C.T.* 1981, **19**, 119) this volume contains a comprehensive index to its wide range of subjects, and a helpful glossary of some of the more technical terms used in LC.

Whilst obviously reflecting the diversity of style of the various authors, all of whom are established chromatographers, the papers included have all been reviewed and adapted to ensure greater consistency of grammar, nomenclature and trade-mark usage than is usual in works of this type. Coverage varies from general discussion of the applications of LC in clinical chemistry and amino-acid analysis to specific methods for the analysis of particular drugs and their metabolites in biological fluids and tissues. These, along with contributions on separation, identification and quantification of carbohydrates, lipids, nucleic acid fragments, neurochemicals and prostaglandins and on peptide mapping and LC enzyme assays, demonstrate the vast scope of the subject. However, there is perhaps a little too much emphasis, three out of 22 papers, on the biological application, albeit fairly wide-ranging, of radially-compressed columns, a concept developed and marketed by the Symposium organizers.

A valuable source of interesting and useful information to workers in many areas who may not yet have considered the technique of LC, this book also represents a good work of reference, particularly with its extensive index, for the established chromatographer.

BOOKS RECEIVED FOR REVIEW

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Environmental Carcinogens—Selected Methods of Analysis. Vol. 4. **Some Aromatic Amines and Azo Dyes in the General and Industrial Environment.** Edited by L. Fishbein, M. Castegnaro, I. K. O'Neill & H. Bartsch. IARC Scient. Publ. no. 40. International Agency for Research on Cancer, Lyon, 1981. pp. xiii + 347. Sw. fr. 60.00.

Cocarcinogenesis and Biological Effects of Tumor Promotors (Carcinogenesis: A Comprehensive Survey, Volume 7). Edited by E. Hecker, N. E. Fusenig, W. Kunz, F. Marks & H. W. Thielmann. Raven Press, New York, 1982. pp. xxxii + 664. $93.84.

Cardiovascular Toxicology. (Target Organ Toxicology Series). Edited by E. W. Van Stee. Raven Press, New York, 1982. pp. xii + 388. $61.20.

Molecular Interrelations of Nutrition and Cancer. Edited by M. S. Arnott, J. van Eys & Y.-M. Wang. Raven Press, New York, 1982. pp. xvi + 474. $78.88.

Laboratory Safety: Theory and Practice. Edited by A. A. Fuscaldo, B. J. Erlick & B. Hindman. Academic Press, London, 1980. pp. xiv + 357. £26.20.

Human Cancer Markers. Edited by S. Sell & B. Wahren. The Humana Press Inc., Clifton, NJ, 1982. pp. xx + 428. $69.50. ($59.50 in USA).

Biological/Biomedical Applications of Liquid Chromatography IV (Chromatographic Science Series, Volume 20). Edited by G. L. Hawk. Marcel Dekker, Inc., New York, 1982. pp. xv + 367. Sw. fr. 156.00.

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Environmental Health Criteria. 17. **Manganese.** Published under the joint sponsorship of UNEP, ILO and WHO. World Health Organization, Geneva, 1981. pp. 110. Sw. fr. 8.00 (available in the UK through HMSO).

Environmental Health Criteria. 19. **Hydrogen Sulfide.** Published under the joint sponsorship of UNEP, ILO and WHO. World Health Organization, Geneva, 1981. pp. 48. Sw. fr. 6.00 (available in the UK through HMSO).

Carcinogens in Industry and the Environment. Edited by J. M. Sontag. Marcel Dekker, Inc., New York, 1981. pp. xi + 761. Sw. fr. 225.00.

Microenvironments in Haemopoietic and Lymphoid Differentiation (Ciba Foundation Symposium 84). Edited by R. Porter & J. Whelan. Pitman Books Ltd, London, 1981. pp. xi + 348. £22.50.

Side Effects of Drugs Annual 6, 1982. Edited by M. N. G. Dukes. Excerpta Medica, Amsterdam, 1982. pp. xviii + 478. Dfl. 150.00.

The Future of Antibiotherapy and Antibiotic Research. Edited by L. Ninet, P. E. Bost, D. H. Bouanchaud & J. Florent. Academic Press, London, 1981. pp. xx + 508. £25.00.

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Information Section

ARTICLE OF GENERAL INTEREST

ARE WE AT RISK FROM LEAD?—PART 2

In the first part of this review (*Cited in F.C.T.* 1982, **20,** 617) we considered the results of studies of the effects of exposure to low levels of lead in man. We turn now to studies in animals. Unlike studies in man, animal studies are not confounded by socio-economic factors, and exposure levels can be carefully controlled. However, a recent review paper makes it clear (Bornschein *et al. CRC Crit. Rev. Toxicol.* 1980, **8,** 101) that they may suffer from several other disadvantages. These include a failure to measure effects on systems other than the CNS, and to consider whether factors such as maternal undernutrition or neonatal growth retardation may have confounded the results. Few animal studies adequately simulate lead exposure conditions in young children, and many do not provide adequate documentation of blood- and tissue-lead levels during treatment. Positive control compounds are rarely used, so that it is impossible to tell whether negative results imply merely that the test set is insufficiently sensitive to detect a defect in performance. Although studies may aim to test effects on cognitive function (learning/memory), in practice it is difficult to distinguish such effects clearly from effects on sensory-motor function, arousal or motivation, which in turn can produce performance differences. The Lawther report (*Lead and Health*, HMSO, London, 1980; pp. 129, £4.50) dismissed all such studies in one sentence, stating that "The period of maximum brain growth in animals varies with the species and it is very difficult to extrapolate from animal models to human infants".

These criticisms apart, there is mounting evidence from tests in various species (rats, mice, dogs, sheep, pigeons and monkeys) that lead at fairly low levels may affect learning and other aspects of CNS function (Bornschein *et al. loc. cit.*). Many of these, including primate studies (which might be expected to reflect most closely the human response) have involved blood-lead (PbB) levels that may be reached in non-occupationally exposed humans.

Monkeys

We have already described a study in which infant rhesus monkeys given lead acetate in milk for 1 yr, sufficient to produce an average PbB level of 30 μg/dl*, showed a suppression of play activity, increased social clinging, and an exacerbated disruption of social behaviour after a change in their play environment (*Cited in F.C.T.* 1981, **19,** 514). Such monkeys (whose PbB level increased progressively from about 10 to 70 μg/dl during the year) also required significantly more trials than controls to finish problems involving reversal learning of visual discrimination tasks, as did another group with PbB levels maintained in the range 40–60 μg/dl. Monkeys with higher PbB levels (70–90 μg/dl) had in addition a pronounced difficulty in attaining a 90% correct response in the first of a series of reversals, due to increases in errors and in balking (Bushnell & Bowman, *Pharmac. Biochem. Behav.* 1979, **10,** 733). Monkeys whose average PbB during the first year of life had been 31·71 or 65·17 μg/dl were retested for spatial cue reversal learning at 4 yr of age (at which stage their PbB levels had declined to mean values of 5–6 μg/dl compared with 4 μg/dl for the control group). The high-lead group was still significantly retarded in acquisition of the original discrimination and of most reversals, and the low-lead group was retarded on the first reversal (*idem J. Toxicol. envir. Hlth* 1979, **5,** 1015).

A similar deficit in reversal learning was reported in cynomolgus monkeys, dosed orally with 500 μg lead (as the acetate)/kg from day 1 of life for 800 days. This treatment produced PbB levels that rose from 20 to a peak of 35–70 μg/dl at about 200 days, then dropped to 20–50 μg/dl by 400 days and remained in this range (generally below 40 μg/dl) throughout the test period. At 2–3 yr of age they were given a two-choice non-spatial form-discrimination test, which involved distinguishing between a cube and a triangle cut from wooden blocks. The treated monkeys learnt the original acquisition and the first reversal more quickly than the controls, but performed worse than controls in subsequent reversals (Rice & Willes, *J. envir. Path. Toxicol.* 1979, **2,** 1195). The same monkeys showed no effects on motor activity, but their performance in a test of schedule-controlled behaviour was altered by lead treatment (Rice *et al. Toxic. appl. Pharmac.* 1979, **51,** 503).

Sheep

Slowed learning has also been reported in lambs exposed to lead only during the prenatal period (Carson *et al. Archs envir. Hlth* 1974, **29,** 154). Ewes were fed finely powdered elemental lead at an average level of 2·3 or 4·5 mg/kg/day for 5 wk before breeding and throughout gestation, doses which produced mean PbB levels of 18·6 ± 2·9 and 34·8 ± 9·9 μg/dl respectively (cf. 4·7 ± 0·9 μg/dl in controls). No signs of lead toxicosis were evident in the ewes. At 2–4 wk of age the control, low- and high-dose lambs had mean PbB levels of 6, 17 and 25 μg/dl respectively, and these

*dl = 100 ml.

levels had fallen to 4, 9 and 14 μg/dl at 10–12 wk. The lambs were weaned at 3 months and were thus no longer exposed to lead from the dams' milk. When tested at 10–15 months of age, the high-lead lambs required significantly more days to learn visual discrimination problems than did the controls. The low-lead animals took slightly longer than the controls to learn some problems, but the differences were not statistically significant.

Rats

In rats, effects on learning ability have been demonstrated at even lower levels. In one such study rats were given drinking-water containing 0, 50, 300 or 1000 ppm lead acetate from weaning at 20–22 days of age, and were fed simultaneously on rat chow of unmeasured lead content resulting in PbB levels at 150 days of age of approximately 5, 6, 26 and 42 μg/dl respectively. After 35 days of exposure behavioural training was begun in which they were assessed on their responses to a fixed-interval food-reinforcement schedule (involving a food reward for the first lever-press response occurring at least 30 sec after the preceding food delivery). Exposure to 50 and 300 ppm increased response rates (frequency of lever pressing) and intersubject variability, while latency, or time to first response in the interval, decreased. At 1000 ppm there was initially a decrease in response rates and an increase in latency values. Following termination of exposure at 50 ppm, response rates and latency values gradually returned to control levels. Behavioural effects produced by 50 or 300 ppm were similar in magnitude but varied in time to onset and decline (Cory-Slechta & Thompson. *Toxic. appl. Pharmac.* 1979, **47**, 151).

Why the lowest dose level used in the last study affected behaviour, despite producing PbB levels not significantly greater than those in control rats, is uncertain. As such levels were measured only at 150 days, it is possible that higher body levels resulted earlier during the treatment procedure. This is fairly plausible since lead absorption from the gut is far greater in young than in adult animals. Alternatively, even 50 ppm lead may have some degree of toxic effect on the animals, which might have a secondary effect on behaviour. However, another study has also suggested that lead at levels of 50 ppm and even less in the drinking-water may affect the learning ability of rats, without producing obvious signs of toxicity. Rats given 0, 25 or 50 ppm lead as the acetate for 35 days, beginning at 23 days of age, showed no stunting of growth or weight loss, but both treated groups displayed impaired learning ability when tested in a Hebb–Williams closed-field maze (Giest & Mattes, *Physiol. Psychol.* 1979, **7**, 399). Unfortunately PbB levels were not measured in this study but as the animals were fed on rat chow they were probably comparable with those reported by Cory-Slechta & Thompson (*loc. cit.*).

Cory-Slechta *et al.* (*Toxic. appl. Pharmac.* 1981, **60**, 78) subsequently investigated the performance of rats (five/group) given 100 or 300 ppm lead acetate (54·6 or 163·9 ppm lead) in drinking-water from weaning. At 55 days of age they were trained to press a lever for food reward, after which a schedule was imposed, in which only lever pressing for longer than a certain time was rewarded. Lead-treated groups showed more variable responses than controls, but on average their response duration was shorter, and they paused for longer before responding. No dose-response relationship was apparent at the two levels used. PbB levels were not measured, but brain levels at 95 days of age were in the range 40–142 ng/g at 100 ppm and 320–1080 ng/g at 300 ppm (cf. 14–26 ng/g in controls).

That the learning ability of adult rats may be impaired by neonatal treatment with lead, despite a subsequent absence of abnormal exposure, was demonstrated by Hastings *et al.* (*Neurobehav. Toxicol.* 1979, **1**, 227). Lactating rats were given 200 or 2000 ppm lead acetate (109 or 1090 ppm Pb) in their drinking-water for 21 days, treatments which produced average milk levels of 21 ± 2 and 139 ± 11 μg Pb/dl and PbB levels in the offspring of 29 ± 5 and 65 ± 25 μg/dl respectively, compared with 11 ± 4 μg/dl in the blood of controls. Brain-lead levels were very similar to those in blood. The offspring were then weaned onto tap water (<0·05 ppm Pb) and laboratory chow (*c.* 0·5 ppm Pb). At 120 days of age the low-lead animals took about 50% longer than the controls, whose mothers had been given either tap water or sodium acetate, to learn a simultaneous visual discrimination task and the high-lead group took even longer. However, at 270 and 330 days when the same rats were also tested in a successive visual discrimination task and in a go no-go discrimination task no significant differences from controls in either of these tests were found.

The effects of lead exposure from the prenatal period onwards were assessed by Winneke *et al.* (*Archs Toxicol.* 1977, **37**, 247) who fed female rats a diet containing 1380 ppm lead acetate (754 ppm Pb) from 60 days before mating until weaning and fed their offspring on the same diet after weaning. Average PbB levels in the mothers were 24·2 μg/dl before mating and 31·2 μg/dl after weaning, whereas in their offspring levels were 26·6 and 28·5 μg/dl at 16 and 190 days of age respectively (cf. 1·7 μg/dl in control offspring at 16 days). The level of lead given was sufficient to reduce the proportion of animals that became pregnant and the size of litters, but the offspring were heavier than the controls (probably because of the reduced litter size). Between 100 and 200 days of age, a total of 40 male offspring were tested in two visual discrimination learning tasks. In the 'easy' task there was no difference from controls, but in the 'difficult' task only one of the ten treated animals learnt the problem within 27 days, compared with eight of ten controls. The rats were also subjected to an open-field test, in which those given lead showed an increase in ambulation, rearing and grooming.

More general effects of lead on neurobehavioural development were investigated in a two-generation rat study (Cahill *et al.* in *Biological Effects of Low Level Radiation*; International Atomic Energy Agency, Vienna. 1976. **2**, 65; Reiter *et al. Envir. Hlth Perspect.* 1975, **12**, 119). Rats were given 5 or 50 ppm lead as the acetate in drinking-water for 40 days prior to mating, after which treatment of pregnant females was continued throughout gestation and lactation, and the offspring (F_1) and their progeny (F_2) were similarly exposed from weaning throughout adult-

hood (F_1 dams and F_2 female offspring were removed from the study at weaning). PbB levels in the F_1 dams were 11, 13 and 26 μg/dl respectively in the control, 5 and 50 ppm groups, while the F_2 neonates showed levels of 8, 11 and 20 μg/dl at birth and 5, 6 and 10 μg/dl at 180 days. Mean body weights of the F_2 offspring during the developmental period were not significantly lower than controls, and indeed males at 50 ppm were significantly heavier on days 15 and 18. However, relative brain weight of the offspring at birth was significantly reduced at both levels. Significant delays were noted in the development of the righting reflex at 5 and 50 ppm and in eye opening at 50 ppm, but there was no difference in the development of the startle response. Adult males at both levels showed a significant reduction in locomotor activity in a residential maze, and did not respond to amphetamine injection with the normal degree of hyperactivity. Brain catecholamine levels in the high-dose-group offspring were significantly affected at 28 and 180 days, but not in the manner expected for hypoactive animals. However EEG measurements on anaesthetized F_1 adult males showed changes at the 5 ppm level that were consistent with reduced activity. However, no such effect was seen in the 50 ppm F_1 adults, nor in the F_2 adults at either level. Other studies in rats have also suggested that low-level lead treatment reduces rather than increases activity (Sobotka & Cook, *Am. J. ment. Defic.* 1974, **79**, 5; Verlangieri, *Pharmac. Biochem. Behav.* 1979, **11**, 95) in contrast with the suggestion that hyperactivity may be associated with lead exposure in children (*cited in F.C.T.* 1982, **20**, 617).

Behavioural changes were also evident in rats that had been exposed to lead only via their mother's milk. The dams were given drinking-water containing 200 ppm lead acetate (109 ppm Pb) from parturition to weaning at 21 days, after which the pups were given normal rat chow and tap water. At 20 days the milk contained a mean level of 21 μg Pb/dl, whereas mean PbB concentrations in the offspring were 29, 5 and 9 μg/dl at 20, 60 and 270 days respectively (cf. 11, 4 and 9 μg/dl in control rats). Brain-lead levels were similar to PbB levels at 20 days, but in both groups were higher than PbB levels at 60 and 270 days. When the offspring were tested for shock-elicited aggression at 60 days of age, the lead-exposed group showed significantly less aggressive behaviour. However, they did not differ in their acquisition and subsequent reversal of a successive brightness discrimination task, on which they were started at approximately 90 days of age. Wheel running activity was not assessed in these rats, but another group of offspring from dams given the higher level of 1000 ppm lead acetate showed less activity than controls only in the first 2 hr, and their activity over the remainder of the 21-day test period (beginning at 30 days of age) was unaffected (Hastings *et al. Pharmac. Biochem. Behav.* 1977, **7**, 37).

A similar treatment regime was subsequently used to investigate other facets of neurobehavioural development. Pups from dams that had been given 200 ppm lead acetate in the drinking-water from parturition to weaning had mean PbB levels of 21·7, 25·2 and 2·5 μg/dl at 10, 21 and 60 days of age respectively while brain levels at these stages were 6·3, 12·5 and 6·9 μg/dl. In controls, lead levels at 60 days were 3·0 μg/dl in blood and 3·2 μg/dl in brain. The lead-treated pups showed no effects on body weight, but their time to eye opening was significantly ($P < 0.05$) delayed, confirming the findings of Reiter *et al.* (*loc. cit.*). The time taken by the pups to right themselves when placed head downwards on a slope was increased at the 10% but not the 5% level of significance. Subjecting the pups to a maximal electroshock seizure test decreased the ability of lead-treated 10–14-day-old pups to maintain suspension from a taught wire by forepaw grip, but did not affect the appearance of the auditory startle response or the air-righting reflex of lead-treated pups (Overmann *et al. Neurotoxicology* 1979, **1**, 25). Lead also caused more severe seizures in response to electroshock treatment by day 16, and this increased severity of response was still evident at day 60, 39 days after cessation of exposure (Fox *et al. ibid* 1979, **1**, 149).

In a comprehensive study involving the administration of 0·5, 5, 25, 50 or 250 ppm lead as the acetate in drinking-water to mothers for 6–7 wk before mating and to their offspring for up to 9 months after birth, the 25 ppm level was sufficient to affect performance of an operant task in adults, and the development of surface- and air-righting in the offspring was delayed at 50 and 250 ppm. Locomotor development was unaffected except for an increase in pivoting at 14 days of age in animals given 250 ppm, and post-weaning activity levels were unaltered. Motor co-ordination in a rotorod test was also unchanged. However, these rats were fed a semi-purified diet and not rat chow as in most such studies. Gastro-intestinal absorption of the lead in the drinking-water would be expected to be facilitated by such a regime, and this is evidenced by the high PbB levels attained. Median PbB levels in the offspring given 0, 25 and 50 ppm lead were 4, 37 and 57 μg/dl at day 1 and had fallen to 3, 22 and 35 μg/dl respectively by day 11 (Grant *et al. Toxic. appl. Pharmac.* 1980, **56**, 42). The blood levels attained were sufficient to affect other systems besides the CNS; the age at vaginal opening was significantly delayed at 25 ppm or more, increased ALA excretion and kidney damage were evident even at 5 ppm, and kidney weights were increased at 0·5 ppm or more (Fowler *et al. ibid* 1980, **56**, 59).

To determine at what stage lead extends its most severe effect in rats, Crofton *et al.* (*Life Sci.* 1980, **26**, 823) conducted cross-fostering experiments with the offspring of dams that had been given 200 ppm lead as the chloride in their drinking-water from 2 wk prior to breeding until weaning at 21 days after parturition. This treatment produced mean PbB concentrations in the dams of 37·8 μg/dl on day 18 of gestation and 35·5 μg/dl at weaning. Some of the pups were left with their mothers for 21 days from birth to weaning, while others were suckled by non-exposed dams, and a third group born of non-exposed dams was fostered onto exposed dams. Mean PbB levels in these groups of pups at weaning were 29·9, 14·5 and 36·0 μg/dl respectively compared with 4·8 μg/dl for the control group. Body weight and food and water consumption were unaffected in both dams and pups. However, in pups exposed *in utero* and during lactation, the development of exploratory and locomotor activity (assessed at 5–21 days of age) was significantly

delayed, by roughly 1 day from day 16 onwards. Litters treated only prenatally with lead showed the same delay, whereas those exposed only postnatally did not differ from unexposed controls. Differences in levels of activity followed the same pattern.

The behavioural effects observed in the last study could be correlated with delays in synaptogenesis and biochemical development of the cerebral cortex. Fifteen-day-old pups from dams exposed to the same concentration (i.e. 200 ppm) of lead chloride both pre- and postnatally (until weaning at 21 days of age) were found to have reduced synaptic densities in the cerebral cortex, and their synaptic figures displayed a less mature profile. Cerebral cortical slices from treated pups exhibited enhanced respiratory responses to a 3–30 mM increase in K^+ concentration; this was associated with a higher rate of glucose uptake but not with a change in lactic acid output, suggesting uncoupling of oxidative phosphorylation. Central cortical concentrations of amino acids, including ALA, were unaltered. Serum thyroid hormone levels were also unaffected, suggesting that the effect of lead on brain development is direct rather than mediated through the thyroid (McCauley *et al. Neuropharmacology* 1979, **18,** 93). There was also a delay in the normal increase in cytochrome content of the cerebral cortex in the male offspring of dams given 5, 30 or 200 ppm lead as the chloride in their drinking-water until weaning (Bull *et al. ibid* 1979, **18,** 83). At weaning these pups had PbB levels of 0·12, 0·21 and 0·36 μg/g, compared with 0·08 μg/g in controls. Effects on cytochrome accumulation rate were most marked at 10–15 days, and had recovered by 30 days. A decrease in this rate was evident even at the lowest level of treatment in 15-day-old rats, although only at 200 ppm did the decrease become statistically significant. The lack of effect on brain ALA, or on cytochrome accumulation after 15 days, suggested that the earlier delay was not the result of a specific inhibition of haem synthesis, but rather represented a more general effect on cerebral cortical development.

Conclusion

Animal studies have revealed that PbB levels in the range 20–35 μg/dl are sufficient to affect learning ability, activity and neurological development, sometimes well after exposure has been terminated. In rat studies, effects have been claimed even at levels below 10 μg/dl. Although the relevance of such animal models to man is undoubtedly questionable, it is obvious that if such data had been presented to support the use of a food additive or pharmaceutical, lead would never have passed the starting post.

[C. Rostron—BIBRA]

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ABSTRACTS AND COMMENTS

Pharmacokinetics of saccharin in man

Sweatman, T. W., Renwick, A. G. & Burgess, C. D. (1981). The pharmacokinetics of saccharin in man. *Xenobiotica* **11**, 531.

Saccharin and its sodium and calcium salts are at present the only intense artificial sweeteners permitted for use in food in the UK although this may soon change following the FACC report on sweeteners (Food Additives and Contaminants Committee Report on the Review of Sweeteners in Food. FAC/REP/34. HMSO. London. 1982; pp. iii + 61. £3.90). However, the safety of saccharin has long been a controversial topic. Given at high doses it has induced tumours of the urinary tract in male rats and promoted the action of known bladder carcinogens in rats (*Cited in F.C.T.* 1982, **20**, 129). However, epidemiological studies have failed to demonstrate a significant and consistent association between saccharin ingestion in humans and an increased risk of bladder cancer (*Food Chemical News* 1979, **21** (41), 68; *Cited in F.C.T.* 1982, **20**, 129; FACC Report on the Review of Sweeteners in Food. FAC/REP/34. HMSO. London, 1982). It has recently been reported that the high dietary levels necessary to induce an increased incidence of bladder tumours in rats are associated with altered pharmacokinetics due to saturation of renal tubular secretion (Sweatman & Renwick, *Toxic. appl. Pharmac.* 1980, **55**, 18). Previous studies on the fate in humans of orally administered saccharin have shown almost complete absorption followed by the rapid excretion of the unchanged compound in the urine (Ball *et al. Xenobiotica* 1977, **7**, 189; Byard *et al. Fd Cosmet. Toxicol.* 1974, **12**, 175). Sweatman *et al.* (cited above) now report a detailed investigation of the pharmacokinetics of saccharin in human volunteers dosed orally and intravenously.

Three adult males were given 10 mg sodium saccharin dihydrate kg body weight intravenously and, 2 wk later, the same dose during a course of probenecid taken in 500-mg doses 12 and 2 hr before and 2 hr after the intravenous dose. Blood and urine samples were collected at intervals for up to 48 hr following the saccharin doses. The same subjects were also given an oral dose (2 g) of saccharin, both after an overnight fast and, 2 wk later, following a standardized breakfast. Blood and urine samples were taken at intervals for up to 96 hr and faeces were collected for 96 hr following the saccharin doses.

The plasma saccharin levels decreased rapidly after intravenous dosing, with a half-life of about 70 min, and recovery of the dose in the urine was complete within 48 hr. Concurrent probenecid treatment significantly decreased the rate of elimination and the plasma and renal clearances but again there was quantitative recovery of the dose within 48 hr. Plasma levels of saccharin following oral administration took longer to reach their peak when the subjects breakfasted first and the peak levels were lower than those attained in fasted volunteers. Approximately 90% of the oral dose was recovered in the urine and up to 8% was present in the faeces within 96 hr irrespective of whether or not breakfast had been taken before dosing and it was calculated that about 85% of the dose was absorbed. The results obtained with concurrent administration of probenecid (a competitor for, and inhibitor of, renal tubular secretion of organic anions) indicated that renal tubular secretion is the major mechanism for excretion of circulating saccharin. Following the oral dose there was variation between volunteers in their ability to eliminate saccharin from the plasma. It is suggested that some individuals may retain unabsorbed saccharin in the gut lumen for a substantial period and that saccharin in the lower bowel may act as a depot of absorbable material. The authors interpret the plasma concentration-time curve following oral administration as an increase to peak determined by absorption from the stomach and upper intestine, an initially rapid decline in plasma levels resulting from either declining absorption rates or from elimination and a variable late, slow decline determined by absorption from the lower gut.

The authors compare the results of this study with those of a previous investigation in rats (Sweatman & Renwick *loc. cit.*) and conclude that absorption of orally administered saccharin is similar in both species and that the sweetener is rapidly eliminated from the blood largely by the kidneys via renal tubular secretion. In rats this process has been found to become saturated at plasma concentrations of 200–300 μg saccharin/ml, but in man no saturation was observed at plasma levels of 50–60 μg/ml. They conclude therefore that there is little possibility of saturation of the renal clearance route of excretion at 'normal' exposure levels since the doses given in the present study were at least 20 times those estimated as the average daily intake in the USA (*Food Chemical News* 1979, **21** (39), 20).

BHA carcinogenic in rats

Ito, N., Hagiwara, A., Shibata, M., Ogiso, T. & Fukushima, S. (1982). Induction of squamous cell carcinoma in the forestomach of F344 rats treated with butylated hydroxyanisole (rapid communication). *Gann* **73**, 332.

Previous long-term feeding studies in rats and carcinogenicity studies in mice have not demonstrated any carcinogenic activity of the widely used antioxidant butylated hydroxyanisole (BHA; JECFA, WHO Fd Add. Ser. 1980, no. 15, p. 87). These long-term feeding studies in rats were all reported before 1962.

In the study cited above groups of 50–52 6-wk-old F344 rats of each sex were fed diets containing 0, 0·5 or 2% BHA for 104 wk. Surviving animals were fed the basal diet for a further 8 wk and were then killed for gross and histopathological examination. Rats

that died or were killed when moribund during the study were also examined.

The mean body weights of rats in the higher dose groups were reduced compared with the controls. Mortality was significantly increased in treated [high dose?] males and females at wk 80 and 72, respectively, but at the end of the experiment survival ranged from 64 to 69% in all groups except the high-dose females (78%).

Treatment-related pathological changes were seen only in the stomach. Hyperplasia of the forestomach was observed in all but one of the rats in the higher dose groups and in 20–25% of those given the lower dose. Forestomach papillomas, characterized by marked proliferation of basal cells and keratin-forming elements, occurred in virtually all of the rats given the 2% BHA diet. At the lower dose forestomach papillomas were observed in only one rat of each sex. Squamous-cell carcinomas of the forestomach occurred only in the high-dose groups; 29% of the females and 35% of the males were affected. Infiltrative cancerous cells were seen in the vascular lumina or muscular wall of the forestomach and the fatty tissue of the omentum, and two metastases were recorded. Forestomach lesions were not reported in any of the control animals.

[Tumours of the forestomach are uncommon in the rat but may be caused by the nematode parasite *Gonglyonema neoplasticum*, particularly in animals deficient in vitamin A (Altman & Goodman, in *The Laboratory Rat*, Vol. I, p. 334. Edited by H. J. Baker, J. R. Lindsey & S. H. Weisbroth. Academic Press, New York, 1979), and by a number of orally administered chemical carcinogens such as polycyclic aromatic hydrocarbons (Zedeck, *J. envir. Path. Toxicol.* 1980, **3** (5 & 6), 537). BHA has not shown any genotoxicity in a range of *in vivo* and *in vitro* studies (JECFA *loc. cit.*). A nongenetic mechanism might be involved. The squamous epithelium of the forestomach would be one of the first tissues in direct contact with orally administered BHA and the high incidence of hyperplasia in the treated rats is consistent with an acute tissue response which in the high-dose groups has progressed to neoplasia. However no such hyperplastic response was reported in the earlier feeding studies of BHA. Histological changes were observed in the intestinal mucosa of rats after *in situ* perfusion with 2 mg BHA/ml medium (Fritsch *et al. Toxicology* 1975, **4**, 341).

It is also of interest that when given in conjunction with carcinogens BHA has inhibited the neoplastic effects of some chemicals (*Cited in F.C.T.* 1974, **12**, 779). This protective effect might be associated with a modification of carcinogen metabolism resulting from enzyme induction by BHA (Benson *et al. Cancer Res.* 1978, **38**, 4486; JECFA *loc. cit.*; Pascal, *Cah. Nutr. Diét.* 1979, **14**, 271); the inhibition by BHA of benzo[*a*]pyrene-induced forestomach neoplasia (Wattenberg *et al. Cancer Res.* 1980, **40**, 2820) has been reported to be due to enhancement of glutathione-*S*-transferase activity (Sparnins & Wattenberg, *J. natn. Cancer Inst.* 1981, **66**, 769). In these studies lower doses of BHA (0·5 or 1% in the diet) were administered for only a few weeks.

Assuming that a fuller report of this work stands up to peer review, what is the significance of these findings to man? BHA does appear to be carcinogenic under the (at present sparsely reported) experimental conditions of this study. However, BHA cannot exert precisely the same effect in man as it does in rats, since the stomach of man does not contain a squamous epithelium. Detailed mechanistic studies may be required to prevent the direct extrapolation that will inevitably be made from animal carcinogen to suspected human carcinogen. Already the Japanese have announced their intention to ban the use of BHA in most foodstuffs (Committee on Technical Barriers to Trade, notification 82 84, 19 May 1982).]

Cholesterol and colon cancer

Miller, S. R., Tartter, P. I., Papatestas, A. E., Slater, G. & Aufses, A. H., Jr (1981). Serum cholesterol and human colon cancer. *J. natn. Cancer Inst.* **67**, 297.

Cancer of the large bowel is the second most common malignant tumour in Western countries, with approximately 20,000 new cases being registered each year in England and Wales (cited by Farrands *et al. Lancet* 1981, **I**, 1231). Although the cause for the high rate of colon cancer is not entirely clear, dietary fat, and in particular cholesterol, has been implicated as an aetiological factor (*Cited in F.C.T.* 1980, **18**, 208; Jain *et al. Int. J. Cancer* 1980, **26**, 757; Thornton, *Lancet* 1981, **I**, 1081). However, Dyer *et al.* (*J. chron. Dis.* 1981, **34**, 249) found no significant relationship between serum cholesterol levels and colorectal cancer in groups studied in Chicago. In the study cited above, serum cholesterol levels were investigated in patients with no known cancers and in patients having various stages of colon cancer.

In 133 case-control pairs matched for age (within 5 yr) and sex, serum cholesterol levels were found to be significantly lower in cancer patients than in the controls, values of 188 + 42 mg/100 ml (mean ± 1 SD) and 213 ± 42 mg/100 ml, respectively, being recorded. The mean difference (25 mg/100 ml) was highly significant in both men and women. However, when patients with early tumours were compared with controls, the difference was not found to be significant, whereas cases with advanced tumours had significantly lower serum cholesterol levels than controls.

In the second part of this investigation, 130 case-case pairs, matched for age and sex, were studied to compare the serum cholesterol levels in patients that had advanced tumours with those that had early tumours. A significantly lower serum cholesterol level was seen only in female patients with more advanced disease, the mean difference being 16 mg/100 ml.

The authors conclude that the observed differences between patients and controls may partially reflect the metabolic influence of advancing disease, since there were no significant differences in serum cholesterol levels between controls and cases with early tumours. They also point out that it is difficult to evaluate how much the decrease in cholesterol is directly attributable to greater weight loss with more advanced disease since data on weight loss were not available. The authors cite reports that have suggested that individuals developing colon cancer have the ability to remove cholesterol from the plasma

pool and excrete it (Wynder & Reddy, *Dig. Dis.* 1974, **19**, 937) and that changes limited to dietary cholesterol have a small effect on serum cholesterol (Key *et al. Metabolism* 1965, **14**, 759). They therefore suggest that the level of serum cholesterol may not be the most appropriate parameter for future investigations of colon carcinogenesis; additional dietary studies of cholesterol intake along with further analysis of steroid metabolites in the large bowel may be of more value.

Another look at the carcinogenicity of 2,4,5-T

Eriksson, M., Hardell, L., Berg, N. O., Möller, T. & Axelson, O. (1981). Soft-tissue sarcomas and exposure to chemical substances: a case-referent study. *Br. J. ind. Med.* **38**, 27.

Phenoxy acids are present in many of the herbicides used in agriculture, horticulture and forestry. A case-control study in northern Sweden suggested that exposure to phenoxy acids and chlorophenols may be associated with a six-fold increase in the risk of developing soft-tissue sarcoma although the role played by impurities such as dioxin or dibenzofuran could not be ascertained (*Cited in F.C.T.* 1980, **18**, 541). The same group of workers has now conducted a further case-control study of soft-tissue sarcomas with the aim of confirming the earlier findings and obtaining information on the effects of different phenoxy acids. This study was carried out in southern Sweden where exposure to phenoxy acid herbicides is likely to be through their agricultural use rather than through forestry, as in the north.

Each of 110 cases (38 deceased) with soft-tissue sarcomas was compared with two carefully selected controls for exposure to phenoxy acids other than 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), to phenoxy acids including 2,4,5-T and to chlorophenols. Exposure was assessed initially by a self-completed questionnaire which was followed up by a telephone interview by a 'blind' interviewer and contact with employers etc. if this was judged necessary. Individuals exposed to phenoxy acids for a total of less than 1 day were considered to be unexposed, and separate analyses were performed for individuals exposed to phenoxy acids for more or less than 30 days. Risk ratios for soft-tissue sarcomas calculated for the various exposure groups were as follows: phenoxy acids or chlorophenols 4·7; phenoxy acids except 2,4,5-T 4·2; phenoxy acids including 2,4,5-T 17·0 (>30 days 8·5, ≤30 days 5·7); all exposures to phenoxy acids 6·8; chlorophenols 3·3. The authors conclude that their results indicate that exposure to phenoxy acids and chlorophenols might constitute a risk factor in the development of soft-tissue sarcomas and that this risk relates not only to 2,4,5-T which, like certain chlorophenols, may contain polychlorinated dibenzodioxins and dibenzofurans but also to other phenoxy acids such as 2,4-dichlorophenoxyacetic acid, 4-chloro-2-methylphenoxyacetic acid and the analogous phenoxypropionic acids which are less prone to contain such contaminants. They also conclude that individuals with low exposure to chlorophenols (defined as a maximum of one continuous week or repeated short-term exposures amounting at the most to 1 month in all) are not subject to an increased risk of developing soft-tissue sarcomas. Smoking habits and exposure to organic solvents, asbestos, glass fibre, power saws and various pesticides did not appear to be associated with an increased risk of developing soft-tissue sarcomas.

[The results of this study are similar to those of the previous investigation (*Cited in F.C.T.* 1980, **18**, 541) which was criticized on a number of counts by the UK Advisory Committee on Pesticides (*Further Review of the Safety for Use in the U.K. of the Herbicide 2,4,5-T*; December 1980; pp. 72). The methods used in the present study were very thorough in many respects and the authors have gone to considerable lengths to assess exposure to phenoxy acids and other compounds. They have also taken great care in selecting the controls. However some of the Committee's criticisms are probably also applicable to the present study. The accuracy with which workers (or their relatives or colleagues) can recall the type or duration of exposure to chemicals is likely to be limited, and, once again, the types of tumours observed covered a wide range.

In a recent review Coggon & Acheson from the MRC Environmental Epidemiology Unit (*Lancet* 1982, **I**, 1057) also point out the possibility of bias resulting from differences in the recall of distant events, particularly between patients and healthy controls. They report, however, that one of the Swedish authors (Hardell, *Scand. J. Work envir. Hlth* 1981, **7**, 119) has compared the cases with soft-tissue sarcomas with a control group of patients with colonic cancer and obtained a similar (five-fold) increased risk of soft-tissue sarcoma after exposure to phenoxy acids and chlorophenols. Coggon & Acheson (*loc. cit.*) conclude that "there is suggestive evidence of a biological association between phenoxy herbicides (or their contaminants) and soft-tissue sarcomas".]

Aluminium and impaired breeding in wild birds

Nyholm, N. E. I. (1980). Evidence of involvement of aluminium in causation of defective formation of eggshells and of impaired breeding in wild passerine birds. *Envir. Res.* **26**, 363.

Severe eggshell defects and reduced clutch sizes indicated a breeding impairment among birds nesting along the shores of two lakes in Swedish Lapland. Such impairment was not noticed in birds breeding further from the shores of these lakes nor in birds breeding along the shore of a lake in a similar environment further north. Extensive analysis of various tissues indicated that neither biocides such as DDT or polychlorinated biphenyls nor heavy metals such as cadmium, chromium, copper, lead or mercury were likely to be causal factors.

The medullary tissue of humeri from nineteen female pied flycatchers (*Ficedula hypoleuca* Pallas) were subjected to multi-element analysis. Specimens were obtained during the laying phase (when the birds were about to lay their second egg) or during the incubation period. Thirteen of the birds laid at least one egg with a defective shell and were classified as

'affected'. Four birds nesting far from any lake and two birds nesting along the shore of the northern lake showed no signs of impaired breeding and were classified as 'normal'. The affected birds suffered from incomplete filling of the bone marrow cavity during the laying phase and laid increasingly defective eggs. Small clutch sizes and a decreased number of developing egg follicles demonstrated the lower laying capacity of the affected birds. Two of the affected birds laid only one egg each, had no developing egg follicles and were found dead in their nests. The defective eggshells were more porous and were made of coarser crystals than were the normal eggshells and they had incorporated blood cells which indicated that intrauterine bleeding had occurred during eggshell formation.

Under normal circumstances, calcium and phosphorus (probably in the form of apatite) are the principal elements present in medullary tissue and these become depleted during the incubation period. In the affected birds, the levels of calcium and phosphorus were relatively low and varied widely throughout the tissue, suggesting abnormal deposition and/or mobilization of apatite. Aluminium was present in the medullary tissue of the humeri of the affected birds but none was found in 'normal' birds. The aluminium levels were similar during the laying phase and incubation, suggesting that the aluminium was not subject to the calcium mobilization mechanism which operates during the laying phase. It is possible that aluminium exerts a toxic effect by interfering with normal phosphate metabolism, perhaps as a result of interactions between aluminium and enzyme systems that require other metals. Such disruption of phosphate metabolism could result in low bone-marrow levels of apatite, and thus decreased levels of calcium available for eggshell production. Low calcium availability may also inhibit the development of ovarian follicles. Phosphate depletion could also occur by binding of orally ingested aluminium with dietary phosphate resulting in the excretion of unabsorbed aluminium phosphate.

Although neither the origin nor transport route of the aluminium found in the tissues of affected birds has been confirmed, the author suggests that acidic precipitation may leach out relatively high levels of aluminium from the gneiss, which is the prevalent bedrock in the area in which the affected birds were found. The area around the northern lake where impaired breeding was not noted consists largely of calcareous deposits which have a high buffering capacity and therefore a low risk of aluminium leaching. The postulated route of transfer of aluminium to the birds is by the consumption of the insects that swarm along the lake shores. However, although it was confirmed that such insects make up a significant proportion of the birds' diet, no attempt was made to assess the aluminium content of these insects.

Flare-up dermatitic reactions induced by ingested nickel

Christensen, O. B., Lindström, C., Löfberg, H. & Möller, H. (1981). Micromorphology and specificity of orally induced flare-up reactions in nickel-sensitive patients. *Acta derm.-vener., Stockh.* **61**, 505.

Nickel is said to be responsible for more instances of allergic dermatitis than all the other metals combined (Fischer, *Contact Dermatitis*, 2nd Ed., p. 96. Lea & Febiger, Philadelphia, PA, 1973) and is one of the most common causes of contact dermatitis (*Cited in F.C.T.* 1976, **14**, 217). Activation of hand dermatitis in nickel-sensitive patients and flare-up reactions at earlier patch-test or contact-dermatitis sites following ingestion of nickel have been reported (Christensen & Möller, *Contact Dermatitis* 1975, **1**, 136; Cronin *et al.* in *Nickel Toxicology*, p. 149. Edited by S. S. Brown and F. W. Sunderman, Jr. Academic Press, London, 1974). The micromorphology and specificity of flare-up reactions induced by oral ingestion of nickel some 4–7 wk after patch testing have now been investigated.

Patch testing with nickel sulphate (5% in petrolatum) was carried out on five adult women, all of whom had been hypersensitive to nickel for several years and were periodically affected by hand eczema. Patch testing with benzalkonium chloride (1% in water) and an intradermal test with tuberculin 2TU served as controls. These controls provided examples of substances known to induce toxic dermatitis and a delayed hypersensitivity reaction, respectively. The tests were read at 72 hr after application and the margins of the inflammatory reactions carefully marked. When the reactions had virtually disappeared 4–7 wk later punch biopsies were removed from the benzalkonium chloride and nickel test sites and from the palms of the hands and examined by light microscopy and direct immunofluorescence. The patients were examined closely for 24 hr following oral ingestion of 5·6 mg nickel (as nickel sulphate) and punch biopsies were then taken from the test sites and palms. Eczematous reactions at the nickel test sites with erythema, infiltration, papules and/or vesicles were observed in all five patients 10–24 hr after oral provocation. The areas of the reaction sites were 2–3 times greater than the original patch-test reactions. Flare-ups of hand eczema were evident in four patients and the fifth reported itching of the palms. Flare-ups also occurred at sites of earlier contact dermatitis. Three of the patients were certain that they had had no dermatitis in these areas for the previous 2–4 yr. No clinical or histopathological changes were elicited at the benzalkonium chloride or tuberculin test sites following oral ingestion of nickel, indicating that the oral antigen had no effect on the inflammatory process *per se*, nor on any type IV reaction other than nickel allergy. The authors suspect that degranulation of the mast cells plays some part in the flare-up reactions but doubt that humoral antibodies play a pathogenic role since neither immunoglobulins of the IgG, IgA and IgM class, nor complement 3 nor fibrinogen could be detected at the test sites using the direct immunofluorescence technique.

[This paper highlights the fact that contact sensitizers are capable of eliciting reactions even when administered by routes other than dermal. It seems that tests in guinea-pigs do not reflect the situation in man. The authors of the present study cite a test (Polák & Turk, *Clin. exp. Immun.* 1968, **3**, 253) in

which flare-up reactions could be consistently provoked in guinea-pigs 2 wk after patch testing, but not after 3 months. In this species dense infiltrates of polymorphonuclear leukocytes were observed at the flare-up sites, but few such leukocytes were observed in the women in the present study.]

The non-carcinogenicity of 1-naphthylamine

Purchase, I. F. H., Kalinowski, A. E., Ishmael, J., Wilson, J., Gore, C. W. & Chart, I. S. (1981). Lifetime carcinogenicity study of 1- and 2-naphthylamine in dogs. *Br. J. Cancer* **44,** 892.

No evidence of carcinogenicity was found in six dogs given 1-naphthylamine (1-NA) at an oral dose level of 15 mg/kg body weight, 5 days/wk for 9 yr (*Cited in F.C.T.* 1981, **19,** 792). The 1-NA in this case contained only 0·038–0·04% 2-naphthylamine (2-NA), in contrast to the 3–6% usually present in the commercial material until the last decade. The findings thus suggested that 2-NA contamination may have been responsible for the bladder papillomata found previously in two dogs given 1-NA (Bonser *et al. Br. J. Cancer* 1956, **10,** 533) and for the excess of bladder cancer in workers exposed to commercial 1-NA (Case *et al. Br. J. ind. Med.* 1954, **11,** 75). Nevertheless, 1-NA is mutagenic in bacteria after metabolic activation, and positive results were also obtained in some yeast and *in vitro* mammalian cell assays (*Evaluation of Short-Term Tests for Carcinogens; Report of the International Collaborative Program*, pp. 68 & 77. Edited by F. J. de Serres & J. Ashby. Elsevier/North-Holland Inc., New York, 1981).

The mutagenicity of 1-NA after metabolic activation may be ascribed to its *N*-hydroxy metabolite (*N*-hydroxy-1-NA), which is itself directly mutagenic in bacteria (Belman *et al. Cancer Res.* 1968, **28,** 535; *Cited in F.C.T.* 1966, **4,** 472; McCoy *et al. Envir. Mutagen.* 1981, **3,** 499). Furthermore, implantation of stearic acid pellets containing 20% *N*-hydroxy-1-NA into the bladders of mice produced significantly more local tumours than did stearic acid alone (Boyland *et al. Br. J. Cancer* 1964, **18,** 575). *N*-Hydroxy-1-NA also caused more local tumours after ip injection into rats than did the equivalent metabolite of 2-NA (Belman *et al. loc. cit.*; Radomski *et al. Cancer Res.* 1971, **31,** 1461). Some tumours at other sites were also found in rats treated ip with *N*-hydroxy-1-NA, and three bronchogenic tumours and one hepatoma occurred in 58 mice given a single sc injection of *N*-hydroxy-1-NA during the 24 hr after birth (Radomski *et al. loc. cit.*). Like many carcinogens, *N*-hydroxy-1-NA binds covalently to DNA, RNA and protein (Kadlubar *et al. Cancer Res.* 1978, **38,** 3628). However, whereas >12 DNA adducts/10^8 nucleotides could be detected in the urothelium of two dogs given ^{3}H-labelled 2-NA (8 mg/kg body weight), none (above the detection limit of 1/10^8 nucleotides) could be found after administration if 8·5 mg ^{3}H-labelled 1-NA/kg body weight (*idem, Carcinogenesis* 1981, **2,** 467).

Purchase *et al.* (cited above) now describe a study in which beagle dogs were treated orally 5 days/wk with 400 mg highly-purified 1-NA, containing only 5 ppm 2-NA (group 1), 1-NA to which 0·5 or 6% 2-NA had been added (groups 2 and 3 respectively) or pure 2-NA (group 4). Groups 1–3, comprising four dogs of each sex per group, were treated for up to 109 months and survivors were killed at 128 months, while treatment of group 4 (three males and two females) ended after 34 months, and all of the dogs were dead by 47 months. The daily dose levels (5 days/wk) were equivalent to about 18·5 mg total naphthylamine/kg for males and 21–22 mg/kg for females, and groups 1, 2 and 3 received approximately 1×10^{-4}, 0·1 and 1·1–1·25 mg 2-NA/kg/day respectively. Groups of four male and four female dogs were used as vehicle-treated controls.

Transient increases in plasma levels of the enzymes alanine transaminase (ALT), alkaline phosphatase (ALP) and ornithine carboxyl transferase (OCT) were seen in group 1, with a more marked response in groups 2 and 3. Group 4 showed high ALT activities, with less effect on OCT, but ALP levels were normal. Blood urea levels were also increased in group 4 animals. Haematuria occurred in all dogs in group 4, and in one of the dogs from each of groups 3 and 1. No treatment-related effects on body weight were found, and only in the urinary system were there gross or histopathological lesions that were considered to be treatment-related. All of the dogs in group 4 had large cauliflower-like masses involving most of the bladder mucosa. Histological examination revealed these to be transitional-cell carcinomas. Two of the group 3 animals had solitary papilliform masses (early carcinomas) while two of the dogs from group 2 had solitary haemangiomas arising in the submucosa and protruding into the bladder lumen. The group-1 dog that had developed haematuria showed focal cystitis with dilation of the submucosal blood vessels and associated haemorrhage. Two of the controls and one dog in group 3 also displayed focal epithelial hyperplasia of the bladder wall. Hyperplasia of the epithelium of the ureter was seen in single dogs in groups 2 and 3 and in three dogs in group 4. The only statistically significant differences in tumour incidence were seen in group 4, in which there were increased incidences of bladder tumours and of animals bearing malignant tumours. Larger numbers of thyroid and skin tumours were seen in groups 1, 2 and 3 than in the controls, but the differences were not significant.

It was concluded that the low incidence of bladder cancer in dogs given 1-NA with 6% added 2-NA explained the carcinogenicity of impure 1-NA in earlier studies. The significance of haemangiomas in two of the animals given 1-NA with 0·5% added 2-NA (group 2) was uncertain, since similar lesions did not appear to have been reported in previous studies. The localized vascular dilation observed in the bladder submucosa of one dog given purified 1-NA may have represented a preangiomatous change, but could not be considered neoplastic. In view of the small size of the treatment groups, the study may not be regarded as proving conclusively the non-carcinogenicity of 1-NA. However, it was calculated that if the haemangiomas in group 2 were taken as evidence of the carcinogenic activity of 2-NA, the daily dose of about 0·1 mg 2-NA/kg that was required to produce them was only 0·5% of the dose of 1-NA given to group 1. 1-NA can thus be considered at least 200 times less potent than 2-NA in inducing bladder cancer in dogs.

Quercetin: no carcinogenicity in hamsters

Morino, K., Matsukura, N., Kawachi, T., Ohgaki, H., Sugimura, T. & Hirono, I. (1982). Carcinogenicity test of quercetin and rutin in golden hamsters by oral administration. *Carcinogenesis* **3**, 93.

Quercetin, a flavonoid found in many edible plants, has been shown to have mutagenic activity in *Salmonella typhimurium* both with and without microsomal activation (*Cited in F.C.T.* 1979, **17**, 688; Bjeldanes & Chang, *Science, N.Y.* 1977, **197**, 577), to induce cell transformation in hamster embryo cells *in vitro* (Umezawa *et al. Toxicology Lett.* 1977, **1**, 175) and to give positive results in the micronucleus test (Sahu *et al. Mutation Res.* 1981, **89**, 69). It has also been reported to be carcinogenic in rats (Pamukcu *et al. Cancer Res.* 1980, **40**, 3468), although no evidence of carcinogenicity was found in another study in this species (Hirono *et al. Cancer Lett.* 1981, **13**, 15). It has been suggested that quercetin could be formed from another common flavonoid, rutin, by hydrolysis in the gut (*Food Chemical News* 1981, **22** (44), 15), but no increased tumour incidence was observed in rats fed rutin at 1, 5 or 10% in the diet (*ibid* 1982, **24** (10), 10). Rutin itself was not mutagenic to *S. typhimurium* with or without addition of S-9 mix (Bjeldanes & Chang, *loc. cit.*) and gave negative results in the micronucleus test (Sahu *et al. loc. cit.*), although it was mutagenic to *S. typhimurium* in the presence of S-9 mix and fecalase (Tamura *et al. Proc. natn. Acad. Sci. U.S.A.* 1980, **77**, 4961). The study cited above provides no evidence of carcinogenic activity of quercetin or rutin in golden hamsters, nor any indication that quercetin may act as a tumour initiator.

In one experiment, three groups of 20 male and 20 female golden hamsters were fed diets containing 10% quercetin or 10% rutin or were given control diet for 733 days. In the second experiment, five groups of 14–30 hamsters were used, each group containing equal numbers of male and female animals. Two of these groups were treated with either 4% quercetin in the diet for 709 days or control diet for 701 days. Another two groups were given quercetin (1% in the diet) for 351 days and then either control diet or a diet containing 1% croton oil (known to promote tumours in rats and mice). The fifth group was given the control diet for 351 days and then the croton oil diet for 350 days.

In both experiments there were no effects of treatment on body-weight gain or survival at 480 days. In Experiment I the tumours most frequently observed were papillomas of the forestomach and cortical adenomas of the adrenal gland. However there were no significant differences between the treated and control groups in the incidence of these or other tumours. In the second experiment virtually all of the tumours found were forestomach papillomas. Some of these showed expansive growth, but none showed invasive growth. In the three groups given quercetin and/or control diet but not croton oil, the tumours were single sessile polyps of the forestomach which were very similar to those observed in Experiment I. In contrast the majority of the animals with forestomach tumours in the groups given croton oil had multiple sessile polyps. There were no significant differences in the incidences of forestomach tumours between the groups given quercetin but not croton oil and that given the control diet only. The incidence of forestomach papillomas in the group treated with quercetin and croton oil was significantly greater than that in the group fed only control diet. However there was no significant difference between the quercetin/croton oil group and the control diet/croton oil group and thus no evidence that quercetin acts as a tumour initiator.

The authors discuss the conflicting results of the present study and those of Pamukcu *et al.* (*loc. cit.*) in Norwegian strain rats. On the basis of their own and other studies they suggest that Norwegian strain rats may be particularly sensitive to quercetin. However, they do not discuss the question of the genetic activity of quercetin in several *in vitro* systems and the apparent lack of carcinogenicity in laboratory animals.

Testicular effects of 2,5-hexanedione

Chapin, R. E., Norton, R. M., Popp, J. A. & Bus, J. S. (1982). The effects of 2,5-hexanedione on reproductive hormones and testicular enzyme activities in the F-344 rat. *Toxic. appl. Pharmac.* **62**, 262.

n-Hexane has been associated with polyneuropathy in humans occupationally exposed (*Cited in F.C.T.* 1977, **15**, 492; *ibid* 1979, **17**, 313) or practising solvent abuse (*ibid* 1976, **14**, 157). Neurotoxic effects have been induced in experimental animals by exposure to *n*-hexane (*ibid* 1977, **15**, 492) and to 2,5-hexanedione (HDE; *ibid* 1981, **19**, 133; Spencer & Schaumberg, *Proc. R. Soc. Med.* 1977, **70**, 37). HDE is a metabolite of *n*-hexane and of another neurotoxic chemical, methyl *n*-butyl ketone (*Cited in F.C.T.* 1981, **19**, 133). *n*-Hexane and its metabolites have also been reported to induce pathological testicular changes (Krasavage *et al. Toxic. appl. Pharmac.* 1980, **52**, 433; O'Donoghue *et al. ibid* 1978, **45**, 269). The effects of HDE on reproductive hormones and testicular enzyme activities in the rat have now been reported.

Fischer 344 rats were given 1% HDE in their drinking-water—a treatment that induces neurotoxic effects similar to those that occur in animals and humans exposed to *n*-hexane by inhalation. One group of control rats was fed *ad lib.* while another was pair-fed with the HDE-treated group. Six rats from each group were killed after 1, 3 or 6 wk and the livers, testes and blood were taken for hormone and enzyme determinations.

After 6 wk testis weights of the two control groups were similar, but the testes from the HDE-treated rats weighed 64% less than those from the pair-fed controls. Throughout the study, serum testosterone levels were unaffected by HDE treatment, but although serum levels of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were unchanged after 1 and 3 wk, at 6 wk they were significantly above those of pair-fed controls. In rats treated with HDE for 6 wk, serum LH levels 20 min after an injection of gonadotrophin-releasing hormone were not significantly different from those of pair-fed controls, thus indicating that consumption of HDE had no effect on the response of the pituitary to administered gonadotrophin-releasing hormone. Microscopic examination

revealed no abnormalities in the testes of rats exposed to HDE for 3 wk. However at this time the activities of the Sertoli-cell enzymes β-glucuronidase and γ-glutamyl transpeptidase (GGT) were significantly decreased compared with pair-fed controls. After 6 wk the testes were azoospermic; at this time the activities of β-glucuronidase and GGT were increased and those of acid phosphatase and sorbitol dehydrogenase were decreased. In the pair-fed controls themselves, β-glucuronidase activity after 6 wk was significantly decreased compared with the controls fed *ad lib.* The testicular enzyme profile after 6 wk of HDE administration was similar to that observed in the azoospermic testis of rats made unilaterally cryptorchid by surgery. It was considered that these enzyme changes might represent changes in Sertoli-cell function as well as simple loss of germ cells. In the livers of HDE-treated rats the lysosomal enzymes β-glucuronidase and acid phosphatase were decreased at all time intervals but sorbitol dehydrogenase was unaffected.

Since HDE has been reported to induce central nervous system (CNS) effects (Schaumburg & Spencer, *Science, N.Y.* 1978, **199,** 199) one of the aims of the present study was to determine the degree of CNS involvement in HDE-induced testicular effects. The lack of an increase in FSH and LH levels after 1 or 3 wk of treatment indicates that HDE does not act on the CNS to decrease hormonal stimulation of the testes. Further evidence for this comes from the normal response of the pituitary to administered gonadotrophin-releasing hormone after 6 wk of HDE treatment. Instead the significant changes in Sertoli-cell enzymes before the onset of azoospermia suggest a direct toxic effect of HDE on the testis.

Comparative toxicity of two glycol ethers

Miller, R. R., Ayres, J. A., Calhoun, L. L., Young, J. T. & McKenna, M. J. (1981). Comparative short-term inhalation toxicity of ethylene glycol monomethyl ether and propylene glycol monomethyl ether in rats and mice. *Toxic. appl. Pharmac.* **61,** 368.

Exposure to high concentrations of ethylene glycol monomethyl ether (EGME; 2-methoxyethanol) has been associated with CNS and haematological effects in animals and man (*Cited in F.C.T.* 1963, **1,** 256; *ibid* 1964, **2,** 263). Testicular degeneration and desquamation of the germinal epithelium were reported many years ago in rabbits given high doses by injection (Wiley *et al. J. ind. Hyg. Toxicol.* 1938, **20,** 269) and a much more recent Japanese study revealed dose-related testicular atrophy and leucopenia in mice treated orally for 5 wk with 62·5–4000 mg/kg body weight (Nagano *et al. Jap. J. ind. Hlth* 1979, **21,** 29). In mice given 31·25–1000 mg/kg by gavage on days 7–14 of gestation, embryotoxic and/or teratogenic effects were evident at all dose levels (*Cited in F.C.T.* 1982, **20,** 345).

Propylene glycol monomethyl ether (PGME; 1-methoxy-2-propanol) exposure has similarly been associated with CNS depression, although its smell and irritancy at low concentrations tend to protect against this effect during inhalation by man (*ibid* 1970, **8,** 709). High oral or iv doses in animals have produced not only CNS effects but also damage to the liver, kidney and testes, and delayed ossification occurred in foetuses of rats exposed orally during gestation to 0·8 ml/kg body weight/day (*ibid* 1974, **12,** 161). Early inhalation studies in which animals were repeatedly exposed for 7 hr/day revealed no evidence of adverse effects in male or female rats or guinea-pigs exposed on 141/198 or 130/184 days, respectively, to 1500 ppm, but female rabbits and a female monkey exposed for similar periods developed slight histological lung and/or liver changes at this level. The no-effect level for the last two species was 800 ppm. At 3000 ppm on 141/198 days rats showed slight initial CNS and growth depression and increased liver weights, and at 6000 ppm (81 exposures over 114 days) mortality and, in males, kidney weights were also elevated. Guinea-pigs exposed to 3000 ppm on 130/184 days were unaffected, but at 6000 ppm on 80/113 days there was marked narcosis and growth depression and slight liver damage (Rowe *et al. A.M.A. Archs ind. Hyg. occup. Med.* 1954, **9,** 509).

The effects of inhalation of EGME and PGME have now been further explored by Miller *et al.* (cited above). Rats and mice (5–10 of each sex per group) were exposed to 100, 300 or 1000 ppm EGME or 300, 1000 or 3000 ppm PGME for 6 hr/day for 5 days, were untreated for the following 2 days, and then were exposed for a further 4 days. They were killed 18 hr after the last exposure, except for some PGME-exposed animals which were allowed to survive for a further 6 wk.

At the highest level of EGME there was growth depression in rats, and a decrease in absolute and relative thymus and testes weights in both species. There were also decreases in packed cell volume, red-blood-cell counts, haemoglobin and white-blood-cell counts (except in female mice in which only the last parameter was significantly affected), and in serum total protein, albumin (males only) and globulin and urinary specific gravity in rats. At 300 ppm effects on body-weight gain, thymus weight, haematology and serum protein levels were still evident in rats, and haematology and (female) thymus weight were also affected in mice. At 100 ppm the only significant changes were decreases in weight gain in female rats and in white-blood-cell count in male rats. Histopathological examination (of rats only) revealed at 1000 ppm a marked reduction of bone marrow cellularity, severe degeneration of the testicular germinal epithelium, severe lymphoid depletion of the cortex of the thymus, and reduced numbers of lymphoid cells in the spleen and mesenteric lymph nodes. The latter were depleted in areas normally populated by both B-lymphocytes (germinal centres) and T-lymphocytes (medullary cords and paracortical regions). Lymphoid depletion of the thymus was also evident at 300 ppm, but not at 100 ppm.

PGME exposure at 3000 ppm was associated with CNS depression, an increase in relative liver weight in rats and female mice, and with decreased urinary specific gravity and increased urinary pH in rats, suggesting slight alterations in kidney function. Urinary specific gravity in male rats was also significantly affected at 1000 ppm. All of these parameters had returned to normal 6 wk after the last exposure. There were also increases in platelet counts and a lowering of alkaline phosphatase activity in PGME-exposed

rats, but the latter was thought to be related to terminal nutritional status, and both were considered unrelated to treatment. No histopathological effects were evident in rats or mice at any level, even in the liver and kidney.

The tissues conspicuously affected by EGME (bone marrow, testicular germinal epithelium and thymic cortex) all have a relatively high rate of cell division, suggesting that EGME might inhibit mitotic processes. However, the intestinal epithelium and ovarian follicles (which also have a very high rate of cell turnover) appeared unaffected, suggesting that other factors were also involved. The reduction in serum globulins was consistent with the lymph-node depletion in areas normally populated by B-lymphocytes, possibly indicating an impairment of the humoral immune response, while the thymic atrophy and lymph-node depletion in areas normally populated by T-lymphocytes suggested that the cellular immune response was also impaired. The great difference in biological activity between EGME and PGME was considered to illustrate the potential for error when inferences about toxicity are made on the basis of chemical structure alone.

No teratogenicity with methylchloroform

York, R. G., Sowry, B. M., Hastings, L. & Manson, J. M. (1982). Evaluation of teratogenicity and neurotoxicity with maternal inhalation exposure to methyl chloroform. *J. Toxicol. envir. Hlth* **9**, 251.

In an earlier study methylchloroform (MC; 1,1,1-trichloroethane) was concluded to have no significant maternal, embryonic or foetal toxicity in rats or mice inhaling 875 ppm for 7 hr/day on days 6–15 of gestation (*Cited in F.C.T.* 1976, **14**, 649). Trichloroethanol and trichloroacetic acid have been detected in the urine of workers exposed to MC (*ibid* 1976, **14**, 75; Stewart *et al. Archs envir. Hlth* 1969, **19**, 467), although most absorbed MC is excreted unchanged via the lungs (*Cited in F.C.T.* 1969, **7**, 397). York *et al.* (cited above) have now investigated whether longer periods of exposure to MC, which might lead to accumulation of metabolites, have reproductive effects in rats.

Groups of 11–20 Long–Evans rats were exposed to 2100 ± 200 ppm MC 6 hr day, 5 days/wk for 2 wk before mating and/or for 6 hr/day, 7 days/wk on days 0–20 of gestation. Half of the rats in each group were killed on day 21 of gestation and the foetuses were removed for examination. The remaining animals were allowed to give birth, and 4 days after parturition the litters were culled to eight pups, with further culling at weaning to two male and two female pups per litter. The dams were killed and autopsied at weaning. The offspring were observed for up to 12 months, during which time they were subjected to various neurobehavioural tests, and were then killed.

There were no signs of toxicity in the dams exposed to MC. Dams exposed before mating did have significantly greater weight gains during pregnancy than those exposed only during pregnancy and the controls (exposed to filtered air). However, since maternal liver weights and blood clinical chemistry parameters were unaffected by MC exposure, it was considered unlikely that this increased weight gain during pregnancy was a sign of MC-induced toxicity. There were no significant treatment effects on parameters of embryotoxicity except for decreases in foetal body weight in groups exposed during pregnancy. No major malformations were observed in any of the foetuses examined, but there were significantly increased incidences of skeletal and soft-tissue variations in foetuses from the group exposed both before and during gestation. These were considered to be indicative of embryotoxicity (a reversible developmental delay) rather than teratogenicity. Among the offspring, no significant differences between groups were observed in body weight, survival, or performance in neurobehavioural tests, and at autopsy the incidence of gross lesions was similar in the treated and control groups. It is therefore concluded that inhalation exposure of female rats before mating and/or during gestation to 2100 ± 200 ppm MC does not have any persistent detrimental effects on any of the parameters measured.

Aromatic amines and bladder cancer in man

Rubino, G. F., Scansetti, G., Piolatto, G. & Pira, E. (1982). The carcinogenic effect of aromatic amines: an epidemiological study on the role of *o*-toluidine and 4,4′-methylene bis(2-methylaniline) in inducing bladder cancer in man. *Envir. Res.* **27**, 241.

Both benzidine (BZ) and 2-naphthylamine (2-NA) are well-established causes of bladder cancer in occupationally exposed humans (*Cited in F.C.T.* 1970, **8**, 210; *IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Man*, Vol. 1, p. 80 and Vol. 4, p. 97. IARC, Lyon, 1972 and 1974). However, for many aromatic amines definitive evidence of carcinogenicity has come only from animal studies. These include *o*-toluidine (*Cited in F.C.T.* 1981, **19**, 799) and 4,4′-methylene bis(2-methylaniline) [3,3′-dimethyl-4,4′-diaminodiphenylmethane; DDDM]. Rats given the latter compound orally developed tumours of the liver, lung, skin and mammary gland (*IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Man*, Vol. 4, p. 73. IARC, Lyon, 1974; Stula *et al. Toxic. appl. Pharmac.* 1975, **31**, 159). Furthermore, when six dogs were given an oral dose of 100 mg DDDM on 3–5 days/wk for 11 wk, and subsequently treated with 50 mg on 5 days/wk for up to 7 yr, the three animals that survived for more than 5 yr all showed hepatocellular carcinomas, and two also had primary lung tumours (*idem. J. envir. Path. Toxicol.* 1978, **1** (3), 339).

Several case reports and epidemiological studies suggesting that *o*-toluidine may cause bladder cancer in man were recently reviewed by the IARC (*IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans*, Vol. 27, p. 155. IARC, Lyon, 1982). It was concluded that as they all involved exposure to other possibly carcinogenic chemicals, *o*-toluidine could not be identified specifically as the responsible agent. One such study, reported briefly in that review (Rubino *et al. Arh. Hig. Rada Toksikol.* 1979, **30**, Suppl. p. 627), has now been described in more detail (Rubino *et al.* cited above). It involved 868 male workers with at least 1 month of

employment between 1922 and 1970 in a dyestuff factory in Northern Italy, who were engaged in manufacturing or using a variety of aromatic amines such as 1- or 2-NA, BZ, fuchsin or safranine T. Open systems were used, which must have resulted in considerable dermal exposure. In comparison with the general Italian population, the group showed a marked excess of bladder cancer (36 observed *v.* 1·23 expected deaths up to the end of 1976) with a mean latent period of 25 yr (range 12–41 yr). The risk tended to increase with duration of exposure, but two deaths occurred after less than 1 yr of exposure. Significant excess mortality was also found for lung, laryngeal, oesophageal and other cancers, and for liver cirrhosis.

The highest excess of deaths from bladder cancer (11 *v.* 0·04 expected) was found among 33 workers who had been engaged in manufacturing both BZ and 1- and 2-NA, and those who had manufactured only 2-NA or BZ were at the next highest risk, with 6/30 and 5/65 of such workers, respectively, dying of bladder cancer. One of the 27 workers engaged only in manufacturing 1-NA died 41 yr after first exposure (cf. 0·05 expected deaths). Bladder cancer caused the deaths of 3/148 who had used BZ or the naphthylamines, and of 5/307 who had intermittent contact with these materials, whereas only 0·24 and 0·44 deaths respectively would have been expected in these groups.

Of 53 men employed solely in manufacturing fuchsin (new fuchsin; Basic Violet 2; C.I. no. 42520) and safranine T (Basic Red 2; C.I. no. 50240) five (cf. 0·08 expected) died of bladder cancer. In one department (A), in which three of the five deaths occurred, toluene was converted to *o*-nitrotoluene and thence to *o*-toluidine and DDDM. In another department (B), in which there were two bladder cancer deaths, the last three materials were heated to produce fuchsin, and a mixture of *o*-toluidine and 2,5-diaminotoluene was oxidized in the presence of aniline to produce safranine T. In the latter process *o*-aminoazotoluene was formed as an intermediate. As five cases of bladder cancer were reported in 1954 among 85 men manufacturing magenta, but not exposed to 1- or 2-NA or BZ (Case & Pearson, *Br. J. ind. Med.* 1954, **11**, 213), and as the commercial production of magenta also involves the use of *o*-toluidine, it is suggested that *o*-toluidine may be the common causal agent. However, DDDM also comes under strong suspicion, and *o*-aminoazotoluene, which produces bladder and other cancers in animals (*IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Man*, Vol. 8, p. 61. IARC, Lyon, 1975) might also have been a causative factor in workers exposed in department B.

Asthma induced by azodicarbonamide dust

Slovak, A. J. M. (1981). Occupational asthma caused by a plastics blowing agent, azodicarbonamide. *Thorax* **36**, 906.

Azodicarbonamide (AC; 1,1′-azobisformamide) is used as a flour-strengthening agent and as a blowing agent in the production of expanded foam plastics. Feeding studies in animals have indicated that AC has low toxicity, and no dermatological problems arising from industrial or bakery use had been reported at the time of a JECFA review in 1965 (*F.A.O. Nutr. Mtg Rep. Ser.* 1967, 40A,B,C). However, more recently, acute respiratory effects have been reported in workers engaged in the grinding of AC (Ferris *et al. J. occup. Med.* 1977, **19**, 424). A survey of workers in a factory manufacturing AC in the form of a fine powder for use in the plastics industry has now been reported.

The AC plant was opened in 1966, and all 151 personnel who were still in employment in 1980 and had been or were currently involved in manufacture, servicing or quality control were surveyed. Twenty-eight (18·5%) of the workers were diagnosed as having developed asthma, predominantly of the late-onset type, as a result of AC exposure. More than half of these workers had developed their symptoms within 3 months of initial exposure to AC, and in 75% the onset of symptoms was within 1 yr of initial exposure. Other symptoms reported included cough, rhinitis, conjunctivitis and rash. Noticeable worsening of their asthma symptoms with continuing exposure was reported by 46% of the affected workers. More than half of the 13 workers who had remained in exposure for more than 3 months after the onset of asthma had developed subjective airways hyperreactivity to common environmental irritants, a condition that persisted for more than 1 month after their removal from AC exposure. In some of these, recovery was still incomplete after several years. No such persistent airways hyperreactivity was reported by workers who were removed from exposure to AC within 3 months after onset of asthma. Smoking habits were not related to development of asthma, and prick tests with common allergens indicated that atopic status was not predictive of AC sensitization. Prick tests with 0·1, 1 or 5% solutions of AC in dimethylsulphoxide were negative in both AC-sensitized and asymptomatic exposed workers. In the asymptomatic exposed workers, who had been in daily contact with AC for more than 1 yr, pre- and post-workshift spirometric measurements provided no evidence of subclinical bronchoconstrictive effects.

The author estimates that annually 8000–10,000 people in the UK plastics and rubber industries come into contact with AC. If such workers develop occupational asthma AC should be suspected, although toluene diisocyanate remains the more frequent sensitizer in these industries (*Cited in F.C.T.* 1980, **18**, 545; *ibid* 1981, **19**, 133; *Respiratory and Immunologic Evaluation of Isocyanate Exposure in a New Manufacturing Plant*, by H. Weill *et al.*; DHHS (NIOSH) Publication No. 81–125, 1981, pp. vi + 152).

Chromate exposure: not just a lung cancer risk?

Sheffet, A., Thind, I., Miller, A. M. & Louria, D. B. (1982). Cancer mortality in a pigment plant utilizing lead and zinc chromates. *Archs envir. Hlth* **37**, 44.

Studies have indicated an increased risk of lung cancer among workers manufacturing chromate pigments, especially zinc chromate (*Cited in F.C.T.* 1979, **17**, 97; *ibid* 1982, **20**, 129). Further evidence of the lung cancer hazard has now been provided by the

results of a detailed cancer mortality study of workers in a chromate pigment plant in the USA.

All males employed at the plant for more than 1 month at any time between 1 January 1940 and 31 December 1969 were included in the study. The total study cohort comprised 1296 whites and 650 nonwhites (including 27 persons of unknown race who were added to both white and nonwhite groups). Vital status (at 31 March 1979) was known for 1104 of the white workers; 211 of these were known to be dead and for 174 the cause of death could be established. The respective figures for the nonwhite workers were 518, 110 and 98. On the basis of work-history records, exposure of the workers was assessed as high (continuous exposure to chemical dust), moderate (occasional exposure to chemical dust or to dry or wet pigments) or low (infrequent exposure to chemical dust or pigments). In almost all parts of the plant individuals who were exposed to any chemicals were also exposed to airborne lead and zinc chromates (in proportions of about 9:1). On the basis of air sampling carried out in later years, it was estimated that during the study period workers in the three categories were exposed to 2, 0·5–2 and <0·1 mg airborne chromium/m^3, respectively.

The observed number of deaths from each cause in the study cohort was compared with the expected number of deaths calculated from race-, cause-, age- and time-specific standard mortality rates for the USA. Deaths from disorders of the circulatory and respiratory systems and from external causes were significantly lower than expected, and contributed to a low overall standard mortality rate. However, there was a statistically significant excess risk of lung cancer among the white workers. Further analysis of the data showed a statistically significant excess of lung cancer in such workers employed for more than 10 yr, and for those with at least moderate exposure to chemicals who had been exposed for 2 yr or more. There was also an excess of lung cancer among the nonwhite workers, and increased incidences of stomach and pancreatic cancers in the total cohort, although the risk ratios in these instances were not significantly increased as determined by most methods of analysis. Cases of lung, stomach and pancreatic cancer accounted for 45 of the 75 cancer cases observed, a proportion that was significantly ($P < 0·01$) above that expected. Of the 14 lung-cancer cases for whom smoking histories were available, 13 smoked and nine smoked heavily. Therefore if exposure to chemicals in pigment-production plants promotes lung cancer, smoking is probably a cofactor—as it is, for instance, in asbestos exposure (*ibid* 1980, **18**, 307). However, since smoking histories were not available for the total cohort it was not possible to determine whether the increase in lung cancer in this study resulted from heavier smoking among the study cohort than in the general population or from combined smoking and chemical exposure.

Therefore this study seems to support an association between pigment manufacture and lung cancer in long-term and/or heavily exposed workers. Chromates may be the responsible compounds, although other chemicals used in the production process, such as nickel compounds, may be implicated. It is recommended that surveillance of workers in pigment-manufacturing plants should be continued, particularly in order to acquire additional data on the incidence of stomach and pancreatic cancers.

Passive smoking reduces lung function in asthmatics

Dahms, T. E., Bolin, J. F. & Slavin, R. G. (1981). Passive smoking. Effects on bronchial asthma. *Chest* **80**, 530.

The existence of allergy to tobacco smoke is not universally accepted, but there is evidence that exposure to tobacco smoke may precipitate asthmatic attacks (*British Medical Journal* 1978, **2**, 253) and reduce lung capability in asthmatics (Bolin *et al. J. Allergy clin. Immunol.* 1979, **63**, 151). However, harmful effects of passive smoking have not been conclusively demonstrated in healthy human beings (Lee. *Fd Chem. Toxic.* 1982, **20**, 223). Dahms *et al.* (cited above) have now reported the results of an acute study on the lung function changes that occur in healthy and asthmatic humans exposed to cigarette smoke.

Ten patients with bronchial asthma and ten control subjects were studied. Both the asthmatics and the controls were nonsmokers. They were exposed to machine-generated cigarette sidestream smoke for 1 hr in a 30-m^3 environmental chamber with controlled temperature, relative humidity, air flow and air turnover. Measurement of the carboxyhaemoglobin level of pre- and postexposure venous blood samples indicated an average carbon monoxide concentration of 15–20 ppm in the chamber over the exposure period. Pulmonary function tests were performed before exposure at 15-min intervals during exposure and, in the case of the asthmatics, 15 min after using a bronchodilator immediately after the postexposure blood samples had been taken. The three parameters assessed were forced vital capacity (FVC), forced expiratory volume after 1 sec (FEV_1), and mean forced expiratory flow during the middle half of the FVC (FEF25–75%). In the asthmatics, significant decreases in the FVC (first observed after 15 min) and in the FEV_1 and FEF25–75% (at 30, 45 and 60 min) demonstrated a linear decrease in pulmonary function over the exposure period. Decreases of about 20% in all three parameters had occurred after 60 min. In eight of the asthmatics pulmonary function had returned to pre-exposure levels 15 min after they had used the bronchodilator; the lung function of the other two had improved but had not reached baseline levels. The non-allergic control subjects showed no decreases in pulmonary function during exposure.

The results of this study indicate that passive smoking increases airways resistance in the lungs of patients with bronchial asthma. The pre-exposure pulmonary function parameters were substantially lower than those of the controls and were less than predicted normal values. Therefore in asthmatics passive smoking would seem to represent an increased risk to an already compromised respiratory system. Whether components of the gaseous phase or particulates in the smoke are the causative agents in such increased airways resistance remains unresolved. The authors also note the possibility that an emotional response to exposure to cigarette smoke may be at

least partially responsible for the pulmonary function changes seen in asthmatic patients. They cite evidence that the exposure conditions used in this study are similar to those sometimes encountered in daily life. Sebben *et al.* (*Archs envir. Hlth* 1977, **32**, 53) reported average carbon monoxide levels of 13·4 ppm in Toronto night clubs (with levels of 20–40 ppm in one poorly ventilated establishment), although it was concluded that it was relatively uncommon to find carbon monoxide concentrations higher than 15 ppm in public places where smoking was permitted.

Good news for Shipham

Inskip, H., Beral, V. & McDowell, M. (1982). Mortality of Shipham residents: 40-year follow-up. *Lancet* **I**, 896.

Early in 1979 the Department of the Environment warned residents of the village of Shipham in Somerset that their environment was contaminated by substantial amounts of cadmium which had been brought to the surface during mining for zinc. Cadmium levels remained high in the village, although the mining had stopped long ago; surveys showed Shipham garden soil to contain cadmium levels in the range 11–998 ppm, whereas in 'normal' areas of the UK levels were generally well below 10 ppm, and studies of agricultural soils indicated mean levels of less than 1 ppm (*Cadmium in the Environment and its Significance to Man*, p. 54. Pollution Paper No. 17. Department of the Environment, HMSO, London, 1980).

Using a non-invasive technique, Harvey *et al.* (*Lancet* 1979, **I**, 551) reported a mean cadmium concentration of 11·0 ppm in the livers of 21 adult volunteers living in the most heavily contaminated area of Shipham. The mean concentration in ten controls was 2·2 ppm. However, Harvey *et al.* observed that they had measured liver-cadmium levels of up to 260 ppm in industrially exposed workers, most of whom were "fit and healthy and without any signs of cadmium toxicity". In a pilot survey of 31 Shipham residents 22 were found to have raised blood cadmium levels, and some had elevated blood pressure and showed evidence of renal tubular damage, effects that were considered to be possibly attributable to exposure to cadmium (Carruthers & Smith, *Lancet* 1979, **I**, 845). A detailed study of the mortality of Shipham residents over 40 years has now been published.

Virtually the entire 1939 populations of Shipham and of a similar nearby village, Hutton, used as a control, were followed up until the end of 1979. In Shipham 278 of the 501 1939 residents died during the study period; in Hutton 215 deaths occurred among 410 residents. Compared with rates for England and Wales, overall mortality rates were low in both villages, and compared favourably with those for the whole of South-west England. [Rates for rural areas of the South-west dating from 1939 were unavailable.] There was an excess of deaths from circulatory disease in both sexes in Shipham and in men from Hutton; the excess in Shipham was from hypertensive and cerebrovascular disease. In women and in both sexes combined the standard mortality ratios (SMRs, ratio of observed to expected deaths) for death from cerebrovascular disease in Shipham were significantly raised ($P < 0{\cdot}05$). For both of these circulatory diseases the SMRs in Shipham were higher than those in Hutton, whereas for other circulatory diseases the reverse was true. The highest SMR was for genito-urinary disease in Shipham men; however although the eight deaths were more than twice the expected number, the result was significant only at the 10% level. The SMRs for nephritis and nephrosis were also elevated, but the numbers involved were very low (two per sex). For both sexes the SMRs for genito-urinary disease and nephritis and nephrosis exceeded those in Hutton. On the other hand, in both villages there was a low SMR for all forms of cancer and for individual cancer sites the SMRs for Shipham did not differ greatly from those for Hutton except when the numbers of cases were very low (and in the case of breast cancer where the SMR for Hutton was exceptionally low). SMRs for respiratory disease were also low in both villages, especially in Shipham where the ratios for both sexes were significantly ($P < 0{\cdot}05$) lowered.

Similar results were obtained when the analysis was repeated considering only those people still living in Shipham in 1946, thus presumably eliminating war evacuees who would be expected to have only a short duration of exposure to high cadmium levels. By using the address recorded in 1939 and cadmium levels in soil measured in 1979 it was possible to assign 70% of the Shipham population to a category of cadmium exposure. Of these, 85% lived in areas with soil cadmium levels >50 μg/g. The all-cause SMRs were similar in high- and low-cadmium areas, and the combined SMR for hypertensive, cerebrovascular and genito-urinary diseases was higher among those in the low-cadmium areas. [The authors admit that only limited information was available about each individual's exposure to cadmium, since the duration of residence before 1939 was unknown and only moves involving transfer to another National Health Service Executive Council or Family Practitioner Committee were recorded.]

Most of the specific diseases with elevated SMRs in Shipham, and showing a difference between Shipham and the control village, are among those that have been linked with cadmium toxicity—renal, hypertensive and cerebrovascular diseases. The major effect of the metal is believed to be on the kidneys (*Cited in F.C.T.* 1979, **17**, 84; *ibid* 1975, **13**, 470) and cadmium exposure has also been linked with hypertension (*ibid* 1977, **15**, 480). However, the results did not provide any evidence of the previously demonstrated associations between cadmium and respiratory disease (*ibid* 1977, **15**, 479; *ibid* 1978, **16**, 288) or cancer (*ibid* 1977, **15**, 479) or indicate the occurrence of bone disorders (*ibid* 1977, **15**, 478). The overall mortality rate in Shipham was well below the national average and the authors consider that although it is difficult to draw conclusions from the relatively small numbers involved in this study, it seems that "if cadmium contamination has any effect on the mortality pattern in Shipham it is slight and does not present a serious health hazard to the residents".

Cosmetic ingredients go further than skin deep

Marzulli, F. N., Anjo, D. M. & Maibach, H. I. (1981).

In vivo skin penetration studies of 2,4-toluenediamine, 2,4-diaminoanisole, 2-nitro-*p*-phenylenediamine, *p*-dioxane and *N*-nitrosodiethanolamine in cosmetics. *Fd Cosmet. Toxicol.* **19**, 743.

The presence in hair dyes and cosmetics of chemicals that have given positive results in lifetime animal feeding carcinogenicity studies has been reported previously (*Cited in F.C.T.* 1977, **15**, 423; *Federal Register* 1978, **43**, 16417; Reznik & Ward, *Fd Cosmet. Toxicol.* 1979, **17**, 493; *Federal Register* 1979, **44**, 23821). *N*-Nitrosodiethanolamine (NDELA) a contaminant of a variety of cosmetics has been shown to penetrate human skin, both excised (*ibid* 1979, **44**, 21365; *Cited in F.C.T.* 1982, **20**, 147) and *in vivo* (*ibid* 1981, **19**, 137). Marzulli *et al.* (cited above) now report the skin penetration capacity of five substances that are found in cosmetics and that have been shown to be carcinogenic in animal studies.

The substances tested were 2,4-toluenediamine (I), 2-nitro-*p*-phenylenediamine (II), NDELA (III), 2,4-diaminoanisole (IV) and *p*-dioxane (V). They were tested on the following sites: abdomens (I, II, IV) or forearms (II, IV) of adult rhesus monkeys; backs of immature Pitman-Moore white pigs (II, III); forearms of adult male human volunteers (I, IV). The substances were radiolabelled (^{14}C) and administered in acetone, methanol or a skin lotion. They were applied to uncovered skin at a dose of 4 μg test material/cm^2 skin over areas ranging from 3 to 15 cm^2. The areas were washed with soap and water 24 hr later and all the urine passed over a 5-day period was collected. Correction factors were obtained by measuring the urinary excretion of ^{14}C following parenteral injection of a known amount of the labelled material. These correction factors were used to account for any test material absorbed but not excreted. Skin penetration ranged from 2·3 $\pm$ 0·4% (mean $\pm$ 2SD) of the applied dose (*p*-dioxane in methanol on the monkey forearm) to 53·8 $\pm$ 15·4% (2,4-toluenediamine in acetone on the monkey abdomen). The skin penetration of 2,4-diaminoanisole and *p*-dioxane were similar (2–5% of the applied dose) while those of 2-nitro-*p*-phenylenediamine and NDELA were higher (ranging from 4–34% of the dose). The results suggested that man and the pig are generally more alike with regard to skin penetration than are man and the monkey, monkey skin being somewhat more permeable. It is difficult to make comparisons however since permeability varies between different areas of skin. With regard to comparison between compounds, it is predicted that skin penetration would be higher when the chemical in question is both water and lipid soluble. The low absorption of *p*-dioxane is explained by its relatively greater volatility causing loss of material by evaporation. Crude calculations using penetration levels estimated from this study and levels of ingredients/contaminants reported to be present in cosmetic formulations indicate absorptions (from 'appropriate use' by humans) of 60 μg *p*-dioxane per application of sunscreen lotion, 18 μg NDELA per hand-lotion application and 1·7 mg 2-nitro-*p*-phenylenediamine or 0·39 mg 2,4-diaminoanisole per hair-dye application. No estimate was made for 2,4-toluenediamine which is apparently no longer used in marketed hair dyes.

Skin sensitization in guinea-pigs and man

Marzulli, F. & Maguire, H. C., Jr (1982). Usefulness and limitations of various guinea-pig test methods in detecting human skin sensitizers—validation of guinea-pig tests for skin hypersensitivity. *Fd Chem. Toxic.* **20**, 67.

As part of the evaluation of the safety of new cosmetics, drugs and other products that will come into contact with human skin it is necessary to determine their potential for inducing allergic contact dermatitis. Traditionally the species of choice for the conduct of skin hypersensitivity tests has been the guinea-pig and while a number of prospective testing methods have been developed there is limited information concerning their usefulness in predicting skin sensitization potential in man. The authors cited above have made a large-scale comparison of test results obtained in guinea-pigs and in man, in an attempt to determine whether animal testing will predict the effects of a compound when used by man.

In the main study, five guinea-pig predictive test methods (the guinea-pig Draize test, the Buehler test, the Magnusson and Kligman maximization test (GPMT), the epicutaneous split-adjuvant test and the cyclophosphamide-induced immunopotentiation test) were used to evaluate 11 substances, most of which have been clinically identified as weak or negligible sensitizers. The results were compared with the findings of predictive tests in man. A subsidiary study involved a limited evaluation, using five of the compounds tested in the main study, of three non-occlusive techniques that did not use complete Freund's adjuvant (CFA). The results of these tests were compared with those from the standard Draize test and the GPMT.

The study points out the merits and limitations of each testing technique for evaluating weak contact sensitizers. The techniques that make use of CFA (GPMT, the epicutaneous split-adjuvant test and the cyclophosphamide-induced immunopotentiation test) showed significantly greater agreement with the findings of tests with humans than did the non-adjuvant techniques (the guinea-pig Draize test and the Buehler test). The GPMT emerged as the best of the adjuvant methods. The GPMT also performed best in the subsidary study.

On the basis of the results, the authors recommend that new compounds be tested first by the Draize guinea-pig technique and then if necessary by the GPMT. The final ranking for allergenicity would be based on the responses achieved from each model. Positive findings in the Draize guinea-pig test would suggest that the compound is a strong skin sensitizer. Negative findings in both the Draize and the GPMT would indicate that the chemical is not likely to be a significant sensitizer in man, while positive results in the GPMT but negative findings in the Draize test would suggest that the compound is likely to be a weak or moderate skin sensitizer in man.

[The authenticity of the technique described as the Buehler method in this study has been disputed (Buehler, *Fd Chem. Toxic.* 1982, **20**, 494) on the grounds that the animals were not restrained to ensure occlusivity of the test site and that specified

procedures to select the dose of and vehicle for the test substances were not followed. However Maguire & Marzulli (*ibid* 1982, **20**, 495) are confident that their study provided a valid comparison of various test methods, including the Buehler technique.]

Tween 80 immunosuppression

Barnett, J. B. (1981). Immunosuppressive effects of Tween 80 on mice. *Int. Archs Allergy appl. Immun.* **66**, 229.

Immunotoxicology is a comparatively new science which has been steadily attracting interest as more and more chemicals are shown to be capable of affecting the immunological systems. Tween 80 (polyoxyethylene sorbitan monooleate), an emulsifier, is yet another example of a commonly used chemical being implicated in the suppression of the immunological response. Barnett (cited above) reports that recent work in his laboratory (Barnett & Bryant, *Int. Archs Allergy appl. Immun.* 1980, **63**, 145; Bryant & Barnett, *ibid* 1979, **59**, 69) has suggested that the primary IgE and IgG_1 was suppressed in mice pretreated with Tween 80 followed by an immunizing dose of ovalbumin adsorbed to aluminium hydroxide (an antigen-adjuvant combination known to produce high levels of IgE and IgG_1). In the present study, the specificity of this suppression was further investigated by determining the effect of Tween 80 on antibody-producing cells (by using the haemolytic plaque technique) and on the cell-mediated response (as measured by contact hypersensitivity) in Balb C mice intraperitoneally injected on day −1 with either saline (controls) or Tween 80 emulsified in saline.

Those animals destined for the haemolytic plaque assay were immunized on day 0 by the intraperitoneal injection of 2×10^7 sheep erythrocytes (SRBC). On day 5, half of the animals in each group were assayed for the number of IgM antibody-producing cells in their spleens. The remainder were boosted with 2×10^7 SRBC on day 10 and assayed for the secondary IgG response which was quantified on day 14. Compared with the controls, mice pretreated with Tween 80 showed a statistically significantly lower primary IgM response. The treated animals also showed a decreased secondary IgG response, but the difference was not found to be statistically significant.

To investigate Tween 80's effect on cell-mediated immunity, mice were sensitized on day 0 by topical application to the shaved back of 25 μl of an 8% (w/v) solution of oxazolone (OX) in acetone. On day 5, the mice were challenged with 10 μg of a 0·1% OX solution by topical application to the right ear, and the immunological response to OX was then assessed for 3 days by measuring the increase in ear thickness. Control animals showed a slightly but not significantly greater increase in ear thickness than did the mice treated with Tween 80. The observation that the contact hypersensitivity response was not significantly diminished in the treated mice suggests that Tween 80 has no effect on the priming and triggering of T-effector cells.

The authors conclude from these findings that the immunosuppression caused by Tween 80 is restricted to the primary humoral response.

[The amount of Tween 80 required to produce the immunosuppressive effects is not given.]

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LETTERS TO THE EDITOR

PERSISTENCE OF TCDD IN MONKEY ADIPOSE TISSUE

Sir,—The highly toxic 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a potential environmental contaminant found in some herbicides and in incinerator effluents, is retained in the liver and fat in rats (Rose *et al. Toxic. appl. Pharmac.* 1976, **36**, 209), hamsters (Olson *et al. ibid* 1980, **56**, 78) and cattle (Jensen *et al. J. agric. Fd Chem.* 1981, **29**, 265) with a half-life of the order of weeks. However, in rhesus macaques, Van Miller *et al.* (*Fd Cosmet. Toxicol.* 1976, **14**, 31) observed that only a few percent of a single dose of tritiated TCDD appeared in the urine and faeces during the first week; they did not make later measurements.

We found easily measurable levels of TCDD in the adipose tissue of a rhesus macaque 2 yr after a preliminary experiment intended to explore short-term retention and excretion of unmetabolized TCDD and induction of hepatic aryl hydrocarbon hydroxylase (AHH) after a single oral dose. Although the findings in a single animal are at best indicative, the design of definitive experiments on the pharmacokinetics of TCDD in monkeys should take into account a probable prolonged retention in these animals.

An adult female rhesus macaque (*Macaca mulatta*) was given by stomach tube 1 μg TCDD (dissolved in corn oil)/kg body weight. At intervals thereafter, aryl hydrocarbon hydroxylase (AHH) activity was estimated in homogenates of needle biopsy samples of liver by the fluorometric method of Nebert & Gelboin (*J. biol. Chem.* 1968, **243**, 6242). The concentrations of TCDD in samples of fat, faeces and urine were measured by packed column gas chromatography–high resolution mass spectrometry using a method previously validated for beef adipose tissue (Gross *et al. Analyt. Chem.* 1981, **53**, 1902).

The monkey lost 50% of its body weight during the 3 months after the dose of TCDD, but at no time showed hair loss, swelling and reddening of the eyelids, or elevation of the nails—all characteristics of TCDD poisoning in rhesus macaques (Allen *et al. Fd Cosmet. Toxicol.* 1977, **15**, 401; McConnell *et al. Toxic. appl. Pharmac.* 1978, **43**, 175). Hepatic AHH activity was elevated on day 2, reached a peak on day 8, and was falling toward the pretreatment level on day 32 (Table 1).

The level of TCDD in the faeces was high for 4 days, then fell to very low or undetectable levels (Table 1); evidently a substantial fraction of the TCDD dose was not absorbed or was promptly excreted, unchanged, into the gut. No TCDD was detected in the urine except for small amounts in the first 2 days and in two later samples.

The concentration of TCDD in adipose tissue rose steadily during the 2 months after the dose (Table 1). However, the animal was losing weight, and the rise in TCDD in adipose tissue probably represented concentration in a shrinking fat depot. The animal was obese at the start of the experiment, and she lost 40% of her body weight during the 2 months after the dose. If this loss occurred

Table 1. *Body weight, concentrations of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in fat, urine, and faeces, and hepatic aryl hydrocarbon hydroxylase (AHH) activity in a female rhesus macaque given a single oral dose of 1 μg TCDD/kg body weight*

Days after dosing	Body weight (kg)	TCDD (ppt)* in				Hepatic AHH activity (% of baseline)
		Faeces	Urine	Adipose tissue	Liver	
0	7·4	ND (6)	ND (6)	ND (40)		100
1	—	28,900 (1200)	3·3 (2)	—		—
2	7·3	22,200 (740)	3·9 (3)	220 (60)	—	207
3	—	22,200 (450)	ND (3)	—	—	—
4	7·2	2030 (190)	ND (5)	340 (35)	—	244
6	—	ND (170)	ND (4)	—	—	—
8	6·9	ND (2)	2·4 (1)	450 (170)	—	264
11	—	ND (1)	1·1 (1)	—	—	—
16	6·5	100 (70)	ND (7)	500 (25)	—	151
22	—	33 (25)	ND (4)	—	—	—
32	5·8	ND (20)	ND (1)	890 (70)	—	132
45	—	21 (8)	ND (1)	—	—	—
64	4·4	—	—	2000 (95)	—	—
715	7·6	—	—	100 (10)	15 (10)	—

ND = Not detectable

*Values in parentheses indicate limits of detection. t = 10^{12}.

primarily in adipose tissue, total body fat was reduced roughly five-fold, and this factor corresponds to the increase in the concentration of TCDD in fat between days 4 and 64. After 2 yr, the body weight of the monkey was about the same as it was 4 days after the dose, and the concentration of TCDD in fat was roughly 25% of the day-4 level. Thus, the apparent half-life of TCDD in fat in this animal was about 1 yr.

Too little liver tissue was available from the needle biopsies for measurement of TCDD; only the larger sample taken at 2 yr was analysed. However, if the level of hepatic AHH activity is an indication of the amount of TCDD in the liver, much of the compound stored in that organ early on must have been soon excreted or translocated. This inference is in agreement with the finding of Van Miller *et al.* (*loc. cit.*) that 7 days after a dose of tritiated TCDD to rhesus macaques, most of the radioactive label was in fat-containing tissues (adipose tissue, skin and muscle), and not in the liver.

Furthermore, we speculate that the toxicity of TCDD for rhesus macaques does not correlate well with the amount sequestered in body fat, because the monkey began to gain weight 3 months after the dose and has been healthy since. The potential late adverse effects for rhesus macaques of long-term TCDD persistence in adipose tissue remain to be investigated.

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W. P. McNULTY,
Laboratory of Pathology,
Oregon Regional Primate Research Center,
Beaverton, OR 97006.

K. A. NIELSEN-SMITH,
Oregon Graduate Center,
Beaverton, OR 97006

and

J. O. LAY, JR. D. L. LIPPSTREU, N. L. KANGAS,
P. A. LYON and M. L. GROSS,
Department of Chemistry,
University of Nebraska,
Lincoln, NE 68588, USA.

MODIFIED STARCHES

Sir.—In the recent paper by Hodgkinson *et al.* (*Fd Chem. Toxic.* 1982, **20**, 371), the following statement is made (p. 381):

> "The more substituted starch used in this study (E1422; DS = 0·08) had, in the case of most of the measured parameters, more effect on mineral metabolism than the less substituted preparation (E1414; DS = 0·05), but this difference could be due to differing effects of phosphate and adipate substitution rather than to variations in the degree of substitution".

It should be noted that there are two types of substitution in these products. The predominant one is with acetyl or acetate substituents. In the case of E1414, the degree of substitution (DS) is 0·05, which corresponds to 1·76% acetate substitution. The 0·08 DS in E1422 corresponds to 2·79% acetate.

This is in contrast to the insignificant substitution levels found in crosslinking treatments involving the introduction of phosphate or adipate groups. Such groups are present at much lower levels of substitution than the acetate groups. The level of phosphate introduced in making the E1414 used in this study is estimated to run at about 0·038% and the level of adipate introduced in making E1422 is estimated to be 0·037% or less. This is only about 1/75 of the acetate substitution in E1422.

These levels of substituents introduced by crosslinking through phosphate or adipate moieties are insignificant in terms of their effect on digestibility and caecal enlargement, and probably on mineral metabolism. This is borne out by studies on distarch phosphate and on acetylated distarch adipate containing minimal levels of acetyl groups. *In vitro* studies have shown that both are digested to the same extent; *in vivo* studies have shown that both are similar to unmodified food starches in digestibility (Joint FAO/WHO Expert Committee on Food Additives, *WHO Fd Add. Ser.* 1974, no. 5).

As shown by Walker & El Harith (*Annls Nutr. Aliment.* 1978, **32**, 671), crosslinking within the limits permitted by regulation for food starch modified has no significant effect on caecal enlargement.

There is no evidence that the type of crosslink at the level found in food starch modified has any effect on digestibility or caecal enlargement.

In view of these points, it should be safe to conclude that the minor differences in those measured parameters noted by the authors are the effect of differences in the level of acetyl substitution, not the effects of the adipate or phosphate crosslink.

O. B. WURZBURG,
Advisor,
National Starch and Chemical Corp.,
Box 6500,
Bridgewater, NJ 08807, USA

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RAPID COMMUNICATION

TOXIC OIL SYNDROME IN SPAIN

W.N. Aldridge and T.A. Connors

MRC Toxicology Unit, MRC Laboratories, Woodmansterne Road, Carshalton, Surrey SM5 4EF, England

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INTRODUCTION

In May 1981 and the following months an epidemic of interstitial pneumonitis (20,000 cases) occurred in Spain. Following this a number approaching 1000 developed a disease syndrome of an unique type. The characteristic lesion is a widespread vasculitis with accompanying neurological, sclerodermal and other disorders. The patients with this condition show little or no recovery.

Over the last 17 months various reports, papers and abstracts have been prepared or have appeared in the open literature (see References).

In June 1981 an association between the pneumonia and the consumption of rapeseed oil (RSO) denatured with aniline was discovered (Tabuenca, 1981). Since then many laboratories in Spain and several in other countries have examined the toxicity of samples of oil to several species of animal and in various *in vitro* tests. From the time when the Spanish Government offered olive oil for suspect toxic oil, guaranteed authentic patient-related samples of oil have been extremely difficult to obtain. Also the "refining" process has not been defined with certainty, thus making it difficult to repeat the process.

EXPERIMENTAL STUDIES

The following is a brief statement of the results of some experiments carried out in these laboratories.

Toxicity of various rapeseed oils

Oil samples from Spain (ZQ, MC-1 and 48-P1-1905) and RSO purchased in the UK and refined after addition of 2% aniline were given in divided doses totalling 7-23 g/kg to rats, mice and hamsters. No signs of toxicity or changes in blood picture occurred at any dose level.

Toxicity of anilides

The anilides of formic, linoleic, erucic, oleic and linolenic acids have been synthesized and analysed by high-resolution capillary gas chromatography with flame ionization. The anilides were given orally to rats as a 1% solution in RSO (from the UK) in doses ranging from 500 to 1500 mg anilide/kg body weight. No signs of toxicity nor morphological changes that could be associated with the anilides were seen.

Analysis of oils

Analysis by high-resolution gas chromatography of the fatty acids derived from various samples of RSO from Spain in comparison with RSO and olive oil purchased in the UK indicated that the samples from Spain varied widely in composition. All the RSO from Spain contained higher concentrations of free fatty acids. The anilide content of the oils was also very variable, being in some as high as 1500 ppm and in some undetectable.

All of the four RSO samples from Spain gave, on the gas-chromatographic tracings, a number of peaks not seen in RSO or olive oil purchased in the UK, indicating substances present in concentrations below 200 ppm. These have not yet been identified.

Administration of RSO to rats deficient in vitamin E and selenium

A deficiency of vitamin E and selenium in cows on a diet rich in unsaturated fats leads to a condition called nutritional degenerative myopathy (McMurray & Rice, 1981). Even though the Spanish toxic syndrome does not appear to be a primary myopathy, others have suggested that lipid peroxidation of membranes is involved in the disease (Fournier, Efthymiou & Lecorsier, 1982; *New Scientist*, 1982; Pestana, 1982; Pestana & Munoz, 1982; Picot, 1982; WHO, 1981c). In an attempt to increase the sensitivity of the rat, its defences against peroxidation and free-radical attack were reduced by vitamin E and selenium deficiency. RSO, refined RSO, RSO + 2% aniline followed by refining and Spanish RSO (47-Pl-1868) were given to groups of rats either with established deficiencies or supplemented with vitamin E and selenium. Analysis showed that the deficient rats had 5-10% of normal plasma vitamin E and 10-20% of normal selenium. The oil from Spain was given orally at 0.1, 0.2 and 0.3 ml/day for wk 1, 2 and 3 respectively. The other oils were given at 5, 10 and 20% in the diet for wk 1, 2 and 3, respectively.

No signs of toxicity other than those due to vitamin E and selenium deficiency were observed. No histological abnormalities were seen that could be attributed to dosing with either the 'case-related' oil samples from Spain or with the refined rapeseed oil that had contained 2% aniline. Rats on a vitamin E and selenium-deficient diet regained their normal plasma-vitamin E status when fed with RSO preparations from the UK; those given the Spanish oil remained deficient.

Cell culture

In experiments in which RSO (a refined UK sample or a Spanish sample, either 47-Pl-1868 or 48-Pl-1905) was added to confluent and dividing HeLa cells, none of the oils (5 µg/ml) were toxic to confluent HeLa cells. The results with dividing cells were variable and it was concluded that this was due to the physical properties of oil-in-water emulsions, e.g. restriction of oxygen supplies to the cells.

CONCLUSIONS

There is at present no satisfactory experimental model of the disease. A recent report (Pestana & Munoz, 1982) has suggested that the rabbit may be a useful model, and that the disease in man is an eosinophil-mediated cytotoxicity probably directed by specific antibodies towards anilides that could be incorporated in the cellular membranes (Murphy & Vodyanoy, 1982). There is some evidence for an immunopathological mechanism (Tabuenca, 1981; Vicario, Serrano-Rios, San Andrés & Arnaiz-Villena, 1982) and there is some against (Brostoff, Blanca, Boulton & Serrano, 1982). The blood eosinophilia seems to be an early response during the involvement of the lung (Gilsanz, 1982). The latest report (Pestana & Munoz, 1982) suggests synergistic effects of anilides and peroxidative status resulting from the poor nutritional quality of the oil mixtures.

The experiments described here have not produced an experimental model for the human disease using synthetic anilides, oil samples from Spain or RSO from the UK untreated, refined or refined after addition of 2% aniline. The experiments with vitamin E- and selenium-deficient rats, although yielding a few interesting findings, have not resulted in any serious signs of toxicity.

The unusual nature of the disease (Gilsanz, 1982; Ministerio de Sanidad, 1981; Tabuenca, 1981) makes it very likely that even isolated cases would have been recognized before this epidemic (M. Serrano-Rios, personal communication 1982). It also seems likely that refined RSO denatured with aniline has been consumed without any apparent ill effect for some years prior to this episode, although it has proved difficult to obtain evidence for or against this. The distribution of the early cases of poisoning (Fig. 1) suggests the possibility that they all arose from one batch of oil or from one method of refining.

Taking all these observations together it is at least possible that the refining of aniline-denatured RSO is not a toxic process in itself but requires some further contamination to produce a mixture responsible for the unique disease syndrome in humans. If one or more components of the mixture were of limited stability, the mixture might initially be highly toxic but might lose its toxicity over the months following the start of the epidemic. If true this is obviously relevant to the present testing of stored oils from Spain.

If there is truth in these suggestions, then there is urgent need for a reappraisal of the epidemiological data (Aldridge, 1982). Further investigations are necessary to test the "one batch and another chemical contaminant" hypothesis, to establish whether aniline-denatured RSO had been consumed in Spain prior to the epidemic, to establish a common aetiology between the early lung involvement and later manifestations of the disease and to obtain the maximum information available on dose-response for the early and late clinical findings.

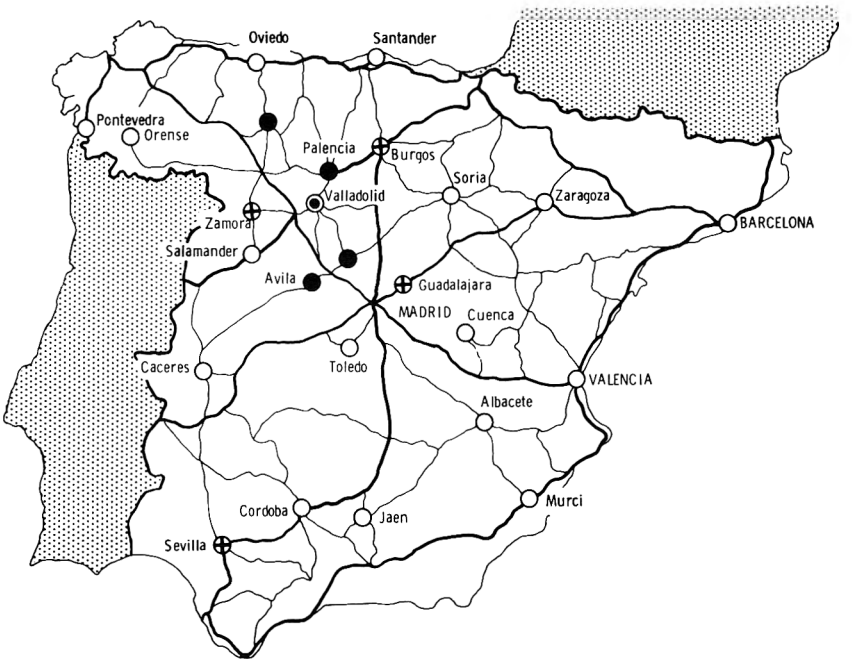

Fig. 1. Distribution of cases up to 30 June 1981; ⊙, 798; ●, 242-487; ⊕, 65-142; ○, 1-25 (WHO, 1981a).

In recent years international interest in the toxicological consequences of exposure to chemicals has increased, largely stimulated by serious accidents. When the exposure of man to chemicals leads to serious disease, the international scientific community should be mobilized in order to expand knowledge about the mechanisms of toxic reactions in man.

After an accident, *rapid* action is required to involve scientists who can (1) identify and quantify exposure and dosimetry and (2) advise on immediate and long-term biological and chemical parameters to be followed up in exposed man and animals. The European Medical Research Council and its Advisory Subgroup in Toxicology have recently provided a list of scientists in many European countries who are willing to act quickly and identify the scientists best suited for the particular accident. This action followed by an effective co-ordination of the resulting research should be actively supported and effected by the international scientific community concerned with toxicology.

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MEETING ANNOUNCEMENTS

FOOD CHEMISTRY CONFERENCE

A conference entitled "Euro Food Chem II" is to be held at the National Research Council, Rome, Italy on 15–18 March 1983. The subject of the conference will be the elucidation of chemical reactions that occur during food processing and storage. Further information can be obtained from Euro Food Chem II, Società Italiana di Scienza dell' Alimentazione, Viale Monte Oppio, 24-I-00124 Rome, Italy.

SEMINAR ON PERCUTANEOUS PENETRATION

A seminar on the percutaneous penetration of drugs, cosmetics and potentially toxic agents, sponsored by the FDA and the Society of Cosmetics Chemists, will take place in Washington, DC on 27 April 1983. The major topics for discussion will be methodology, the relevance of experimental methods, the choice of animal models, vehicle effects, and theory. Further details are available from either Dr R. Bronaugh, Food and Drug Administration, HFF-164, 200 "C" Street, S.W., Washington, DC 20204, or Dr H. Maibach, University of California Medical School, San Francisco, CA 94143, USA.

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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 Chemical Toxicology*:

Characterization of impurities in commercial lots of sodium saccharin produced by the Sherwin–Williams Process. I. Chemistry. By R. M. Riggin and G. W. Kinzer.

Characterization of impurities in commercial lots of sodium saccharin produced by the Sherwin–Williams Process. II. Mutagenicity. By R. M. Riggin, W. L. Margard and G. W. Kinzer.

Oral daily intake of cadmium, lead, manganese, copper, chromium, mercury, calcium, zinc and arsenic in Belgium: a duplicate meal study. By J. P. Buchet, R. Lauwerys, A. Vandevoorde and J. M. Pycke.

Metabolic profile of caecal micro-organisms from rats fed indigestible plant cell-wall components. By I. R. Rowland, A. Wise and A. K. Mallett.

Protection by indole-3-carbinol against covalent binding of benzo[*a*]pyrene metabolites to mouse liver DNA and protein. By H. G. Shertzer.

Changes in preneoplastic response to aflatoxin B_1 in rats fed green beans, beets or squash. By J. N. Boyd, N. Misslbeck and G. S. Stoewsand.

Indirect immunoperoxidase localization of aflatoxin B_1 in rat liver. By J. J. Pestka, J. T. Beery and F. S. Chu.

Distribution of ^{14}C-labelled acrylamide and betain in foetuses of rats, rabbits, beagle dogs and miniature pigs. By G. J. Ikeda, E. Miller, P. P. Sapienza, T. C. Michel, M. T. King, V. A. Turner, H. Blumenthal, W. E. Jackson III and S. Levin.

Promoting effects of various chemicals in rat urinary bladder carcinogenesis initiated by *N*-nitroso-*n*-butyl-(4-hydroxybutyl)amine. By S. Fukushima, A. Hagiwara, T. Ogiso, M. Shibata and N. Ito.

N-Nitroso-*N*-methyloctadecylamine in hair-care products. By J. B. Morrison, S. S. Hecht and J. A. Wenninger.

Subchronic feeding study of grape colour extract in beagle dogs. By P. J. Becci, F. G. Hess, M. A. Gallo, W. D. Johnson and J. G. Babish (Short paper)

Reproduction study of grape colour extract in rats. By P. J. Becci, F. G. Hess, J. G. Babish, M. A. Gallo and K. A. Voss. (Short paper)

Subchronic feeding study of carnauba wax in beagle dogs. R. A. Parent, G. E. Cox, J. G. Babish, M. A. Gallo, F. G. Hess and P. J. Becci. (Short paper)

Reproduction and subchronic feeding study of carnauba wax in rats. By R. A. Parent, T. A. Re, J. G. Babish, G. E. Cox, K. A. Voss and P. J. Becci. (Short paper)

Effects of subchronic feeding of ginseng extract G115 in beagle dogs. By F. G. Hess, Jr. R. A. Parent, K. R. Stevens, G. E. Cox and P. J. Becci. (Short paper)

The effect of intestinal esterase inhibition on the *in vivo* absorption and toxicity of *di-n*-butyl phthalate. By R. D. White, D. L. Earnest and D. E. Carter. (Short paper)

The prophylactic reduction of aluminium intake. By A. Lione. (Review paper)

[*Contents continued*]

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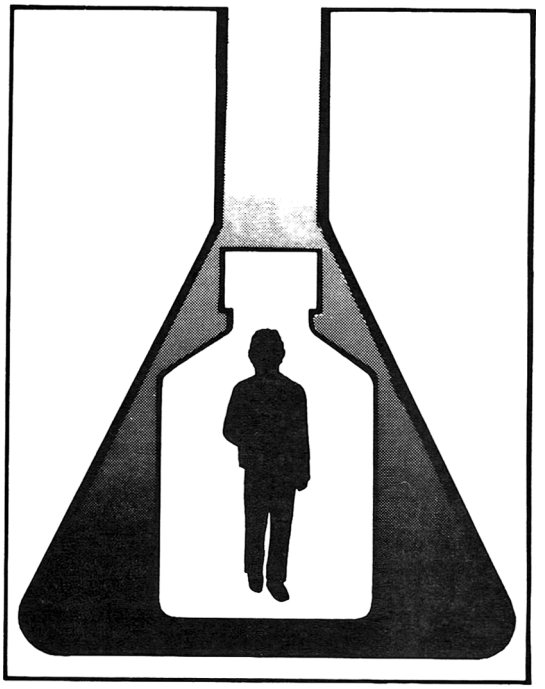

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