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Research Section

ENHANCEMENT OF 1,2-DIMETHYLHYDRAZINE-INDUCED COLON CARCINOGENESIS IN MICE BY DIETARY AGAR

H. P. GLAUERT and M. R. BENNINK

Department of Food Science and Human Nutrition

and

C. H. SANDER

Department of Pathology, Michigan State University, East Lansing, MI 48824, USA

(Received 12 July 1980)

Abstract—The effect of dietary agar on colon carcinogenesis was investigated. The frequency and number of tumours of the colon induced by subcutaneous injections of 1,2-dimethylhydrazine (DMH) was examined in male CF_1 mice fed different levels of agar and fat in the diet. The mice were fed one of four diets: a low-fat, low-fibre control diet, the control diet with added agar, a high-fat diet, or a high-fat, agar-containing diet. The mice were injected with 20 mg DMH/kg body weight weekly for 20 wk. Controls were injected with saline. The mice fed agar had significantly more colon tumours per animal than those fed diets that did not contain agar and there was a slightly increased frequency of malignant tumours in agar-fed mice compared with that in mice given the control diet. Feeding high-fat diets only slightly increased the incidence of malignant tumours and did not significantly affect the number of tumours per animal. No tumours occurred in mice injected with saline. Since it has been suggested that faecal steroids have a co-carcinogenic role in colon carcinogenesis, faecal bile acids and neutral sterols were determined. Feeding agar decreased the faecal neutral sterol and bile-acid concentrations, whereas feeding high-fat diets did not affect the faecal bile-acid concentrations and increased faecal neutral sterol concentrations only when agar was also in the diet. The results of this study show that dietary agar enhances DMH-induced colon carcinogenesis in mice even though the level of faecal steroids is lowered.

INTRODUCTION

Agar, a gum which forms a firm gel at low concentrations, is used in food products as a gelling agent and is used pharmaceutically as a laxative (Selby & Wynne, 1973). Agar is a sulphated polysaccharide derived from the red-purple seaweeds (class Rhodophyceae) and consists primarily of galactose and 3,6-anhydrogalactose, along with glucuronic acid and a significant proportion of half-ester sulphates (Cummings, 1976; Selby & Wynne, 1973). Agar is not digested by humans and thus may be considered a dietary fibre (Cummings, 1976).

Although agar is consumed regularly by humans, there have been few studies on the effects of feeding agar to experimental animals. The effect of dietary agar on cholesterol metabolism has been investigated (Kelley & Tsai, 1978; Tsai, Elias, Kelley, Lin & Robson, 1976). However, studies to examine the effect of agar on intestinal parameters are scarce. Watt & Marcus (1978) showed that Danish agar, after degradation by acid, induces ulcerative colitis when fed to guinea-pigs.

Carrageenan, another sulphated polysaccharide derived from a seaweed, has deleterious effects on the colon (Watt & Marcus, 1973). Degraded and native carrageenan induce ulcerative colitis when administered orally to several species (Grasso, Sharratt, Carpanini & Gangolli, 1973; Watt & Marcus, 1973). Also, degraded carrageenan induces colorectal tumours (Wakabayashi, Inagaki, Fujimoto & Fukuda, 1978) and rectal squamous metaplasia (Fabian, Abraham, Coulston & Golberg, 1973) when fed to rats, while native carrageenan enhances colon carcinogenesis induced by methylnitrosourea (MNU) and azoxymethane (AOM) in rats (Watanabe, Reddy, Wong & Weisburger, 1978).

Because of the structural similarity between agar and carrageenan, we investigated the effect of dietary agar on 1,2-dimethylhydrazine(DMH)-induced colon carcinogenesis in CF_1 mice fed two different levels of dietary fat. DMH induces colon tumours organospecifically after injection into experimental animals (Deschner & Long, 1977; Newberne & Rogers, 1973). The tumours induced are histologically similar to colon tumours seen in humans (Deschner & Long, 1977; Newberne & Rogers, 1973). We also determined the levels of faecal neutral sterols and bile acids to learn if the effect of agar may be related to the effects of these chemicals, since faecal steroids have been hypothesized to play a co-carcinogenic role in colon carcinogenesis (Reddy, Weisburger &Wynder, 1978). We found that agar enhanced DMH-induced colon carcinogenesis and decreased faecal sterol concentrations.

EXPERIMENTAL

Chemicals. Agar (bacto-agar) was obtained from Difco Laboratories, Detroit, MI; DMH was obtained

from Aldrich Chemical Co., Milwaukee, WI; bile-acid and neutral sterol standards were obtained from Steraloids, Inc., Wilton, NH.

Colon carcinogenesis. Four-week-old male CF_1 mice (ARS Sprague–Dawley, Madison, WI) were housed individually in stainless-steel hanging wire cages. The animal room was temperature- and humidity-controlled and a 12-hr light–dark cycle was used. Mice were randomly assigned to one of four groups of 28 mice each. Each group was given one of four diets (Table 1): (1) fibre-free control diet; (2) agar diet, consisting of the control diet plus 8% added agar (i.e. 8 g of agar were added to 100 g of the control diet); (3) high-fat diet; (4) a high-fat, agar diet, consisting of the high-fat diet plus 8% added agar. Each diet had the same energy:protein ratio (we assumed that no calories were derived from the agar). Vitamins were added to the diet to meet at least 150% of the National Academy of Sciences/National Research Councils requirements (NAS/NRC, 1972). Water and food were provided *ad lib.*

After they had been fed the diets for 1 wk, the mice were given weekly sc injections of DMH. Before injection, DMH dihydrochloride (1 mg/ml) was dissolved in saline, and the pH was adjusted to 6·8. Twenty-three mice in each dietary group were injected with 20 mg DMH dihydrochloride/kg body weight for 20 wk. The remaining 5 mice in each group were given an equivalent amount of saline. The mice were weighed weekly during the experiment. The mice were killed by cervical dislocation 21 wk after the start of the experiment. The large intestine from the ileocaecal valve to the anus was opened, examined visually for lesions, and placed in 10% buffered, neutral formalin. All suspected tumours were removed, embedded in paraffin wax, sectioned at six μm, and then stained with haematoxylin and eosin for microscopic examination. The lesions were classified according to the degree of cellular hyperplasia, dysplasia, or anaplasia and to the presence or absence of spread below the basement membrane. Data were analysed using the chi-square test for tumour incidence and analysis of variance for tumour number.

Faecal neutral sterol and bile-acid analysis. Twenty-four hour faecal collections for neutral sterol and bile-acid analysis were made in wk 10 and 11 after the start of DMH injections. Neutral sterols were extracted from dried faecal samples from each mouse by a slight modification of the procedure described by Evrard & Janssen (1968). Sterols were extracted with petroleum ether after saponification with 20% KOH in ethylene glycol. [^{3}H]Cholesterol was added as an internal standard to account for incomplete recoveries and 5α-cholestane was added to the extracted sterols as an internal standard for gas–liquid chromatography (GLC). The neutral sterols were silylated (Grundy, Ahrens & Miettinen, 1965) and the trimethyl silylyl (TMS) ethers were separated by GLC with a Varian Model 3700 gas chromatograph equipped with a flame ionization detector. The GLC conditions were as follows: injector temperature, 250°C; detector temperature 270°C; column temperature, 255°C. The neutral sterols were separated on a 1·8-m long, 3·2-mm diameter stainless-steel column packed with 3% OV-17 on 100/120 Gas Chrom Q. Cholesterol, coprostanol and coprostanone standards were silylated and chromatographed using 5α-cholestane as an internal standard. The sterols were quantitated with a Varian CDS 111 integrator.

Bile acids were extracted from faeces as described by Grundy *et al.* (1965). The faecal pellets from four or five mice were pooled for analysis. [^{14}C]Glycocholic acid was added as an internal standard to account for incomplete recoveries. After extraction, bile acids were separated from fatty acids by the method of Makita & Wells (1963). Bile acids were methylated by the addition of excess etheral diazomethane. The methyl esters of the bile acids were then dried, silylated and separated by GLC. 5α-Cholestane was used as the internal standard for GLC. GLC con-

Table 1. *Composition of diets*

Dietary component	Level of component (g/100 g diet) in			
	Control diet	Control + agar diet	High-fat diet	High-fat + agar diet
Casein	20·0	18·5	25·0	22·7
Methionine	0·3	0·3	0·4	0·3
Safflower oil	2·0	1·8	2·5	2·3
Vitamin mixture*	1·2	1·1	1·5	1·4
Mineral mixture†	6·0	5·6	7·5	6·8
Glucose	70·5	65·3	43·1	39·2
Agar	—	7·4	—	9·1
Tallow	—	—	20·0	18·2
Total ingredients ...	100·0	100·0	100·0	100·0
kcal g protein ...	18·7	18·8	18·8	18·8

*The vitamin mixture contained (per 100 g of mix): retinol acetate, 8333 IU; vitamin D, 2125 IU; α-tocopherol acetate, 330·0 mg; choline, 16·6 g; thiamin-HCl, 50·0 mg; riboflavin, 62·5 mg; pyridoxine, 20·8 mg; niacin, 150·0 mg; biotin, 4·2 mg; folic acid, 100·0 mg; vitamin B_{12}, 83·3 μg; inositol, 25·0 mg; *p*-aminobenzoic acid, 166·7 mg; menadione, 100·0 mg; ascorbic acid 416·7 mg.

†The mineral mixture contained (g per 100 g of mix): calcium acetate H_2O, 6·23; calcium diphosphate $2H_2O$, 28·28; dipotassium phosphate, 28·19; ferric citrate $5H_2O$, 2·42; magnesium sulphate $7H_2O$, 9·97; zinc sulphate, 0·30; potassium iodide, 0·64; sodium diphosphate $12H_2O$, 14·50; sodium chloride, 9·46.

Table 2. *Incidence and classification of tumours of the colon in CF_1 mice injected with 1,2-dimethylhydrazine and fed high- or low-fat diets with or without added agar for 21 wk*

Parameter	Results for mice fed			
	Control diet	Control + agar diet	High-fat diet	High-fat + agar diet
Percentage of mice with colon tumours	77	73	86	95*
Percentage of mice with malignant colon tumours	50	68	68	77
Mean no. colon tumours/mouse†	1·9	4·2	2·3	6·1
Mean no. colon tumours/tumour-bearing mouse†	2·4	5·7	2·7	6·4
Mean no. colon tumours/tumour-bearing mouse classified as:				
Hyperplasia	0·1			0·1
Dysplasia	0·8	0·6	0·9	1·2
Adenocarcinoma†	1·5	5·1	1·8	5·1

Significantly different ($P < 0·05$ by chi-square test) from the corresponding value for mice fed the control + agar diet.

†Mice that were fed diets that contained agar had significantly ($P < 0·05$ by analysis of variance) more tumours than those that were given diets without added agar.

The values are for groups of 23 mice. Five other mice in each dietary group were injected with saline and no tumours of the colon developed in any of these mice.

ditions were the same as above except that the column temperature was held initially at 210°C for 10 min, then increased by 12°C/min to 270°C and held at 270°C until the last TMS ether was eluted. The bile-acid TMS ethers were separated on a 1·8-m long, 3·2-mm diameter stainless-steel column packed with 3% SP-2100 on 100/120 Supelcoport. We have reported bile acids as the sum of all bile acids rather than as individual bile acids because of the possible oxidation of hydroxyl groups while drying the faecal samples and because of the production of artefacts during alkaline hydrolysis (Lepage, Fontaine & Roy, 1978). The faecal sterol data were analysed using standard analysis of variance statistics.

RESULTS

Mice that were injected with DMH gained less weight during the experiment than controls; mice that were fed high-fat diets gained significantly ($P < 0·05$) more weight than mice fed low-fat diets. The addition of agar to the diet did not affect weight gain.

Colon carcinogenesis

Only animals given DMH injections developed colorectal tumours (Table 2). The tumour incidence in the mice fed the high-fat, agar diet was significantly ($P < 0·05$) greater than the tumour incidence in the mice fed the agar diet. The tumour incidence among

Table 3. *Neutral sterol and bile-acid concentrations in faeces of CF_1 mice fed high- or low-fat diets with or without added agar for 21 wk*

Type of diet	Total neutral sterol concn (mg/g faeces)	Degradation of neutral sterols (%)*	Total bile-acid concn (mg/g faeces)	Faecal wt (g/day)
DMH-treated mice				
Control	14·4	29	22·1	0·23
Control + agar	2·9†	30	12·0†	0·79
High-fat	14·4	35	14·4	0·45
High-fat + agar	6·4†‡	21	14·4†	0·92
Saline-treated mice				
Control	14·2	31	27·6	0·24
Control + agar	2·8†	29	11·5†	0·61
High-fat	13·7	26	24·2	0·36
High-fat + agar	6·8†‡	23	12·8†	0·82

DMH = 1,2-Dimethylhydrazine

*Neutral sterol degradation was calculated as follows: (coprostanol + coprostanone)/(cholesterol + coprostanol + coprostanone) × 100.

†There was a significant difference ($P < 0·05$ by analysis of variance) between all of the values for rats given diets containing agar and all of those for rats given diets without agar.

‡Significantly different (‡$P < 0·05$ by analysis of variance) from corresponding value for mice fed the low-fat, agar-containing diet.

Values are means for groups of 23 (DMH-treated) or five (saline-treated) mice.

the other dietary groups was similar. The incidence of malignant tumours was slightly greater ($P < 0.2$) in mice fed the agar or high-fat diets compared with that in mice fed the control diet; however, there were no significant differences in the incidence of malignant tumours between mice given the high-fat, agar diet, the agar diet or the high-fat diet. The number of tumours per mouse and the number of tumours per tumour-bearing mouse were not increased in the mice fed high-fat diets (compared with mice fed low-fat diets) but were increased significantly ($P < 0.05$) in the agar-fed mice. All malignant tumours were adenocarcinomas, and were confined to the epithelial layer (carcinoma *in situ*). Dysplastic and hyperplastic lesions were also seen. The dysplastic group was considered premalignant whereas the hyperplastic lesions were considered to be an initial response to DMH. Most tumours were found in the lower half of the colon and were often located in close proximity to lymphoid nodules.

Faecal neutral sterol and bile acid analysis

Feeding agar had the general effect of decreasing faecal neutral sterol concentrations (Table 3). We have reported neutral sterol concentrations as the sum of all the neutral sterols since degradation of neutral sterols was similar between groups. Faecal neutral sterol and bile-acid concentrations were significantly ($P < 0.05$) reduced in mice fed agar. Neutral sterol concentrations were significantly ($P < 0.05$) higher in agar-fed mice on high-fat diets compared with those in agar-fed mice on low-fat diets. However, the fat content of the diet did not affect neutral sterol concentrations in mice fed agar-free diets. The dietary fat level did not affect faecal bile-acid concentrations.

DISCUSSION

This experiment shows that dietary agar enhances the development of DMH-induced colon tumours in male CF_1 mice. Although agar-fed mice showed only a slight increase ($P < 0.2$) in the incidence of malignant tumours, the number of tumours per mouse, number of tumours per tumour-bearing mouse, and number of adenocarcinomas per tumour-bearing mouse were all significantly greater ($P < 0.05$) in mice that were fed agar than in those that were not. The increase in the malignant tumour incidence in agar-fed mice is relatively modest, but the two-fold increase in tumour number is similar to significant differences reported in other studies of the effect of diet on colon carcinogenesis. For example, Reddy, Weisburger & Wynder (1974) reported an increase from 0·78 to 1·63 tumours per rat when the dietary fat level was increased from 4 to 20%, and Barbolt & Abraham (1978) found that dietary bran significantly decreased the number of tumours per rat from 6·4 to 1·8 in DMH-induced colon carcinogenesis.

The effect of other dietary fibres on chemically-induced colon carcinogenesis varies with the particular fibre. Carrageenan, as stated earlier, enhances both AOM- and MNU-induced colon carcinogenesis (Watanabe *et al.* 1978). The role of certain dietary fibres in chemically-induced colon carcinogenesis is unclear. Dietary alfalfa enhances MNU-induced colon carcinogenesis, but does not affect AOM-induced colon carcinogenesis (Watanabe, Reddy, Weisburger & Kritchevsky, 1979). Pectin inhibits AOM-induced colon carcinogenesis (Watanabe *et al.* 1979), does not affect MNU-induced colon carcinogenesis (Watanabe *et al.* 1979), and enhances DMH-induced colon carcinogenesis (Bauer, Asp, Oste, Dahqvist & Fredlund, 1980). Other dietary fibres have been shown to inhibit chemically-induced colon carcinogenesis. Dietary wheat bran has been shown to inhibit chemically-induced colon carcinogenesis in several studies (Barbolt & Abraham, 1978; Watanabe *et al.* 1979; Wilson, Hutcheson & Widerman, 1977). Dietary cellulose has been shown to inhibit DMH-induced colon carcinogenesis in two studies (Freeman, Spiller & Kim, 1978; Spiller, Kim & Freeman, 1980). However, Ward, Yamamoto & Weisburger (1973) found that dietary cellulose did not affect AOM-induced colon carcinogenesis. Dietary fibres are thought to inhibit colon carcinogenesis by reducing the concentration of bile acids, neutral sterols, or other materials in the colon. Several bile acids have been shown to act as tumour promoters in the rat colon (Narisawa, Magadia, Weisburger & Wynder, 1974; Reddy, Narisawa, Weisburger & Wynder, 1976; Reddy, Watanabe, Weisburger & Wynder, 1977). However, dietary agar also lowered the faecal neutral sterol and bile-acid concentrations. Therefore, dietary agar must have enhanced tumorigenesis by another mechanism.

Dietary agar may enhance colon carcinogenesis by acting directly on the colon mucosa. Using scanning electron microscopy, we have obtained preliminary evidence that dietary agar produces surface irregularities in rat colon mucosa (Glauert, Sander, Sanger & Bennink, 1979). These changes in the epithelial surface could allow faecal constituents that may be co-carcinogens or promoters (such as bile acids) to enter the cell in greater amounts. Cholestyramine, an ion-exchange resin that when fed to rats has been shown by scanning electron microscopy to produce discontinuities in the epithelial layer of the colon (Cassidy, Grund, Lightfoot, Vahouny, Gallo, Kritchevsky, Story & Treadwell, 1978), also enhances chemically-induced colon carcinogenesis (Nigro, Bhadrachari & Chomchai, 1973).

In the present study mice that were fed the high-fat diet had only a slightly higher ($P < 0.2$) malignant tumour incidence and had no significant change in tumour number compared with mice fed the low-fat diet. This finding is different from those of several other studies, in which it has been shown that chemically-induced colon carcinogenesis is enhanced when the fat content of the diet is raised. Reddy *et al.* (1975) found that increasing the dietary fat level from 0·5 or 5 to 20% enhanced DMH-induced colon carcinogenesis. The type of fat (animal *v.* vegetable) does not alter the enhancing effect of high levels of dietary fat (Reddy, Narisawa, Vakusich, Weisburger & Wynder, 1976), and high-fat diets enhance colon carcinogenesis when any one of three carcinogens—DMH, MNU, or methylazoxymethanol acetate—is used (Reddy, Watanabe & Weisburger, 1977). Nigro, Singh, Campbell & Pak (1975) showed that adding beef fat to a stock diet enhances AOM-induced colon carcinogenesis. In our experiment, the observation that high-fat diets did not raise faecal neutral sterol and bile-acid concentrations

may explain why the high-fat diets did not enhance colon carcinogenesis. Also, we isocalorically substituted fat for carbohydrate, whereas in other studies fat was substituted for carbohydrate on a weight basis (gram for gram) or fat was added to a stock diet.

Another possible reason for differences between the results of our study and previous studies is that we used mice whereas most other studies have been done using rats. The induction of colon tumours in mice by DMH has been less well studied than in rats, especially with regard to the effects of nutrition on DMH-induced colon carcinogenesis. The induction of tumours in mice by DMH appears to be strain-specific. CF_1 (Deschner & Long, 1977; Thurnherr, Deschner, Stonehill & Lipkin, 1973), Swiss (Toth, Malick & Shimizu, 1976), BALB/c (Clapp, Bowles, Satterfield & Klima, 1979) and ICR/Ha (Evans, Hauschka & Mittleman, 1974) mice are susceptible to DMH-induced colon carcinogenesis, whereas DBA/2 and C57BL/Ha mice are resistant to DMH-induced colon carcinogenesis (Evans *et al.* 1974). In CF_1 mice, DMH induces adenocarcinomas and areas of dysplasia in the colon (Deschner & Long, 1977). In rats, DMH induces adenomas and adenocarcinomas in the colon (Newberne & Rogers, 1973; Sunter, Appleton, Wright & Watson, 1978). Although studies examining the effects of butylated hydroxyanisole and butylated hydroxytoluene on chemically-induced colon carcinogenesis have been done with mice (Clapp *et al.* 1979; Wattenberg & Sparnins, 1979), all previous studies of the effects of fat, fibre and protein have been done with rats.

Several conclusions can be drawn from the present study. First, dietary agar enhanced DMH-induced colon carcinogenesis in male CF_1 mice: the tumour incidence was slightly higher and the number of tumours per mouse was doubled in mice fed agar. Second, agar's effect was not mediated by neutral sterols or bile acids—the faecal concentrations of these metabolites were lower in mice fed agar.

In this study we have shown that the ingestion of agar can cause adverse effects in the colon. Therefore, the consumption of agar by humans may be deleterious, and the biological effects of agar should be examined further. Also, the results of this study further point out the heterogeneity of dietary fibres. Dietary fibres should not be treated as a homogeneous group, particularly since components and substituents of the fibre determine their biological properties.

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REFERENCES

Barbolt, T. A. & Abraham, R. (1978). The effect of bran on dimethylhydrazine-induced colon carcinogenesis in the rat. *Proc. Soc. exp. Biol. Med.* **157,** 656.

Bauer, H. G., Asp, N. G., Oste, R., Dahlqvist, A. & Fredlund, P. E. (1980). Effect of two kinds of pectin and guar gum on 1,2-dimethylhydrazine induced colon tumors in the rat. *Eur. Surg. Res.* **12,** 41.

Cassidy, M. M., Grund, B., Lightfoot, F., Vahouny, G., Gallo, L., Kritchevsky, D., Story, J. & Treadwell, C. (1978). Alterations in topographical ultrastructure of rat jejunum and colon induced by feeding with alfalfa and cholestyramine. *Fedn Proc. Fedn Am. Socs exp. Biol.* **37,** 543.

Clapp, N. K., Bowles, N. D., Satterfield, L. C. & Klima, W. C. (1979). Selective protective effect of butylated hydroxytoluene against 1,2-dimethylhydrazine carcinogenesis in BALB/c mice. *J. natn. Cancer Inst.* **63,** 1081.

Cummings, J. H. (1976). What is fiber? In *Fiber in Human Nutrition.* Edited by G. A. Spiller & R. J. Amen. p. 1. Plenum Press, New York.

Deschener, E. E. & Long, F. C. (1977). Colonic neoplasms in mice produced with six injections of 1,2-dimethylhydrazine. *Oncology* **34,** 255.

Evans, J. T., Hauschka, T. S. & Mittleman, A. (1974). Differential susceptibility of four mouse strains to induction of multiple large-bowel neoplasms by 1,2-dimethylhydrazine. *J. natn. Cancer Inst.* **52,** 999.

Evrard, E. & Janssen, G. (1968). Gas–liquid chromatographic determination of human fecal bile acids. *J. Lipid Res.* **9,** 226.

Fabian, R. J., Abraham, R., Coulston, F. & Golberg, L. (1973). Carrageenan-induced squamous metaplasia of the rectal mucosa in the rat. *Gastroenterology* **65,** 265.

Freeman, H. J., Spiller, G. A. & Kim, Y. S. (1978). A double-blind study on the effect of purified cellulose dietary fiber on 1,2-dimethylhydrazine-induced rat colonic neoplasia. *Cancer Res.* **38,** 2912.

Glauert, H. P., Sander, C. H., Sanger, V. L. & Bennink, M. R. (1979). Influence of dietary agar and tallow on colon carcinogenesis. *Fedn Proc. Fedn Am. Socs. exp. Biol.* **38,** 714.

Grasso, P., Sharratt M., Carpanini, F. M. B. & Gangolli, S. D. (1973). Studies on carrageenan and large-bowel ulceration in mammals. *Fd Cosmet. Toxicol.* **11,** 555.

Grundy, S. M., Ahrens, E. H., Jr & Miettinen, T. A. (1965). Quantitative isolation and gas–liquid chromatographic analysis of total fecal bile acids. *J. Lipid Res.* **6,** 397.

Kelley, J. J. & Tsai, A. C. (1978). Effect of pectin, gum arabic and agar on cholesterol absorption, synthesis, and turnover in rats. *J. Nutr.* **108,** 630.

Lepage, G., Fontaine, A. & Roy, C. C. (1978). Vulnerability of keto bile acids to alkaline hydrolysis. *J. Lipid Res.* **19,** 505.

Makita, M. & Wells, W. W. (1963). Quantitative analysis of fecal bile acids by gas–liquid chromatography. *Analyt. Biochem.* **5,** 523.

Narisawa, T., Magadia, N. E., Weisburger, J. H. & Wynder, E. L. (1974). Promoting effect of bile acids on colon carcinogenesis after intrarectal instillation of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in rats. *J. natn. Cancer Inst.* **53,** 1093.

National Academy of Sciences/National Research Council (1972). *Nutrient Requirements of Laboratory Animals No. 10.* 2nd Ed. NAS/NRC, Washington, DC.

Newberne, P. M. & Rogers, A. E. (1973). Animal model: DMH-induced adenocarcinoma of the colon in the rat. *Am. J. Path.* **72,** 541.

Nigro, N. D., Bhadrachari, N. & Chomchai, C. (1973). A rat model for studying colonic cancer: effect of cholestyramine on induced tumors. *Dis. Colon Rectum* **16,** 438.

Nigro, N. D., Singh, D. V., Campbell, R. L. & Pak, M. S. (1975). Effect of dietary beef fat on intestinal tumor formation by azoxymethane in rats. *J. natn. Cancer Inst.* **54,** 439.

Reddy, B. S., Narisawa, T., Vukusich, D., Weisburger, J. H. & Wynder, E. L. (1976). Effect of quality and quantity of dietary fat and dimethylhydrazine on colon carcinogenesis in rats. *Proc. Soc. exp. Biol. Med.* **151,** 237.

Reddy, B. S., Narisawa, T., Weisburger, J. H. & Wynder, E. L. (1976). Promoting effect of sodium deoxycholate on colon adenocarcinomas in germ-free rats. *J natn. Cancer Inst.* **56,** 441.

Reddy, B. S., Watanabe, K. & Weisburger, J. H. (1977). Effect of high-fat diet on colon carcinogenesis in F344 rats treated with 1,2-dimethylhydrazine, methylazoxymethanol acetate, or methylnitrosourea. *Cancer Res.* **37,** 4156.

Reddy, B. S., Watanabe, K., Weisburger, J. H. & Wynder, E. L. (1977). Promoting effect of bile acids in colon carcinogenesis in germ-free and conventional F344 rats. *Cancer Res.* **37,** 3238.

Reddy, B. S., Weisburger, J. H. & Wynder, E. L. (1974). Effects of dietary fat level and dimethylhydrazine on fecal acid and neutral sterol excretion and colon carcinogenesis in rats. *J. natn. Cancer Inst.* **52,** 507.

Reddy, B. S., Weisburger, J. H. & Wynder, E. L. (1978). Colon cancer: Bile salts as tumor promoters. In *Carcinogenesis.* Vol. 2. *Mechanisms of Tumor Promotion and Cocarcinogenesis.* Edited by T. J. Slaga, A. Sivak & R. K. Boutwell. p. 453. Raven Press, New York.

Selby, H. H., & Wynne, W. H. (1973). Agar. In *Industrial Gums.* Edited by R. L. Whistler. p. 29. Academic Press, New York.

Spiller, G. A., Kim, Y. S. & Freeman, H. J. (1980). Protective effects of purified cellulose fiber diets in rat colonic neoplasia induced with 1,2-dimethylhydrazine. *Fedn Proc. Fedn Am. Socs exp. Biol.* **39,** 1118.

Sunter, J. P., Appleton, D. R., Wright, N. A. & Watson, A. J. (1978). Pathological features of the colonic tumours induced in rats by the administration of 1,2-dimethylhydrazine. *Virchows Arch. B. Zellpath.* **29,** 211.

Thurnherr, N., Deschner, E. E., Stonehill, E. H. & Lipkin, M. (1973). Induction of adenocarcinomas of the colon in mice by weekly injections of 1,2-dimethylhydrazine. *Cancer Res.* **33,** 940.

Toth, B., Malick, L. & Shimizu, H. (1976). Production of intestinal and other tumors by 1,2-dimethylhydrazine dihydrochloride in mice. *Am. J. Path.* **84,** 69.

Tsai, A. C., Elias, J., Kelley, J. J., Lin, R.-S.C. & Robson, J. R. K. (1976). Influence of certain dietary fibers on serum and tissue cholesterol levels in rats. *J. Nutr.* **106,** 118.

Wakabayashi, K., Inagaki, T., Fujimoto, Y. & Fukuda, Y. (1978). Induction by degraded carrageenan of colorectal tumors in rats. *Cancer Lett.* **4,** 171.

Ward, J. M., Yamamoto, R. S. & Weisburger, J. H. (1973). Cellulose dietary bulk and azoxymethane-induced intestinal cancer. *J. natn. Cancer Inst.* **51,** 713.

Watanabe, K., Reddy, B. S., Weisburger, J. H. & Kritchevsky, D. (1979). Effect of dietary alfalfa, pectin and wheat bran on azoxymethane- or methylnitrosourea-induced colon carcinogenesis in F-344 rats. *J. natn. Cancer Inst.* **63,** 141.

Watanabe, K., Reddy, B. S., Wong, C. Q. & Weisburger, J. H. (1978). Effect of undegraded carrageenan on colon carcinogenesis in F344 rats treated with azoxymethane or methylnitrosourea. *Cancer Res.* **38,** 4427.

Watt, J. & Marcus, A. J. (1978). Comparison of the effects of carrageenans and Danish agar on the colon of guinea pigs. *Proc. Nutr. Soc.* **37,** 39A.

Watt, J. & Marcus, R. (1973). Progress report: Experimental ulcerative disease of the colon in animals. *Gut* **14,** 506.

Wattenberg, L. W. & Sparnins, V. L. (1979). Inhibitory effects of butylated hydroxyanisole on methylazoxymethanol acetate-induced neoplasia of the large intestine and on nicotinamide adenine dinucleotide-dependent alcohol dehydrogenase activity in mice. *J. natn. Cancer Inst.* **63,** 219.

Wilson, R. B., Hutcheson, D. P. & Widerman, L. (1977). Dimethylhydrazine-induced colon tumors in rats fed diets containing beef fat or corn oil with or without wheat bran. *Am. J. clin. Nutr.* **30,** 176.

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SUBCHRONIC EFFECTS OF GUAR GUM IN RATS

S. L. GRAHAM, A. ARNOLD, L. KASZA*, G. E. RUFFIN,
R. C. JACKSON, T. L. WATKINS and C. H. GRAHAM

Bureau of Foods, Food and Drug Administration, Department of Health and Human Services, Washington, DC 20204, USA

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Abstract—Male and female Osborne–Mendel rats, approximately 4 wk old, were fed guar gum for 91 days at levels of 0, 1·0, 2·0, 4·0, 7·5 or 15·0% in the diet. Body weights, organ weights, haematology, clinical chemistry and histology were the criteria studied. Guar gum significantly reduced body weights in females of all the treated groups and in males on the 7·5 and 15% dietary levels. Utilization of the gum was poor and, at the 15% level, there was some reduction in bone-marrow cellularity. Serum-glucose values and kidney weights were the only other parameters showing dose-related effects.

INTRODUCTION

Guar gum is obtained from the guar plants *Cyamopsis tetragonaloba* and *C. proraloides*. These plants have been grown for centuries in India and Pakistan and the seeds have been used for human and animal food. Guar gum is used in many commercial food products as a binder or thickening agent and in other food products as a texturizing agent. It is one of the food substances that has been generally recognized as safe (GRAS) under the provisions of the *Code of Federal Regulations* (21 CFR 121.101, revised 1 January 1972).

Guar gum is a polysaccharide composed of mannose and galactose residues and has an average molecular weight in the range 200,000–300,000 (Glicksman, 1969). The polysaccharide consists of a straight chain of D-mannopyranose units with D-galactopyranose units attached to every other D-mannopyranose residue in the chain (Smith & Montgomery, 1959).

In the United States, guar gum was first used in food products in 1949, and data show that the usage for food purposes in 1970 was 4·3 million pounds. It is used in amounts between 1·13 and 0·005% in foods ranging from breakfast cereals to fish products and formulas, respectively (Subcommittee on Review of the GRAS List, 1972). The Joint FAO/WHO Expert Committee on Food Additives (1970) established a temporary acceptable daily intake (ADI) of 0–125 mg/kg body weight for humans. A 60-kg individual, on this basis, could consume 7500 mg guar gum each day.

Booth, Hendrickson & DeEds (1963) fed guar gum to rats at a level of 6% in the diet for 91 days and found that the weight gains were not significantly below those of the control group. No significant alterations in haemoglobin or in erythrocyte and leucocyte counts were observed. In this same study, digestibility and utilization of several polysaccharides were compared to that of dextran. Guar gum was 76% digestible and utilization, based on weight gain, was very low (+1).

Kratzer, Rajaguru & Vohra (1967) compared the effects of various polysaccharides on weight gain, food consumption, nitrogen retention and fat absorption in chicks, and determined the metabolizable energy of the feed, which contained 1270 ICU vitamin D_3/kg. They found that guar gum (2%) depressed growth and significantly reduced nitrogen retention, and also reduced fat absorption and metabolizable energy. These responses persisted, even in the presence of high dietary fat and protein. The Stanford Research Institute (1972), under a contract with the FDA, conducted mutagenicity tests with guar gum. The gum did not elicit a measurable mutagenic response in the host-mediated assay using Salmonella as the test organism and the rat as host, but *Saccharomyces cerevisiae* showed an increased mitotic recombination frequency when the cells were exposed to concentrations of either 1 or 5%. The Food and Drug Research Laboratories (1973), also under a contract with the FDA, conducted teratological tests in three species of animals (hamsters, mice and rats) and all were negative. No carcinogenic or allergenic activities of guar gum have been reported.

Several gums were selected from the GRAS list and are being investigated either because of increased usage or the lack of toxicity data. In our subchronic studies we have attempted to select dietary levels that will approximate or exaggerate daily human ingestion. The present study reports the acute oral LD_{50} and the effects of 90-day oral administration of guar gum to Osborne–Mendel rats. The highest dietary level used (15·0%) represents for rats an intake of 15,000 mg/kg/day (Association of Food and Drug Officials, 1959), over 100 times the 125 mg/kg/day acceptable daily intake.

EXPERIMENTAL

Materials. Jaguar® A-20-D (pure guar gum), Lot no. JAG-A20-DF-15-06-149-1, obtained from Stein Hall Co., Inc., New York, was used in both the acute

*Present address: Toxicology Branch, Division of Pesticide and Toxic Substances, Environmental Protection Agency, Washington, DC 20060.

and subchronic studies. The gum was a cream to light-tan powder and the viscosity of a 1% solution was equal to at least 2600 cP in 2 hr. Mesh analyses showed a minimum of 99% through US no. 140 mesh and of 83% through no. 200 mesh. There was an 8–13% loss on drying. The diets for subchronic feeding tests were prepared by adding guar gum to ground Purina Laboratory Chow (Ralston Purina Co., St. Louis, MO) and blending in a food mixer.

Acute toxicity. The acute oral toxicity of guar gum was determined in adult Osborne–Mendel rats. Guar gum, as a 0·2 g/ml suspension in corn oil, was administered by stomach-tube to groups of ten male and ten female rats. The rats were fasted overnight before treatment; their fasted weights averaged 450 g for males and 225 g for females. The oral administration of the calculated total dose required multiple equal applications (not exceeding three) separated by periods of at least 2 hr. The surviving rats were observed for 14 days after treatment. The LD_{50} was calculated by the probit procedure (SAS Institute, Inc., 1979).

Subchronic toxicity. Six groups of 130 Osborne–Mendel rats, approximately 4 wk old and evenly divided by sex, were fed guar gum at 0, 1·0, 2·0, 4·0, 7·5 or 15·0% dietary levels for 13 wk. The rats were housed individually and were allowed food and water *ad lib.* Deaths were recorded and clinical observations made daily, and weekly records were kept of animal weights and food consumption. An analysis of variance (ANOVA) technique (Steel & Torrie, 1960) was performed on the initial body weights (weights at week 0), separately by sex, and an analysis of covariance (ANACOVA) technique (Steel & Torrie, 1960) was used to adjust the body weights at wk 4, 8 and 12 for initial weight. After 13 wk, 40 male and 40 female rats were retained for a study of the teratogenic potential of guar gum, which will be reported in a separate publication. The remaining survivors (approximately 25 males and 25 females per dietary level) were killed by CO_2 asphyxiation and exsanguinated *via* the posterior vena cava. Blood samples were collected from ten male and ten female rats in each group. Haematological tests performed included haemoglobin and haematocrit determinations, and erythrocyte, total and differential leucocyte, and platelet counts. An ANOVA technique and the least significant difference (LSD) multiple comparison procedure (Steel & Torrie, 1960) were used to determine whether differences existed between the mean values for the control and treatment groups.

Serum clinical chemistry tests were performed to determine concentrations of glucose, urea nitrogen (BUN), creatinine, Na^+, K^+, Cl^-, CO_2, uric acid, total protein, albumin, globulin, calcium, phosphate, cholesterol, triglycerides, total bilirubin, thyroxine (T_4) and cholesterol esters, and activities of alkaline phosphatase, glutamic–oxalacetic transaminase (SGOT), glutamic–pyruvic transaminase (SGPT) and lactic dehydrogenase (LDH). For each sex separately, and LSD multiple comparison procedure was used to determine whether differences existed between the means for the controls and those for the other groups. A regression analysis (Steel & Torrie, 1960) for linear dose response was also performed.

At autopsy, following gross examinations, all livers, kidneys, spleens, hearts and testes were removed and weighed. For each sex separately, an ANACOVA technique was used to adjust the organ weight for body weight and to determine whether the adjusted means for the treated groups differed from those of the controls. In addition, a curvilinear regression technique was used to determine whether the adjusted treatment means followed a linear trend.

Histopathological examinations of the control group (44 rats) and the group fed 15% guar gum (36 rats) consisted of a full screen of tissues (brain, pituitary, adrenal, thyroid, parathyroid, thymus, pancreas, heart, lung, oesophagus, stomach, small intestine, large intestine, liver, spleen, bone with marrow, lymph node, skeletal muscle, kidney, urinary bladder, testis, prostate, seminal vesicle or ovary, uterus, salivary gland, skin and eye) with any additional tissues showing gross abnormality. A short screen of tissues (adrenal, thyroid, heart, lung, kidney, liver, bone marrow and testis) and all other tissues that showed gross pathological abnormalities were examined in the groups receiving the 1 or 4% dietary levels. Only the liver was examined in animals fed the 2 or 7·5% levels. All groups were observed for gross pathological changes and livers from ten animals in each group were stained with Oil Red O (for fat).

RESULTS

Acute toxicity

The oral LD_{50} of guar gum for male and female rats was calculated to be 7·06 g/kg with 95% confidence limits of 5·79–8·57 g/kg. The LD_{50} for males was 7·35 g/kg with 95% confidence limits of 5·22–10·73 g/kg, and the value for females was 6·77 g/kg with confidence limits of 4·50–9·65 g/kg.

Subchronic toxicity

Mortality. Of the 300 animals originally designated for pathological examination, only 258 (127 males and 131 females) were submitted because the humidity in the room housing the animals suddenly changed on day 8 of the experiment (for 1 day only) and, as a result, 23 males and 19 females died. These deaths occurred between wk 2 and wk 5 and were evenly distributed among the groups. As far as could be ascertained clinically, the surviving animals were not adversely affected. Growth curves and food intake were maintained consistently during this period and immediately afterwards.

Food consumption and body weights. Male rats fed the 7·5 and 15% dietary levels consumed, respectively, 4·9 and 8·8% less diet than the controls. Average daily doses of guar gum for the males fed the 1, 2, 4, 7·5 and 15% levels were 580, 1187, 2375, 4561 and 10,301 mg/kg/day, respectively. Female rats at the same dose levels had consumption values that were 0·3, 4·1, 4·2, 1·2, and 0·3%, respectively, lower than the female controls, and average daily doses were 691, 1362, 2762, 5770 and 13,433 mg/kg/day.

Table 1 shows the effect of guar gum ingestion on the body weights of rats. There were no significant differences ($P > 0·05$) among the group means for the initial body weights. After 4 wk, male and female rats fed guar gum at the 7·5 and 15% levels had body weights that were significantly less than control

values. Males on the 2% dietary level also had significantly smaller weights ($P < 0.01$). After 8 wk, males fed the 7·5 and 15% levels and females fed the 4, 7·5 and 15% levels had body weights that were significantly decreased from the respective control weights. The body weights of male rats after 12 wk on the diet were significantly decreased ($P < 0.001$) at both the 7·5 and 15% dietary levels, and the weights of females were significantly reduced at all dietary levels.

Haematology. Haematocrit values for males were less than control values at all test levels, but the decrease was of borderline significance. Haemoglobin determinations and erythrocyte and leucocyte counts were all within the control range. Regression analysis for the determination of linear dose response for the haematological values of males showed that the haematocrit means appeared to follow a negative linear trend; the slope was significant ($P < 0.05$) and there were no departures from linearity.

In females, haemoglobin levels and erythrocyte counts were significantly decreased ($P < 0.05$) only at the 4% dietary level; values were 15·0 g/dl and 6.66×10^6/mm^3, respectively, compared with control values of 15·7 g/dl and 7.57×10^6/mm^3. The slopes of the lines for the haemoglobin and haematocrit response parameters were not significant. Other haematological parameters in females were not significantly different from control values.

Clinical chemistry. Both sexes showed common changes in serum CO_2, total protein and LDH values. CO_2 values were significantly higher ($P < 0.01$) than control values in both males and females at all dose levels. Total protein was decreased ($P < 0.01$) in males at all dose levels but was increased ($P < 0.05$) in females at the 4% level and was decreased at the 7·5 ($P < 0.05$) and 15% ($P < 0.01$) levels. LDH activity was also significantly decreased in males at all dose levels and in females at the 1, 2 and 7·5% levels. Only female rats on the 2, 4, 7·5 and 15% dose levels showed significant changes in cholesterol and triglyceride values. Regression analysis on all clinical chemistry values showed that, except for glucose, there appeared to be no linear dose response for either male or female rats. The glucose values for males (217, 220, 200, 187, 182 and 157 mg/dl for the 0, 1, 2, 4, 7·5 and 15% dose levels, respectively) appeared to follow a linear trend, and the slope of the line was significant ($P < 0.05$); there were no deparartures from linearity ($P > 0.10$). No linear trend for the glucose values of females was observed and the slope was not significant ($P > 0.10$).

Organ weights. In males, the only adjusted mean organ weights that showed significant changes were the liver and kidneys. The liver weights at each dose level were significantly less than the mean for the control males (12·78, 11·22, 11·08, 11·39, 11·61 and 11·92 g for the 0, 1, 2, 4, 7·5 and 15% dose levels, respectively). Kidney weights at the 7·5 and 15% levels were significantly less (2·82 g, $P < 0.05$ and 2·69 g, $P < 0.001$, respectively) than that for the controls (3·02 g). The kidney weight was also decreased (to 2·88 g) at the 4% level, but the value was of borderline significance. Curvilinear regression analysis indicated that the kidney weights, adjusted for body weight, appeared to follow a linear trend; the slope of the line was significant ($P < 0.05$) and the adjusted means did not appear to scatter excessively about the regression line ($P > 0.10$). Liver weights of females at the 1, 4 and 15% levels (7·17, 7·10 and 7·11 g, respectively) were each significantly less ($P < 0.05$) than that for the control females (7·6 g), but the figure (7·3 g) for the females on 7·5% guar gum did not differ significantly from the control value. The adjusted mean for females at the 2% level was considered to be of borderline significance.

Histological examination. With the possible exception of the bone marrow in male rats fed the 15% level, no tissue examined had consistent histopathological alterations that could be attributed to the ingestion of guar gum. There was a suggestion of regressive changes in the bone marrow; at the 15% level, seven of 15 males and five of 21 females had a grade 3 (moderate) bone-marrow cellularity. Although this degree of cellularity is within normal limits, the fact

Table 1. *Mean body weights of rats fed 0–15·0% guar gum in the diet for 0, 4, 8 and 12 wk*

Dietary level (%)	Mean body weight (g) at wk			
	0	4	8	12
		Males		
0	50·5 (65)	210·5 (62)	339·7 (62)	404·5 (62)
1·0	50·2 (65)	211·4 (64)	341·1 (64)	415·9 (64)
2·0	54·7 (65)	195·8** (64)	332·9 (62)	396·9 (62)
4·0	49·5 (65)	203·6 (63)	334·4 (63)	394·9 (63)
7·5	51·2 (65)	186·2*** (61)	310·9*** (61)	370·8*** (61)
15·0	50·8 (65)	167·6*** (55)	279·1*** (55)	343·6*** (54)
		Females		
0	47·7 (65)	151·5 (62)	211·3 (62)	251·9 (62)
1·0	46·6 (65)	156·3 (62)	210·8 (62)	243·9* (62)
2·0	48·2 (65)	150·0 (63)	205·9 (63)	233·2*** (63)
4·0	47·4 (65)	147·2 (61)	204·3* (61)	231·0*** (61)
7·5	47·6 (65)	145·6* (62)	199·5*** (62)	225·8*** (62)
15·0	48·4 (65)	133·2* (60)	184·2*** (60)	210·3*** (60)

Values are means for the numbers of animals indicated in parentheses. Those with asterisks differ significantly from the control value: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

that results for several animals in this particular group fell at the lower end of the normal range suggested a subtle or borderline effect. The marrow atrophy was characterized by a proportional reduction of all cellular elements.

DISCUSSION

The data show that guar gum, at both the 7·5 and 15% dietary levels, caused significant decreases in mean body weights of male rats. At all test levels the mean body weights of females were significantly lower than that of the female controls. Since weight gain was significantly reduced without drastic reductions in food consumption, it is probable that the utilization of guar gum by the rats in this study was poor. Booth *et al.* (1963) found that guar gum, fed to rats at 6% of the diet, reduced body weights, and the utilization was low compared with that of dextran.

Haematology was negative, and of 22 different clinical chemistry parameters, glucose values of males showed the only consistent dose-related trend. Kidney weights of males, adjusted for body weight, were the only organ weights that showed a dose-related trend. Liver weights in both male and female rats showed a downward trend, but the decreases were not consistent as the dose level increased.

The lack of more consistent linear trends may suggest that the duration of the feeding was too short for patterns to be established. A longer exposure, with particular attention to the examination of bone marrow and with additional inspection of the liver and splenic extramedullary haematopoiesis, may clarify the suggestion of a reduction of all cellular elements.

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REFERENCES

Association of Food & Drug Officials (1959). *Appraisal of Safety of Chemicals in Foods, Drugs, and Cosmetics.* Association of Food and Drug Officials of the United States, Littleton, CO.

Booth, A. N., Hendrickson, A. P. & DeEds, F. (1963). Physiologic effects of three microbial polysaccharides on rats. *Toxic. appl. Pharmac.* **5**, 478.

Food and Drug Research Laboratories, Inc. (1973). Teratologic Evaluation of FDA 71-16 (Guar Gum) in Mice, Rats, Hamsters and Rabbits. Final report prepared under DHEW contract no. FDA 71-260, Maspeth, NY. NTIS No. PB-223-819/4, National Technical Information Service, Springfield, VA.

Glicksman, M. (1969). *Gum Technology in the Food Industry.* p. 140. Academic Press, New York.

Joint FAO/WHO Expert Committee on Food Additives (1970). Thirteenth Report: Specifications for the Identity and Purity of Food Additives and Their Toxicological Evaluation; Some Food Colours, Emulsifiers, Stabilizers, Anticaking Agents, and Certain Other Substances. *Tech. Rep. Ser. Wld Hlth Org.* **445.**

Kratzer, F. H., Rajaguru, R.W.A.S.B. & Vohra, P. (1967). The effect of polysaccharides on energy utilization, nitrogen retention and fat absorption in chickens. *Poultry Sci.* **46**, 1489.

SAS Institute, Inc. (1979). *Statistical Analysis Systems.* p. 357. SAS Institute, Inc., P.O. Box 100066, Raleigh, NC.

Smith, F. & Montgomery, R. (1959). *The Chemistry of Plant Gums and Mucilages.* ACS Monograph Series No. 141. p. 170. Reinhold Publishing Corp., New York.

Subcommittee on Review of the GRAS List (Phase II) (1972). A Comprehensive Survey of Industry on the Use of Food Chemicals Generally Recognized as Safe (GRAS). Report prepared under DHEW contract no. FDA 70-22. Committee on Food Protection, Division of Biology and Agriculture, National Research Council, National Academy of Sciences, Washington, DC.

Stanford Research Institute (1972). Study of Mutagenic Effects of Guar Gum (FDA 71-16). Report prepared under DHEW contract no. FDA 71-267, Menlo Park, CA. NTIS No. PB-221-815/4, National Technical Information Service, Springfield, VA.

Steel, R. G. D. & Torrie, J. H. (1960). *Principles and Procedures of Statistics.* pp. 106, 112 & 305. McGraw-Hill Book Company, Inc., New York.

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TESTICULAR RESPONSES OF RATS AND DOGS TO CYCLOHEXYLAMINE OVERDOSAGE

R. W. JAMES*, R. HEYWOOD and D. CROOK
Huntingdon Research Centre, Huntingdon, Cambridgeshire, PE18 6ES, England

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Abstract—Cyclohexylamine (CHA), the principal metabolite of cyclamate, was given by oral gavage to male rats (200 mg/kg/day) and male Beagles (250 mg/kg/day) for 9 wk. Subsequently some of these animals were maintained undosed for 13 wk to assess recovery. CHA adversely affected body-weight gain and food consumption in both species. Although a degree of tolerance developed, vomiting tended to occur after CHA administration to dogs. Serum follicle stimulating hormone levels increased and testosterone levels decreased in rats given CHA. No effects were found on the serum luteinizing hormone and testosterone levels in dogs, but reversible effects on sperm morphology were induced in this species. There were no statistically significant ($P > 0{\cdot}05$) effects on the weight of the pituitaries, testes or secondary sex organs of either species. The only lesion detectable by conventional histological examination was focal atrophy of seminiferous tubules in one rat examined 13 wk after cessation of CHA treatment. Quantitative assessment of testicular spermatogenesis showed that CHA administration reduced the counts of pachytene spermatocytes, and of early and late spermatids, in both species. These effects were apparently reversible in dogs but not in rats.

INTRODUCTION

Effects on testicular weight and histology have been associated with prolonged dietary administration of cyclamates to rats (Oser, Carson, Cox, Vogin & Sternberg, 1975; Taylor & Friedman, 1974). Testicular changes occur only after administration of dietary concentrations of at least 3% for 12 months or longer. Such experimental treatment also induces gastrointestinal disturbances, reduced food intake and impaired body-weight gain. Coulston, McChesney & Benitz (1977) found that the testes of rhesus monkeys given oral doses of 200 mg cyclamate/kg/day for 8 yr were histologically normal. While testicular effects have not been observed in short-term rodent or canine studies (Richards, Taylor, O'Brien & Duescher, 1951; Taylor, Richards & Wiegand, 1968) and testicular changes do not occur following chronic feeding at dietary levels of less than 3%, the safety in normal usage of cyclamate is a continuing focus for debate.

The metabolic fate of cyclamate has been studied in both man and animals (Golberg, Parekh, Patti & Soike, 1969; Parekh, Goldberg & Golberg, 1970; Renwick & Williams, 1972). Following continuous ingestion of cyclamate, up to 70% conversion to cyclohexylamine (CHA) occurs. The CHA formed is excreted in urine, mainly unchanged, with trace amounts of cyclohexanone, cyclohexanol and *N*-hydroxycyclohexylamine. The pharmacological characteristics of CHA include release of catecholamines, acetylcholine and histamine together with analgesic, sedative and tranquillizing properties (Miyata, Kasé, Kamikawa, Kataoka, Kikuchi & Touchi, 1969). The compound is a strong base, irritant to skin and mucous membranes, with an oral LD_{50} of 710 mg/kg for rats (Smyth, Carpenter, Weil, Pozzani, Striegel & Nycum, 1969). The potential testicular toxicity and anti-fertility effects of CHA have been examined in rats (Gaunt, Sharratt, Grasso, Lansdown & Gangolli, 1974; Green, Palmer & Legator, 1972; Khera & Stoltz, 1970; Khera, Stoltz, Gunner, Lyon & Grice, 1971; Kroes, Peters, Berkvens, Verschuuren, de Vries & Van Esch, 1977; Mason & Thompson, 1977).

Testicular changes, reduced food intake and impaired weight gain are consistently reproducible at doses of 288–528 mg/kg/day. At lower doses of CHA, conflicting data exist; however, there is a definite 'no effect' level at 30 mg/kg/day. The only effect observed in mating studies has been a slight increase in preimplantation loss when high doses are administered. Cattanach (1976) has reviewed mutagenic studies of CHA and its metabolites. He concluded that the effects observed in rodent breeding experiments could not be ascribed to dominant lethality. Furthermore there was no evidence to implicate CHA as a mutagen in normal usage. The mechanism underlying the testicular responses to overdosing with CHA remains a matter for speculation. Studies to examine the effects of massive doses of CHA on pituitary–testicular hormones, semen characteristics and quantitative assessment of spermatogenesis are described in this paper.

EXPERIMENTAL

Materials. CHA, conforming to the specification of the British Standards Institution (1968), was obtained from Fisons Ltd, Loughborough, Leics. The National Institute of Arthritis, Metabolism and Digestive Diseases (NIAMDD, Bethesda, MD, USA) supplied reagents and follicle stimulating hormone (FSH; NIAMDD-FSH-RP1) and luteinizing hormone (LH; NIAMDD-LH-RP1) standards for radioimmunoassay of FSH and LH. Ovine LH (LER-1056-C2) and

*Present address: Department of Pathology, The Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS.

canine pituitary reference standard (LER-1685-1: 0·025-LH-S1 units/mg) were obtained from Professor L. E. Reichert, Emory University, Atlanta, GA, USA. Ovine LH antibody no. 15 was supplied by Professor G. D. Niswender, Colorado State University, Fort Collins, CO, USA. Testosterone-11α-hemisuccinate, for radio-iodination, was obtained from Steraloids Ltd, Croydon.

Animals. Thirty specific pathogen-free male rats of the CD strain, aged 35 days, were obtained from the Charles River Breeding Laboratories, Manston, Kent. The rats were allowed 1 wk to acclimatize to our laboratory conditions before treatment was begun. The rats were housed in groups of five in suspended wire-mesh cages; room temperature and humidity were regulated at 21 ± 2°C and 50 ± 5%, respectively. Lighting was controlled to provide alternate 12 hr periods of light (08.00–20.00 hr B.S.T) and dark. Spratt's Laboratory Rodent Diet No. 1 (Spratt's Laboratory Services, Barking, Essex) and tap water were freely available.

Four sexually mature Beagle dogs were selected from the colony maintained at Huntingdon Research Centre. The dogs were individually kennelled and apart from routine anthelmintic therapy ('Coopane', Wellcome Veterinary Products, Berkhamsted, Herts) and vaccination against distemper, hepatitis and leptospirosis, no other medications were permitted. Each dog was given 400 g Spratt's Dog Diet (Spratt's Laboratory Services) daily and tap water was freely available.

Experimental design and observations. The 30 rats were allocated to an experimental or a control group, each of 15 rats, so as to give approximately equal initial group mean body weights. A 4·62% (v/v) suspension of CHA in corn oil was prepared once weekly and stored in air-tight smoked glass containers. The CHA suspension was administered by oral gavage at 0·5 ml/100 g body weight so that each rat received 200 mg CHA/kg/day. Control rats were given the same volume of corn oil. CHA was administered to all four dogs as a 3·47% (v/v) suspension in corn oil. Gastric irritation made it necessary to use an incremental gavage regime for the dogs. Initially the CHA suspension was administered to achieve oral doses of 75 mg/kg on days 1 and 2, 75 mg/kg twice daily on days 3 and 4, 150 mg/kg on days 5 and 6, and then 150 mg/kg twice daily on subsequent days. After day 9 the dose was reduced to 125 mg/kg twice daily to avoid marked appetite suppression. Five rats from each group were killed after wk 4 and 9 of treatment and two dogs after wk 9 of treatment. The remaining five rats per group and two dogs were maintained untreated for 13 wk before terminal examination.

For both species, body-weight gain was recorded weekly. Food and water intake were measured daily for dogs and weekly for rats. All of the animals were examined several times a day for evidence of clinical malreaction to CHA administration. Rats that died during the experiment were autopsied in order to establish a possible cause of death.

Testicular measurements and semen examinations were made for the dogs before starting treatment and then repeated after 2, 4 and 8 wk of treatment and again during during wk 4, 8 and 12 of the withdrawal period. The length (l) and breadth (b) of each testis was measured in the scrotum using vernier calipers. The area (mm^2) given by the formula $\pi lb/4$ (Heywood & James, 1978) was chosen as an index of testicular size. Group mean values were calculated on each occasion and the changes from the mean pre-dose values determined. Canine semen samples were obtained using an artificial vagina (Harrop, 1954). The volume, density, motility, sperm concentration and morphology of each ejaculate was recorded (Heywood & Sortwell, 1971). The concentrations of Na^+, K^+ (mequiv/litre), acid phosphatase, alanine aminotransferase (EC 2.6.1.2) and aspartate aminotransferase (EC 2.6.1.1) were determined (mU/ml) as described by James, Heywood & Street (1979).

Serum samples were obtained from five rats per group after 1 day and 4 and 9 wk of treatment and after 13 wk of withdrawl from treatment for determination of FSH, LH and testosterone. Canine sera were examined for LH and testosterone concentrations before dosing began, after 1, 2, 4 and 8 wk of treatment and during wk 4, 8 and 12 of the withdrawal period. During the treatment period serum samples were obtained 4 hr after CHA administration. Sera were stored at −20°C before radioimmunoassay. Rat FSH and LH (ng/ml) were determined using methods supplied by the NIAMDD. Canine LH (ng/ml) was determined using the heterologous double antibody procedure of Niswender, Reichert, Midgeley & Nalbandov (1969) previously validated for canine LH assays by Smith & McDonald (1974). Testosterone (ng/ml) concentrations were measured using the technique of Frankel, Mock, Wright & Kamel (1975), but modified as follows. Testosterone was extracted from sera using a mixture of diethyl ether and petroleum ether (1:4, v/v); the radioligand was testosterone-11α-hemisuccinate iodinated by the 'chloramine T' procedure; separation of free and bound fractions was achieved using 50% saturated ammonium sulphate.

The terminal examinations that were carried out on up to five rats per group after 4 and 9 wk of dosing and 13 wk withdrawal, and on two dogs after 9 wk of dosing and 13 wk withdrawal, comprised full macroscopic post-mortem examination, together with weighing and preservation of pituitary glands, testes (including epididymides), and prostate and seminal vesicles (rats only). Pituitaries were preserved in mercuric formol, testes in formol–saline and the other tissues in buffered neutral formalin. Histological preparations of the preserved organs were stained as follows: pituitaries with periodic acid Schiff (PAS)–Alcian Blue–Orange G (El Etreby & Tüshaus, 1973), testes with PAS, and prostates and seminal vesicles with haematoxylin and eosin.

A quantitative assessment of spermatogenesis was performed using the eight-stage classification of Roosen-Runge (1962). Type B spermatogonia (stage 7), pachytene spermatocytes (stage 5), early (round) spermatids (stage 1), late (elongated) spermatids (stage 8) and Sertoli cells were counted in seminiferous tubules seen in circular cross-section. Counts for each cell type were made in three seminiferous tubules per rat and three seminiferous tubules per testis per dog. Two perpendicular diameters were averaged for each tubule using an optical graticule (Graticules Ltd, Tonbridge, Kent). The criteria of Clermont & Perey

(1957) were used for recognition of rat spermatogenic cells and those of Foote, Swierstra & Hunt (1972) for dog material. 'Crude' counts were corrected for variations in nuclear (Abercrombie, 1946) and tubular (Swierstra & Foote, 1963) diameters.

Subject to the constraints of homogeneity of variance (Bartlett's Test) and variance (F) ratios, statistical comparisons between treatments were made using analysis of variance (ANOVAR). Testicular measurements and semen characteristics of dogs before treatment were compared with subsequent values using paired t-tests. Rat organ weight data were analysed using co-variance analysis with body weight as covariate.

RESULTS

Two rats died during the investigation: one control rat during wk 11 of the recovery period and one rat 8 wk after cessation of CHA treatment. In both cases death was attributable to chronic respiratory disease.

Clinical reactions of rats to CHA administration were manifested as reduced motor and grooming activity during wk 1 of treatment. Dogs given CHA vomited on up to 35% of the dosing occasions during the first 4 wk. Subsequently vomiting occurred after approximately 12% of the dosing occasions. Vomiting occurred between 5 and 120 min after dosing. The dogs were frequently observed to be unusually quiet and tended to pass loose faeces.

Over the 9-wk dosing period dogs showed a 10% loss of body weight and treated rats showed a 25% suppression in the rate of weight gain in comparison with the control rats. The adverse effect on the body weight of dogs was completely reversed during the recovery period; however, at the end of the recovery period rats that had received CHA still showed a 13% suppression in the rate of weight gain in comparison with the controls. Administration of CHA impaired appetite in both species: the dogs showed a 25% reduction and rats a 15% reduction in food intake over the 9 wk of treatment. During the recovery period the dogs regained normal appetite but previously treated rats continued to show a 10% reduction in food consumption. Both species tended to drink slightly increased quantities of water during the dosing period.

Treatment with CHA did not significantly affect the serum LH or testosterone concentrations of dogs (Table 1). All of the values were within normally accepted limits for sexually mature Beagle dogs (James, Crook & Heywood, 1979). Increased FSH and decreased testosterone concentrations in rat sera obtained after 9 wk CHA treatment differed significantly ($P < 0.05$) from control values. There were no statistically significant differences ($P > 0.05$) in these parameters at any other sampling point.

Reductions in the testicular area of dogs given CHA attained statistical significance ($P < 0.05$) after 4 and 8 wk of treatment, when decreases of 24 ± 6 and 26 ± 10%, respectively, occurred. After 8 wk of CHA administration, decreased sperm counts and a significantly increased percentage of abnormal (coiled-tail) spermatozoa were evident in canine ejaculates (Table 2). Normal sperm counts were obtained at all examinations made during the recovery period. Increased numbers of abnormal spermatozoa were evident after 4 and 8 wk withdrawal but not after 12 wk. Seminal plasma showed no significant biochemical effects.

Organ weight data are summarized in Table 3. The pituitary, testicular and prostate weights of dogs examined after 9 wk dosing were marginally lower than after 13 wk of recovery. However, dogs in the first group examined were of lower body weight and all values were within published ranges (James & Heywood, 1979a). Testicular and seminal vesicle weights of rats given CHA were consistently lower than those of control rats at all examinations. Analysis of co-variance with initial and final body weight as

Table 1. *Serum hormone concentrations of rats and dogs given cyclohexylamine (CHA)*

Treatment (no. of animals)	Serum hormone concentration (ng/ml)†		
	Testosterone	LH	FSH
Dogs			
Predosing (4)	1·9 ± 0·5	1·5 ± 0·3	ND
CHA‡ for: 8 wk (4)	1·9 ± 0·8	2·1 ± 0·7	ND
9 wk + 13 wk recovery (2)	2·7 ± 0·1	2·9 ± 0·4	ND
Rats			
9 wk dosing with: corn oil (5)	2·0 ± 0·4	14 ± 15	222 ± 55
CHA§ (5)	0·8 ± 0·2*	10 ± 29	358 ± 91*
13 wk recovery after 9 wk dosing with:			
corn oil (4)	1·1 ± 0·5	35 ± 19	249 ± 32
CHA§ (4)	0·8 ± 0·3	41 ± 40	278 ± 136

LH = Luteinizing hormone FSH = Follicle stimulating hormone
ND = Not determined

†Canine LH is expressed as ng LER-1685-1/ml (limit of detectability <0·5 ng/ml); rat LH is expressed as ng NIAMDD-LH-RP1/ml (limit of detectability <5 ng/ml) and rat FSH is expressed as ng NIAMDD-FSG-RP1/ml (limit of detectability <30 ng/ml). See Experimental section for further details.

‡250 mg/kg/day.

§200 mg/kg/day. Rats given corn oil served as controls.

Values are means ± 1SD and those marked with an asterisk differ significantly (analysis of variance) from the corresponding control mean (*$P < 0.05$).

Table 2. *Semen characteristics of dogs given 250 mg cyclohexylamine/kg/day*

Semen characteristic	Mean ± 1SD (range)		
	Before dosing, N = 4	After 8 wk of dosing, N = 4	After 9 wk of dosing followed by 12 wk of recovery, N = 2
Ejaculate volume (ml)	6·0 ± 6·8 (1·5–15·9)	4·4 ± 3·9 (1·3–10·0)	6·5 ± 4·5 (3·0–9·9)
Motility (0–5)	2·0 ± 0·6 (1–4)	2·0 ± 1·4 (0–3)	2·0 ± 1·4 (1–3)
Density (0–5)	2·5 ± 1·4 (2–3)	2·5 ± 0·6 (2–3)	3·5 ± 0·7 (3–4)
Sperm count: 10^6/ml ejaculate	67 ± 48 (18–125)	29 ± 11 (19–42)	106 ± 49 (71–104)
10^6/ejaculate	241 ± 140 (59–286)	119 ± 81 (25–220)	563 ± 314 (423–703)
Percentage of sperm:			
live	79 ± 13 (68–97)	85 ± 3 (82–88)	87 ± 6 (83–91)
abnormal (coiled tail)	12 ± 7 (5–22)	40 ± 6* (31–46)	9 ± 4 (6–11)

The value marked with an asterisk is significantly different (paired *t*-test) from the mean value before dosing (*$P < 0.05$).

covariates was carried out on the rat data. No statistically significant differences ($P > 0.05$) were apparent.

No histologically detectable lesions were found in any of the dogs' organs. One rat examined after 13 wk withdrawal of CHA showed bilateral testicular atrophy, many seminiferous tubules being devoid of spermatogenic cells and containing syncytia of Sertoli cells. No other histologically recognizable lesions were seen in the rat tissues examined.

Quantitative assessment of testicular spermatogenesis revealed some statistically different decreases ($P < 0.05$) in the number of germ cells present in the seminiferous tubules of rats and dogs given CHA (Table 4). In the dog testes counts of pachytene spermatocytes, and of early and late spermatids after 9 wk of treatment were significantly lower than counts at the end of the recovery period. Significant reductions in the numbers of late spermatids were apparent in CHA-treated rats, both during the dosing and the withdrawal periods. (The rat that developed testicular atrophy was excluded from the examination after 13 wk recovery.) After 13 wk withdrawal, rats previously given CHA were also found to have reduced numbers of pachytene spermatocytes and early spermatids. There were no statistically significant effects ($P > 0.05$) in either species for counts of type B spermatogonia.

DISCUSSION

These results are in agreement with previously published accounts of the effects of overdosage with CHA on testicular function. The doses used in rats (200 mg/kg/day) and dogs (250 mg/kg/day) quantitatively reduced testicular spermatogenesis in both species. The previously published 'no effect' level with regard to the rat testes is about 30 mg/kg/day (Gaunt *et al.* 1974); the present doses are six to seven times higher and were chosen in an attempt to elucidate mechanisms of action rather than to confirm 'no effect' levels.

Although effects on testicular function were apparent they cannot be compared with the potent effects that may be produced with hormonal steroids, or antispermatogenic or cytotoxic agents (Gomes, 1970; Jackson, 1972; Patanelli, 1975). The observed effects of CHA on the semen quality of dogs, the serum FSH and testosterone concentrations of rats and the development of testicular atrophy in one rat 13 wk after cessation of CHA administration are circumstantial rather than conclusive evidence of a testicular effect.

Table 3. *Body and organ weights of rats and dogs given cyclohexylamine (CHA)*

Treatment (no. of animals)	Mean body weight (kg)		Mean weight (g) of			
	Initial	Final	Pituitary (mg)	Testes	Prostate	Seminal vesicle
Dogs						
CHA† for 9 wk (2)	12·0	10·6	68	18·7	7·2	NA
CHA† for 9 wk + 13 wk recovery (2)	13·1	13·2	81	23·8	10·5	NA
Rats						
4 wk dosing with: corn oil (5)	0·150	0·343	11	4·2	0·6	1·5
CHA‡ (5)	0·146	0·293*	9	3·8	0·5	1·0
9 wk dosing with: corn oil (5)	0·146	0·460	11	4·8	0·7	1·4
CHA‡ (5)	0·152	0·375**	11	4·5	0·8	1·2
13 wk recovery after 9 wk dosing with:						
corn oil (4)	0·147	0·549	15	5·0	0·8	2·0
CHA‡ (4)	0·152	0·501	10	4·0	0·8	1·8

NA = Not applicable

†250 mg/kg/day.

‡200 mg/kg/day. Rats given corn oil served as controls.

Values marked with asterisks are significantly different (analysis of variance) from the corresponding control value (*$P < 0.05$; **$P < 0.01$).

Table 4. *Results of quantitative assessment of spermatogenesis for dogs and rats given cyclohexylamine (CHA)*

Treatment (no. of animals)	Mean ± 1SD seminiferous tubule diameter	Mean no.† of germ cells classified as: Type B spermatogonia (stage 7)	Pachytene spermatocytes (stage 5)	Spermatids: Early (stage 1)	Spermatids: Late (stage 8)	Mean no. of Sertoli cells
Dogs						
250 mg CHA/kg/day for 9 wk (2)	181 ± 11**	16 (6·7)	29** (13·5)	68*** (47·3)	61*** (42·5)	24 (11·1)
250 mg CHA/kg/day for 9 wk + 13 wk recovery (2)	219 ± 7	19 (9·1)	39 (15·0)	104 (59·8)	92 (52·9)	29* (11·1)
Rats						
Controls (14)	275 ± 18	37 (16·6)	68 (21·1)	209 (80·6)	177 (77·9)	20 (12·0)
200 mg CHA/kg/day for:						
4 wk (5)	250 ± 11	36 (17·8)	69 (23·6)	189* (80·2)	133*** (64·4)	20 (13·2)
9 wk (5)	275 ± 24	38 (17·1)	75* (23·3)	203 (78·3)	158** (69·5)	21 (12·6)
9 wk + 13 wk recovery (3)‡	271 ± 9	34 (15·6)	52*** (16·4)	143*** (55·9)	93** (41·5)	20 (12·1)

†Values are means for crude counts made in three seminiferous tubules per rat and three seminiferous tubules per testis per dog. Two perpendicular diameters were averaged for each tubule using an optical graticule. Counts corrected for variations in nuclear and tubular diameters are given in brackets. The eight-stage classification of Roosen-Runge (1962) was used to identify stages of spermatogenesis.

‡One rat that developed bilateral testicular atrophy was excluded from the examination.

Values marked with asterisks are significantly different (analysis of variance) from those for the corresponding recovery group (dogs) or control group (rats): $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

However, the findings of the quantitative histometric examinations may suggest an effect of CHA on primary pachytene spermatocytes in both species. This is also in agreement with the finding (Gaunt *et al.* 1974) that rats remained fertile after CHA treatment and no evidence of embryo or foetotoxic effects was obtained. The occurrence of testicular atrophy in a single rat is not readily explained especially since no effects were seen on spermatogonia in the remaining rats or the dogs. Furthermore, spontaneous testicular atrophy does occur in the Sprague–Dawley rat (James & Heywood, 1979b). In the present case it may either have been a spontaneous lesion or it represents increased sensitivity to the effects of CHA in a single rat.

Although obvious effects on body weight and food intake were apparent, the testicular effects are probably not explicable simply on this basis. The studies of Gaunt *et al.* (1974) and Mason & Thompson (1977) included observations on pair-fed and paired-weight rates. It seemed that inanition *per se* did not explain the testicular effects of CHA, although interaction between inanition and chemical action could not be excluded. CHA administration increased the basal metabolic rate in rats, possibly as a result of sympathomimetic effects.

The hormonal responses of rats given CHA were characteristic reactions to depletion of the germinal epithelium (Collins, Collins, McNeilly & Tsang, 1978; Debeljuk, Arimura & Schally, 1973), and are therefore secondary responses and not the primary cause of the testicular effects. The possibility of direct action of CHA on the seminiferous epithelium cannot be excluded. Further investigations should be designed to assess the pharmacokinetics of CHA within the seminiferous epithelium compartment of the blood–testis barrier (Setchell & Main, 1978). The ability of CHA to induce metabolizing enzymes within the testes (Lee & Dixon, 1978) is also worthy of investigation.

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REFERENCES

Abercrombie, M. (1946). Estimation of nuclear population from microtome sections. *Anat. Rec.* **94,** 239.

Blumberg, A. G. & Heaton, A. M. (1970). The occurrence of cyclohexylamine in urines studied for drug use by thin layer chromatography. *J. Chromat.* **48,** 565.

British Standards Institution (1968). Specification for Cyclohexylamine. B.S. 2534:1968.

Cattanach, B. M. (1976). The mutagenicity of cyclamates and their metabolites. *Mutation Res.* **39,** 1.

Clermont, Y. & Perey, B. (1957). The stages of the cycle of the seminiferous epithelium of the rat: practical definitions in PA-Schiff-haematoxylin and haematoxylin-eosin stained sections. *Revue can. Biol.* **16,** 451.

Collins, P. M., Collins, W. P., McNeilly, A. S. & Tsang, W. N. (1978). Plasma FSH, LH and testosterone levels in the male rat during degeneration of the germinal epithelium caused by severe heat treatment or ligation of the vasa efferentia. *J. Reprod. Fert.* **54,** 285.

Coulston, F., McChesney, E. W. & Benitz, K.-F. (1977). Eight-year study of cyclamate in rhesus monkeys (Abstract). *Toxic. appl. Pharmac.* **41,** 164.

Debeljuk, L., Arimura, A. & Schally, A. V. (1973). Pituitary and serum FSH and LH levels after massive and selective depletion of the germinal epithelium in the rat testis. *Endocrinology* **92,** 48.

El Etreby, M. F. & Tüshaus, U. (1973). Differentialfärbung zur Darstellung von Wachstumshormon und Prolaktin produziereden Zellen des Hypophysenvorderlappens. *Histochemie* **33,** 121.

Foote, R. H., Swierstra, E. E. & Hunt, W. L. (1972). Spermatogenesis in the dog. *Anat. Rec.* **173,** 341.

Frankel, A. I., Mock, E. J., Wright, W. W. & Kamel, F. (1975). Characterisation and physiological validation of a radioimmunoassay for plasma testosterone in the male rat. *Steroids* **25**, 73.

Gaunt, I. F., Sharratt, M., Grasso, P., Lansdown, A. B. G. & Gangolli, S. D. (1974). Short-term toxicity of cyclohexylamine hydrochloride in the rat. *Fd Cosmet. Toxicol.* **12**, 609.

Glogner, P. (1970). The metabolism of tolbutamide and cyclamate. *Hum. Genet.* **9**, 230.

Golberg, L., Parekh, C., Patti, A. & Soike, K. (1969). Cyclamate degradation in mammals and *in vitro* (Abstract). *Toxic. appl. Pharmac.* **14**, 654.

Gomes, W. R. (1970). Chemical agents affecting testicular function and male fertility. In *The Testes*. Vol. 3. p. 483. Edited by A. D. Johnson, W. R. Gomes & N. L. Vandermark. Academic Press, New York.

Green, S., Palmer, K. A. & Legator, M. S. (1972). Effects of cyclohexylamine on the fertility of male rats. *Fd Cosmet. Toxicol.* **10**, 29.

Harrop, A. E. (1954). A new type of canine artificial vagina. *Br. vet. J.* **110**, 194.

Heywood, R. & James, R. W. (1978). Assessment of testicular toxicity in laboratory animals. *Envir. Hlth Perspect.* **24**, 73.

Heywood, R. & Sortwell, R. J. (1971). Semen evaluation in the Beagle dog. *J. small Anim. Pract.* **12**, 343.

Jackson, H. (1972). Chemical methods of male contraception. In *Reproduction in Mammals. Book 5. Artificial Control of Reproduction*. p. 67. Edited by C. R. Austin & R. V. Short. Cambridge University Press, Cambridge.

James, R. W., Crook, D. & Heywood, R. (1979). Canine pituitary–testicular function in relation to toxicity testing. *Toxicology*. In press.

James, R. W. & Heywood, R. (1979a). Age-related variations in the testes and prostate of beagle dogs. *Toxicology* **12**, 273.

James, R. W. & Heywood, R. (1979b). Age-related variations in the testes of Sprague–Dawley rats. *Toxicology Lett.* **4**, 257.

James, R. W., Heywood, R. & Street, A. E. (1979). Biochemical observations on beagle dog semen. *Vet. Rec.* **104**, 480.

Khera, K. S. & Stoltz, D. R. (1970). Effects of cyclohexylamine on rat fertility. *Experientia* **26**, 761.

Khera, K. S., Stoltz, D. R., Gunner, S. W., Lyon, D. A. & Grice, H. C. (1971). Reproduction study in rats orally treated with cyclohexylamine sulfate. *Toxic. appl. Pharmac.* **18**, 263.

Kroes, R., Peters, P. W. J., Berkvens, J. M., Verschuuren, H. G., de Vries, Th. & Van Esch, G. J. (1977). Long term toxicity and reproduction study (including a teratogenicity study) with cyclamate, saccharin and cyclohexylamine. *Toxicology* **8**, 285.

Lee, I. P. & Dixon, R. L. (1978). Factors influencing reproduction and genetic toxic effects on male gonads. *Envir. Hlth Perspect.* **24**, 117.

Litchfield, M. H. & Swan, A. A. B. (1971). Cyclohexylamine production and physiological measurements in subjects ingesting sodium cyclamate. *Toxic. appl. Pharmac.* **18**, 535.

Mason, P. L. & Thompson, G. R. (1977). Testicular effects of cyclohexylamine hydrochloride in the rat. *Toxicology* **8**, 143.

Miyata, T., Kasé, Y., Kamikawa, Y., Kataoka, M., Kikuchi, K. & Touchi, T. (1969). Pharmacological characteristics of cyclohexylamine, one of the metabolites of cyclamate. *Life Sci.* **8**, 843.

Niswender, G. D., Reichert, L. E., Midgeley, A. F. & Nalbandov, A. V. (1969). Radioimmunoassay for bovine and ovine LH. *Endocrinology* **84**, 1163.

Oser, B. L., Carson, S., Cox, G. E., Vogin, E. E. & Sternberg, S. S. (1975). Chronic toxicity study of cyclamate: saccharin (10:1) in rats. *Toxicology* **4**, 315.

Parekh, C., Goldberg, E. K. & Golberg, L. (1970). Fate of sodium cyclamate-^{14}C in the rhesus monkey (M. mulatta). *Toxic. appl. Pharmac.* **17**, 282.

Patanelli, D. J. (1975). Suppression of fertility in the male. In *Handbook of Physiology*. Section 7, Vol. 5. p. 245. Edited by R. D. Greep & E. B. Astwood. American Physiological Society, Washington, DC.

Renwick, A. G. & Williams, R. T. (1972). The fate of cyclamate in man and other species. *Biochem. J.* **129**, 869.

Richards, R. K., Taylor, J. D., O'Brien, J. L. & Duescher, H. O. (1951). Studies on cyclamate sodium (Sucaryl sodium), a new noncaloric sweetening agent. *J. Am. pharm. Ass.* **40**, 1.

Roosen-Runge, E. C. (1962). The process of spermatogenesis in mammals. *Biol. Rev.* **37**, 343.

Setchell, B. P. & Mans, S. J. (1978). Drugs and the blood-testis barrier. *Envir. Hlth Perspect.* **24**, 61.

Smith, M. S. & McDonald, L. E. (1974). Serum levels of luteinizing hormone and progesterone during the estrous cycle, pseudopregnancy and pregnancy in the dog. *Endocrinology* **94**, 404.

Smyth, H. F., Carpenter, C. P., Weil, C. S., Pozzani, U. C., Striegel, J. A. & Nycum, J. S. (1969). Range-finding toxicity data: list VII. *Am. ind. Hyg. Ass. J.* **30**, 470.

Swierstra, E. E. & Foote, R. H. (1963). Cytology and kinetics of spermatogenesis in the rabbit. *J. Reprod. Fert.* **5**, 309.

Taylor, J. D., Richards, R. K. & Wiegand, R. G. (1968). Toxicological studies with sodium cyclamate and saccharin. *Fd Cosmet. Toxicol.* **6**, 313.

Taylor, J. M. & Friedman, L. (1974). Combined chronic feeding and three-generation reproduction study of sodium saccharin in the rat (Abstract). *Toxic. appl. Pharmac.* **29**, 154.

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THE EFFECT OF WATER-BORNE NITRATE ON SALIVARY NITRITE

C. L. WALTERS and P. L. R. SMITH
British Food Manufacturing Industries Research Association, Leatherhead, Surrey

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Abstract—The increase in salivary nitrite concentration caused by drinking 0–50 mg nitrate was measured in eight volunteers (over the subsequent 4–6 hr). The magnitude of the increase was dependent on the amount of nitrate consumed and varied between individual volunteers. Significant increases were not usually obtained with nitrate intakes of less than 10 mg. Four of the volunteers had a low relatively constant resting salivary nitrite concentration whereas the remaining four subjects showed much greater variations in nitrite concentration before the nitrate drink. A correlation was obtained between the increase in salivary nitrite concentration after drinking 50 mg nitrate and the rate of fall of the resting nitrite concentration during the period preceding consumption of the nitrate drink.

INTRODUCTION

The importance of environmental factors in the aetiology of gastric cancer has been suggested by geographical variations in its incidence (Pfeiffer, Fodor & Canning, 1973; Wynder, Kmet, Dungal & Segi, 1963). In particular, the nitrate contents of water supplies have been reported to be elevated in areas of high risk both in Colombia (Correa, Haenszel, Cuello, Tannenbaum & Archer, 1975) and in England (Hill, Hawksworth & Tattersall, 1973). This finding has directed attention towards the possibility of *in vivo* formation of *N*-nitroso compounds since amines are widely distributed in the environment.

The saliva of fasting humans contains inorganic nitrite, at concentrations that vary considerably between individuals and this can react with amines to form *N*-nitroso compounds *in vivo* (Harada, Ishiwata, Nakamura, Tanimura & Ishidate, 1975). Parotid duct saliva was found to be free of nitrite (Tannenbaum, Sinskey, Weisman & Bishop, 1974) and therefore oral bacteria may be implicated in its production from nitrate. The ingestion of vegetables or their juices rich in nitrate leads to enhanced nitrate concentrations in the mouth and thereby to increases in salivary nitrite which can persist for some hours (Stephany & Schuller, 1975; Spiegelhelder, Eisenbrand & Preussmann, 1976; Tannenbaum, Weisman & Fett, 1976). Tannenbaum *et al.* (1976) were able to reduce markedly the conversion of nitrate to nitrite in the saliva of volunteers through the application of antiseptic mouth washes.

Until recent years, the main concern about nitrate in water supplies centred on its reduction to nitrite by bacteria within the colonized achlorhydric stomach of the young infant. Foetal haemoglobin is particularly susceptible to oxidation by absorbed nitrite, with the production of methaemoglobin, which is inactive in the transport of oxygen from the lungs to the tissues. In addition, the erythrocytes of the young infant can be deficient in methaemoglobin reductase, an enzyme which reduces the oxidized haem pigment to its active ferrous form. Furthermore, the young infant consumes a large proportion of its food in a liquid form in water and thus the intake of nitrate from this source may be high in relation to blood volume. Fortunately, methaemoglobinaemia resulting from the conversion of dietary nitrate to nitrite can be readily reversed by transfusion with an electron transporting agent such as methylene blue or a reducing agent such as ascorbic acid. Nevertheless, fatalities of young infants have occurred in areas of high-nitrate drinking water and, for this reason, the World Health Organization has recommended a limit on its concentration for continuous use of 11·3 mg/litre as nitrate nitrogen (WHO, 1977). However, the possibility of an epidemiological link between nitrate in drinking water and gastric cancer has highlighted the potential importance of nitrite produced in the saliva as a precursor to carcinogenic nitrosamines and nitrosamides. In experimental animals, for instance, *N*-nitrosamides have proved to be active gastric carcinogens (Sugimura & Fujimura, 1967; Sugimura, Fujimura & Baba, 1970). For this reason, a study has been initiated to determine the effects on salivary nitrite concentrations of the ingestion by a group of volunteers of nitrate in drinking water at levels up to and in excess of the WHO recommended limit. Considerable care has been taken to minimize the effects of nitrate from other dietary sources on the nitrite responses observed.

EXPERIMENTAL

Studies of nitrite levels in the saliva of volunteers. The intake of nitrate overnight by a group of volunteers was restricted as far as possible by the use of distilled water for beverages and by avoidance of foods recognized to contain high nitrate concentrations. Samples of saliva (3–4 ml) were collected from the volunteers over the course of a few minutes at intervals of 30–45 min before and after the consumption of nitrate in 250 ml distilled water within 5 min. The salivary nitrite response to the intake of water without and with the addition of nitrate was

evaluated on at least three occasions for each concentration.

Nitrite determinations were made directly on the salivas without further preparation by a method based on that of Shinn (1941) using as blanks further aliquots of each saliva after treatment with 0·5% sulphamic acid to take account of any colour in the samples.

Determinations of nitrate in whole meals. Meals were weighed and homogenized in an M.S.E. Atomix with 500 ml distilled water. After heating the homogenates for 1 hr on a steam bath they were cooled, centrifuged and the supernatants filtered. Determinations of nitrate in the supernatants as nitrite were made after its reduction with spongy cadmium prepared according to Follett & Ratcliff (1963).

RESULTS

Variations in resting salivary nitrite levels

Figure 1 illustrates the appreciable differences observed between the early morning levels of nitrite in the saliva of six volunteers who ate breakfasts of their own choice. In three of the volunteers, the salivary nitrite concentrations were maintained at relatively constant low levels throughout the morning. Three of the other subjects with higher initial values showed a general fall in nitrite concentration during the morning. At least four of the volunteers showed a rise in salivary nitrite concentration in the afternoon, probably as a result of nitrate consumed in the lunchtime meal, which was not restricted in any way.

Figure 2 shows the salivary nitrite levels of two volunteers throughout three days. Volunteer A, who normally consumes only a very light lunch, showed low levels of nitrite throughout without obvious responses during the post-lunch period. In view of the rises in salivary nitrite levels observed during the afternoons in some volunteers, the nitrate levels of some of the meals available to the volunteers were determined and are shown in Table 1; these included a selection of those which would have been consumed by the volunteers at mid-day. The consumption of these meals would lead to an intake of nitrate comparable with or in excess of the amounts to be studied in drinking water. For this reason, the observations on the latter were restricted to the pre-lunch period, the nitrate intake overnight being restricted as far as possible through, for instance, the use of distilled water for drinking purposes and the avoidance of foods particularly rich in nitrate such as spinach or beets.

Responses of salivary nitrite to intake of nitrate in drinking water

Nitrate in distilled water was consumed by eight volunteers at approximately 10.30 a.m. on at least three occasions for each level of intake. Figure 3 shows the responses in salivary nitrite concentration to the ingestion by a male volunteer of the highest level of nitrate (50 mg nitrate) on five occasions. In general, low (below 5 mg/litre as nitrite) relatively constant nitrite levels were observed in this volunteer when other sources of nitrate were avoided as far as possible prior to challenge with nitrate in drinking water. In each case, elevated salivary nitrite levels resulted from the intake of nitrate in water, the increase being relatively constant in four out of the five instances. On the fifth occasion, a much smaller increase in nitrite concentration resulted for no obvious reason. When the observations were extended beyond the morning period and a mid-day meal was omitted, secondary smaller peaks were apparent.

Figure 4 shows the mean increases up to the highest salivary nitrite level recorded in all eight volunteers prior to the mid-day meal and following the

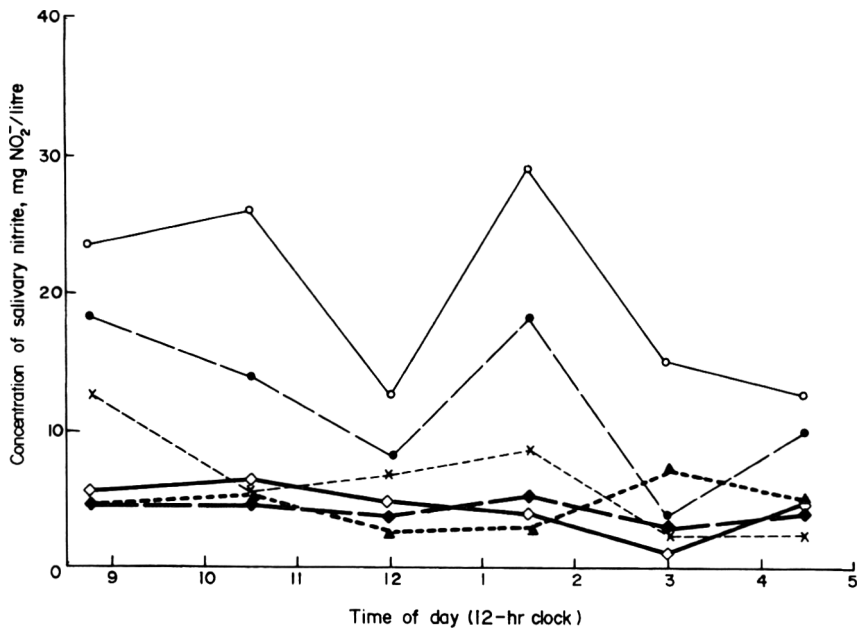

Fig. 1. Salivary nitrite levels of six volunteers during a day on unrestricted diets.

Table 1. *Nitrate contents of meals available to volunteers*

Meal	Weight (g)	Nitrate concentration (mg NO_3^-/kg)	Total nitrate (mg NO_3^-)
Main course			
Ham vol-au-vents, potatoes, beans	324	98	37
Chicken and ham pie, potatoes, mixed vegetables	317	39	12
Plaice, chipped potatoes, peas	305	48	14
Steak and kidney pie, potatoes, carrots	310	39	12
Sliced beef, potatoes, mixed vegetables, Yorkshire pudding	301	47	14
Beef cutlet, chipped potatoes	234	99	23
Dessert			
Ice-cream roll	98	32	3·0
Plum crumble and custard	291	18	5·2
Apple turnover	188	40	7·4

consumption of various amounts of water-borne nitrate. Up to a nitrate intake of about 10 mg NO_3^-, the variations between individuals in the increases in the salivary nitrite level were unlikely to be significant in most cases. Above this nitrate concentration, however, the increases in salivary nitrite levels were more obvious but varied considerably between volunteers. Of the two females included in the group, one showed a large increase in salivary nitrite concentration with increasing levels of nitrate in the drinking water whilst the other showed a far more gradual increase. On the basis of the small numbers of volunteers involved, therefore, it is unlikely that sex has contributed to the considerable variations observed in the responses to water-borne nitrate.

It was noted that four of the eight volunteers selected had a low and relatively constant resting salivary nitrite concentration during the early morning period up to the time of ingestion of the water-borne nitrate. The salivary nitrite level of the remaining four subjects all showed much greater falls during the pre-drink period. A plot of the mean increase in salivary

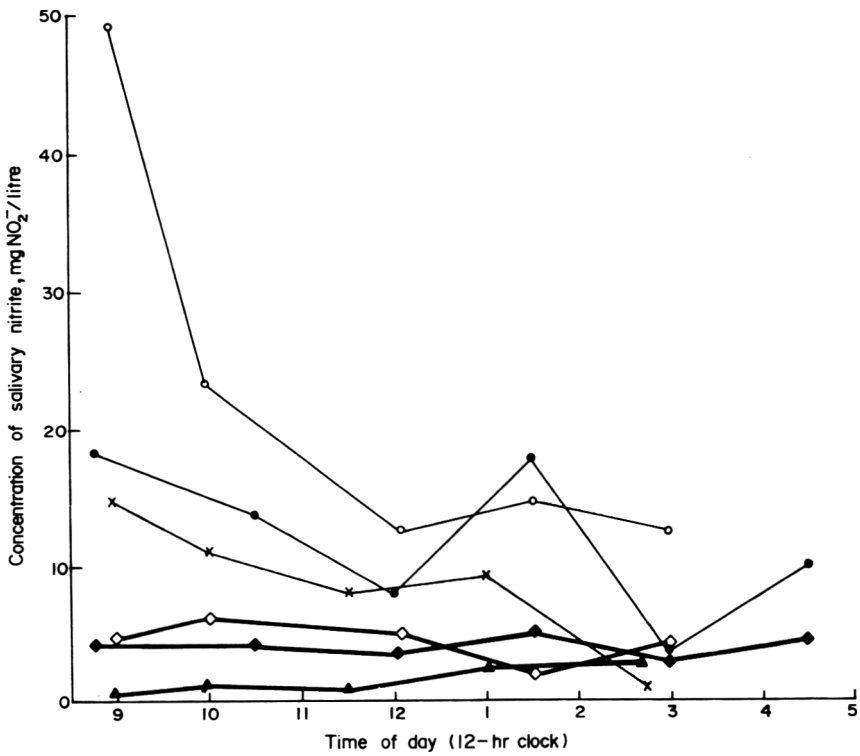

Fig. 2. Salivary nitrite levels in volunteers A (——) and B (——) during three days on unrestricted diets.

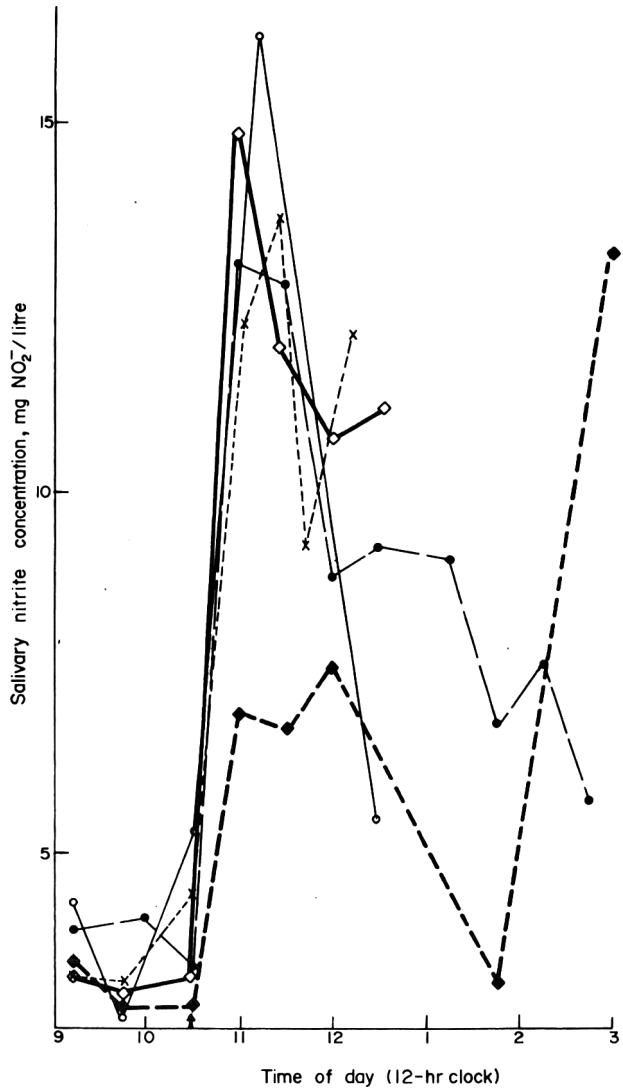

Fig. 3. Salivary nitrite responses to the ingestion of 50 mg nitrate ion by a male volunteer on five occasions.

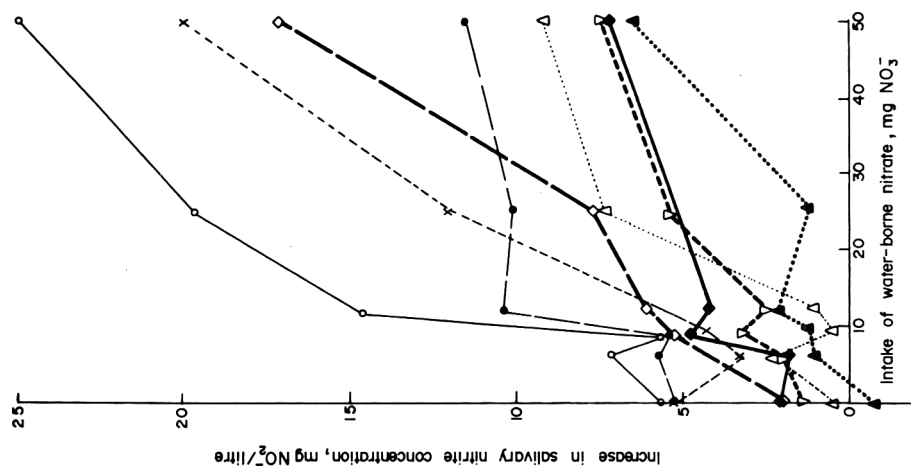

Fig. 4. Relationship of increase in salivary nitrite concentration over 1 hr to intake of water-borne nitrate by eight volunteers.

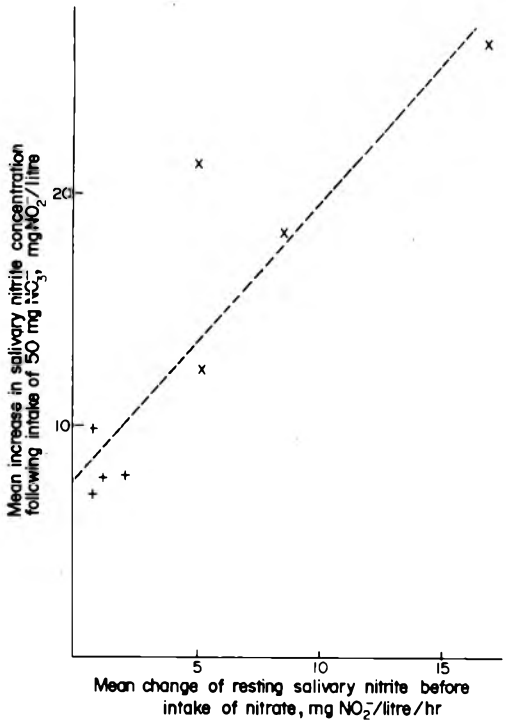

Fig. 5. Relationship of the increase in salivary nitrite concentration over 1 hr produced by the consumption of 50 mg NO_3^- ion to the fall in resting salivary nitrite concentration before consumption of nitrate. Volunteers who were less efficient at converting nitrate to nitrite (+); volunteers who were more efficient (×).

nitrite concentration following the consumption of 50 mg water-borne nitrate by all volunteers against the mean change of resting salivary nitrite in each before the intake of nitrate comprised essentially a straight line (Fig. 5), the coefficient of correlation for the relationship being 0·89. The values for the four volunteers less 'efficient' in converting nitrate to nitrite form a cluster while those for the more 'efficient' volunteers are far more dispersed.

DISCUSSION

The ingestion of nitrate at concentrations in excess of the limit recommended by WHO for continuous use led to elevated levels of nitrite in the saliva but the size of the increase varied considerably between individuals. This finding accords with that of Tannenbaum *et al.* (1976) who used celery juice as the nitrate source. As observed by Spiegelhalder *et al.* (1976) and other workers who used nitrate-rich vegetables and vegetable juices, the nitrite concentration in the saliva is also related to the amount of nitrate in the diet when that nitrate is given in water. Spiegelhalder *et al.* found that the average increase in salivary nitrite concentration relative to nitrate intake in vegetables was 20 mg NO_2^-/litre per 100 mg NO_3^-; this figure was exceeded by some volunteers consuming nitrate in water whilst the response of others was appreciably smaller. Spiegelhalder *et al.* (1976) also observed a threshold input of potassium nitrate in vegetables of the order of 54 mg below which the salivary nitrite concentration was unaltered but this was certainly not the case in all volunteers consuming nitrate in water. Evidence was obtained, however, of second smaller peaks of nitrite concentration resulting from the ingestion of nitrate, in keeping with the observations of Spiegelhalder *et al.* (1976) when they used much larger quantities of nitrate in vegetables or their juices. They ascribed the secondary and following peaks to absorption of nitrite swallowed in the saliva from the stomach into the blood stream where it was re-oxidized by oxyhaemoglobin to nitrate. Thus the nitrate formed was secreted by the salivary glands and reduced once more after a delay by the oral microflora.

The size of the increase in salivary nitrite concentration is probably more important in terms of the production of *N*-nitroso compounds than total nitrite production since the rate of nitrosation of a secondary amine is generally proportional to the square of the nitrite concentration. The variations in the capacity of subjects to convert nitrate ingested in water to nitrite were striking. The relationship of this capacity to the rate of fall in salivary nitrite concentration preceding nitrate intake adds weight to the concept of the participation of the individual's oral microflora in the reduction (Tannenbaum *et al.* 1976). This supposition assumes that the overnight intakes of nitrate by the volunteers were grossly similar, a situation which could not be completely controlled, and that individuals did not vary greatly their storage of nitrate *in vivo*, in the manner proposed by Tannenbaum *et al.* (1978). Nevertheless it is evident that the proportion of the small group of volunteers showing the greatest rates of fall in resting salivary nitrite concentrations were best able to convert water-borne nitrate into salivary nitrite.

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REFERENCES

Correa, P., Haenszel, W., Cuello, C., Tannenbaum, S. & Archer, M. (1975). A model for gastric cancer epidemiology. *Lancet* **ii**, 58.

Follett, M. J. & Ratcliff, P. W. (1963). Determination of nitrite and nitrate in meat products. *J. Sci. Fd Agric.* **14**, 138.

Harada, M., Ishiwata, H., Nakamura, Y., Tanimura, A. & Ishidate, M. (1975). Studies on *in vivo* formation of *N*-nitroso compounds. I. Changes of nitrite and nitrate concentrations in human saliva after ingestion of salted Chinese cabbage. *J. Fd Hyg. Soc., Japan* **16**, 11.

Hill, M. J., Hawksworth, G. & Tattersall, G. (1973). Bacteria, nitrosamines and cancer of the stomach. *Br. J. Cancer* **28**, 562.

Pfeiffer, C. J., Fodor, J. G. & Canning, E. (1973). An epidemiologic analysis of mortality and gastric cancer in Newfoundland. *Can. med. Ass. J.* **108**, 1374.

Shinn, M. B. (1941). Colorimetric method for determination of nitrite. *Ind. Engng Chem. analyt. Edn.* **13**, 33.

Spiegelhalder, B., Eisenbrand, G. & Preussmann, R. (1976). Influence of dietary nitrate on nitrite content of human saliva: possible relevance to *in vivo* formation of *N*-nitroso compounds. *Fd Cosmet. Toxicol.* **14**, 545.

Stephany, R. W. & Schuller, P. L. (1975). De aanwezigheid van nitriet in menselijk speeksel en het *N*-nitrosamine probleem. In Volksgezondheid, Verslagen, Adviezen,

Rapporten 33/34: Berichten RIV 1974 en *Liber Amicorum*. p. 184.

Sugimura, T. & Fujimura, S. (1967). Tumour production in glandular stomach of rat by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. *Nature, Lond.* **216,** 943.

Sugimura, T., Fujimura, S. & Baba, T. (1970). Tumor production in the glandular stomach and alimentary tract of the rat by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. *Cancer Res.* **30,** 455.

Tannenbaum, S. R., Fett, D., Young, V. R., Land, P. D. & Bruce, W. R. (1978). Nitrite and nitrate are formed by endogenous synthesis in the human intestine. *Science, N.Y.* **200,** 1487.

Tannenbaum, S. R., Sinskey, A. J., Weisman, M. & Bishop, W. (1974). Nitrite in human saliva. Its possible relationship to nitrosamine formation. *J. natn. Cancer Inst.* **53,** 79.

Tannenbaum, S. R., Weisman, M. & Fett, D. (1976). The effect of nitrate intake on nitrite formation in human saliva. *Fd Cosmet. Toxicol.* **14,** 549.

WHO (1977). Health Hazards from Drinking Water. Report of a Working Group, London, 26–30 September 1977. WHO Regional Office for Europe, Copenhagen, ICP/PPE/005.

Wynder, E. L., Kmet, J., Dungel, N. & Segi, M. (1963). An epidemiological investigation of gastric cancer. *Cancer, N.Y.* **16,** 1461.

Fd Cosmet. Toxicol. Vol. 19, pp. 303 to 310, 1981
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INDUCTION OF TUMOURS OF THE URINARY SYSTEM IN F344 RATS BY DIETARY ADMINISTRATION OF SODIUM *o*-PHENYLPHENATE

K. HIRAGA and T. FUJII

Department of Toxicology, Tokyo Metropolitan Research Laboratory of Public Health, 24-1, Hyakunincho 3 chome, Shinjuku-ku, Tokyo 160, Japan

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Abstract—Sodium *o*-phenylphenate (OPP-Na), a fungicide approved as a food additive in Japan, was given in pellet diets at dietary levels of 0, 0·125, 0·25, 0·5, 1·0, 2·0 or 4·0% to groups of about 10 male and female F344/Du rats for 13 wk and to male F344/Du rats for 91 wk. In the 13-wk study, urinary bladder tumours developed in one out of ten male rats fed 1% OPP-Na, nine out of ten male rats fed 2% OPP-Na, one out of ten male rats fed 4% OPP-Na and two out of ten female rats fed 4% OPP-Na. Five transitional cell carcinomas were observed in male rats fed 2% OPP-Na and one such carcinoma was found in a male rat fed 4% OPP-Na. In the 91-wk study, tumours of the urinary bladder, renal pelvis and renal papilla developed in one out of 21 rats (5%) fed 0·5% OPP-Na, seven out of 21 rats (33%) fed 1% OPP-Na, 20 out of 21 rats (95%) fed 2% OPP-Na and 17 out of 21 rats (85%) fed 4% OPP-Na. These were all transitional cell carcinomas, except for one carcinosarcoma in the 2% group. A dose-related increase in the incidence of non-neoplastic lesions of the kidney were observed in treated rats. In the 13-wk study, slight to moderate pyelonephritis was observed in six out of ten male rats and one out of ten female rats fed 4% OPP-Na. In the 91-wk study, moderate to severe pyelonephritis was observed in four out of 21 rats (19%) fed 2% OPP-Na and in all of 20 rats fed 4% OPP-Na.

INTRODUCTION

Sodium *o*-phenylphenate (OPP-Na), a fungicide, has been approved as a food additive for citrus fruits in Japan. It has been reported that OPP-Na has no teratogenic or mutagenic effect in mice (Ogata, Ando, Kubo & Hiraga, 1978a; Ogata, Yoshida, Nawai, Ando, Kubo, Hiraga & Masubuchi, 1978b). Neither does OPP-Na have marked suppressive effects on the immune response in mice (Sasaki & Nakao, 1978). No chromosomal aberrations attributable to OPP-Na have been demonstrated in CHO-KI cells (Nawai, Yoshida, Nakao & Hiraga, 1979; Yoshida, Nawai & Hiraga, 1979). The single oral LD_{50} of OPP-Na was 857 mg/kg in male mice, 812 mg/kg in female mice and 1096 mg/kg in male rats (Ogata, Ando, Kubo & Hiraga, 1979; Tayama, Iguchi & Hiraga, 1979). Signs of intoxication were observed in rats fed OPP-Na at 0·125, 0·25, 0·5, 1, 2 or 4% in the diet for 13 wk (Iguchi, Tayama & Hiraga, 1979).

The available information on the long-term toxicity of OPP-Na is limited; in this paper we report the occurrence of tumours of the urinary system in rats fed OPP-Na.

EXPERIMENTAL

Materials. OPP-Na used for the studies was technical-grade Dowicide A (Lot No. MM01044; ≥95% pure) obtained from Dow Chemical Co., MI, USA.

Animals. Male and female F344/DuCrj (Fischer) rats were obtained when 4 wk old from Charles River Japan Inc., Kanagawa. They were housed individually in stainless-steel cages, 40 × 25 × 16·5 cm, with wire-mesh fronts and floors. The cages were suspended from belt-type racks with an automatic water-supply system (*via* a bacteria filter). The animal room was maintained at 24–26°C, and the relative humidity at 50–60%. Incoming air was passed through a prefilter of 85% efficiency and a filter of 99% efficiency at the intake. Room air was changed ten times per hour. Fluorescent lighting was controlled to give 11 hr light (06·00–17·00 hr) and 13 hr dark. No other experiments were carried out in this room.

Administration of materials. OPP-Na was mixed with powdered diet CE-2 (Nihon Clea Co., Ltd, Tokyo), at concentrations of 0·125, 0·25, 0·5, 1·0, 2·0 and 4·0%, and the mixtures were made into pellets. The concentrations of OPP-Na in the pellets 10 wk after preparation were determined by gas chromatography to be 0·09, 0·19, 0·39, 0·90, 1·78 and 4·41%, respectively. Groups of ten male and ten female rats were fed diets containing 0, 0·125, 0·25, 0·5, 1·0, 2·0 or 4·0% OPP-Na for 13 wk. Groups of about 20 male rats were fed diets containing 0, 0·125, 0·25, 0·5, 1·0, 2·0 or 4·0% OPP-Na for 91 wk. Feed and water were supplied *ad lib.* The experiment was begun when the animals were 5 wk old.

Observation. All of the animals were observed twice daily for changes in behaviour. Animals killed when moribund and those dying during the studies were autopsied. Rats surviving to the end of the studies were killed and autopsied.

Pathological examination. The pathological examination consisted of gross and histological examinations of major tissues, major organs and all gross lesions. The tissues were preserved in 10% neutral buffered formalin, embedded in paraffin wax, sectioned, and stained with haematoxylin and eosin. Occasional sections were subjected to special techniques such as periodic acid Schiff, azan or Ayoub-Shklar keratin

Table 1. *Incidence of urinary bladder tumours in F344 rats fed sodium o-phenylphenate (OPP-Na) for 13 wk*

Dietary level of OPP-Na (%)	No. of rats evaluated*	No. of rats with urinary bladder tumour	No. of rats with	
			Papilloma	Transitional cell carcinoma
		Males		
0	10	0	0	0
0·125	10	0	0	0
0·25	10	0	0	0
0·5	9	0	0	0
1·0	10	1	1	0
2·0	10	9	4	5
4·0	10	1	0	1
		Females		
0	10	0	0	0
0·125	9	0	0	0
0·25	9	0	0	0
0·5	9	0	0	0
1·0	10	0	0	0
2·0	10	0	0	0
4·0	10	2	2	0

*Rats examined histologically.

and prekeratin stains for more definitive diagnosis. Urinary bladders were distended as far as possible with formalin. The criteria of Hicks, Wakefield, Vlasov & Pliss (1976) and Mostofi (1973) were used to classify urinary bladder tumours.

RESULTS

No rats died during the 13-wk study. In the 91-wk study, the survivals of rats fed 0, 0·125, 0·25, 0·5, 1, 2 or 4% OPP-Na were 90, 90, 95, 90, 90, 57 and 71%, respectively. The mean intakes of OPP-Na at the 0, 0·125, 0·25, 0·5, 1, 2 and 4% dietary levels were 85, 177, 353, 706, 1384 and 2487 mg/kg/day, respectively, in the males and 87, 177, 352, 706, 1338 and 2431 mg/kg/day in the females in the 13-wk study, and 62, 125, 250, 500, 1000 and 2000 mg/kg/day in the males in the 91-wk study. Haematuria was observed grossly in treated rats from 45 wk after the start of the administration. Haematuria was observed at each dose level although it did not occur continuously at each level. Haematuria was not observed in the controls.

Tumours attributed to the administration of OPP-Na were found in the urinary system (Tables 1 & 2). In the 13-wk study, urinary bladder tumours developed in male and female rats fed OPP-Na (Table 1). There were five malignant tumours among male rats fed 2% OPP-Na and one among those fed 4% OPP-Na. All of these tumours were transitional cell carcinomas. In the 91-wk study, tumours of the urinary bladder, renal pelvis and renal papilla developed in male rats fed OPP-Na (Table 2). All of these tumours were transitional cell carcinomas except for one which was a carcinosarcoma of the urinary bladder and which occurred in a rat fed 2% OPP-Na. The first tumour of the renal papilla and renal pelvis were

Table 2. *Incidence of tumours of the urinary system in male F344 rats fed sodium o-phenylphenate (OPP-Na) for 91 wk*

Dietary level of OPP-Na (%)	No. of rats evaluated†	No. of rats with tumours of the urinary system‡	No. of rats with transitional cell carcinoma of the		
			Renal papilla	Renal pelvis	Urinary bladder
0	20	0(0)	0	0	0
0·125	20	0(0)	0	0	0
0·25	20	0(0)*	0	0	0
0·5	21	1(5)*	1	1	0
1·0	21	7(35)*	0	1	6
2·0	21	20(95)*	1	0	19§
4·0	20	17(85)	10	3	8

†No. of rats that survived for longer than 1 wk and were examined histologically.

‡In brackets, the no. of rats with tumours of the urinary system expressed as a percentage of the no. of rats evaluated.

§A carcinosarcoma, showing both epithelial and connective tissue differentiation was also found in one rat in this dose group.

Values marked with asterisks were used to estimate the regression equation: Y (probit) = 5·65 × (log dose) + 4·75. There was a dose-related increase in the incidence of tumours at dietary levels between 0·5 and 2%.

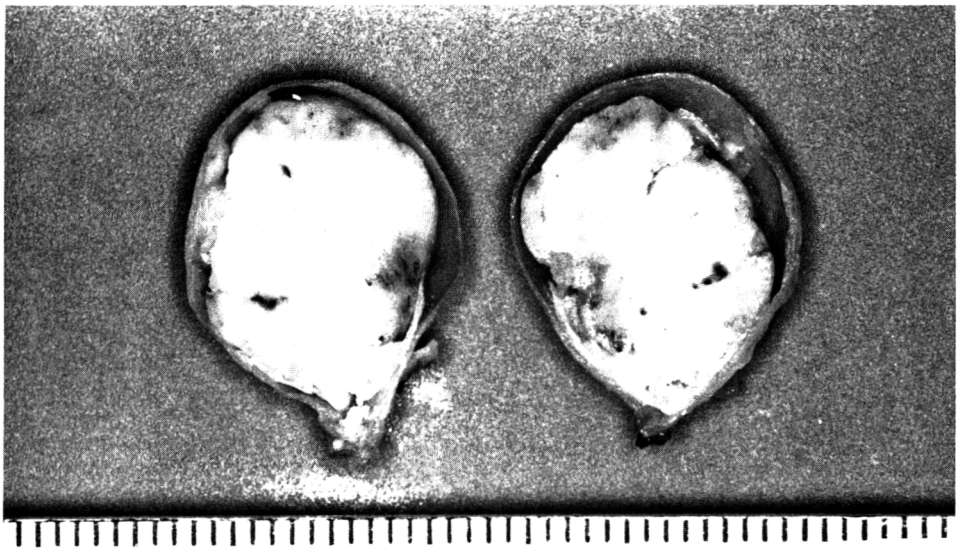

Fig. 1. Gross appearance of the cut surface of a urinary bladder tumour from a male rat fed sodium *o*-phenylphenate at 2% in the diet for 67 wk. Each division represents 1 mm.

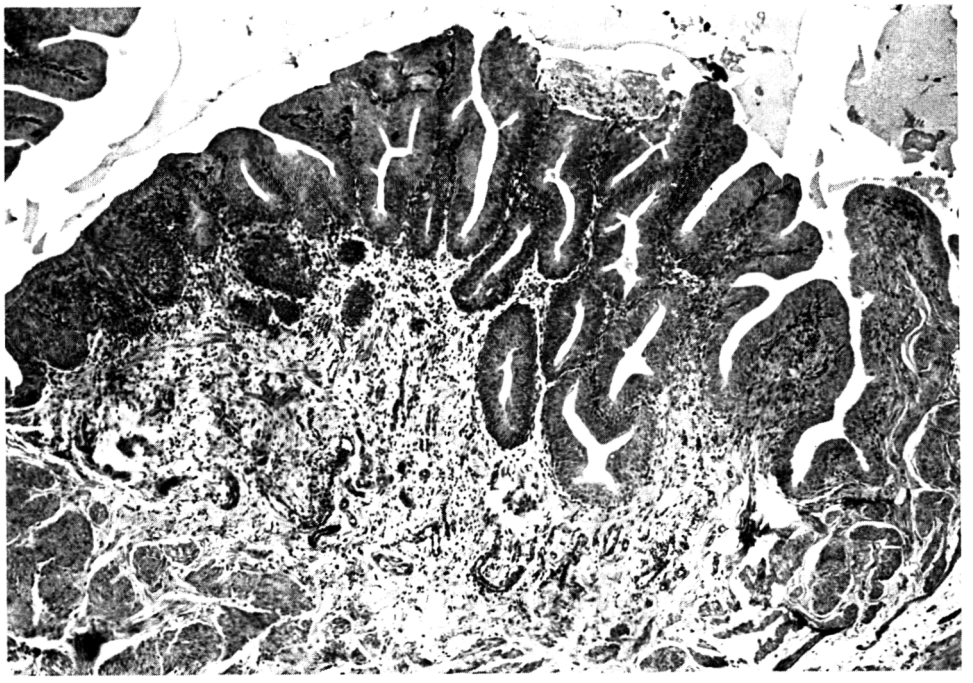

Fig. 2. Transitional cell carcinoma of the urinary bladder of a male rat fed sodium *o*-phenylphenate at 2% in the diet for 13 wk. The carcinoma shows an exophytic growth pattern. Haematoxylin and eosin ×40.

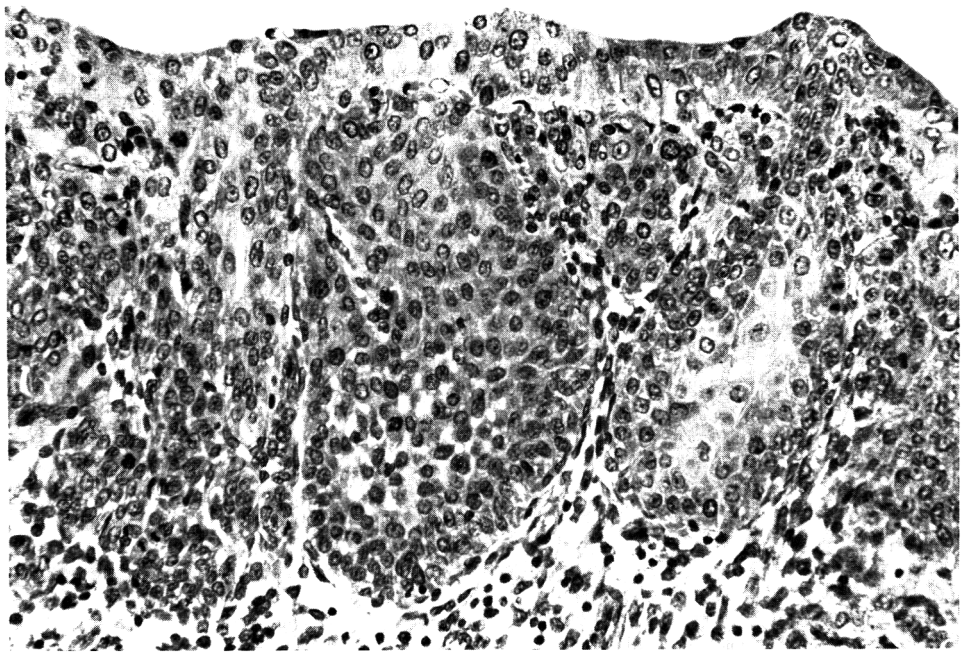

Fig. 3. Transitional cell carcinoma of the urinary bladder of a male rat fed sodium *o*-phenylphenate (2% in the diet) for 13 wk. Haematoxylin and eosin × 200.

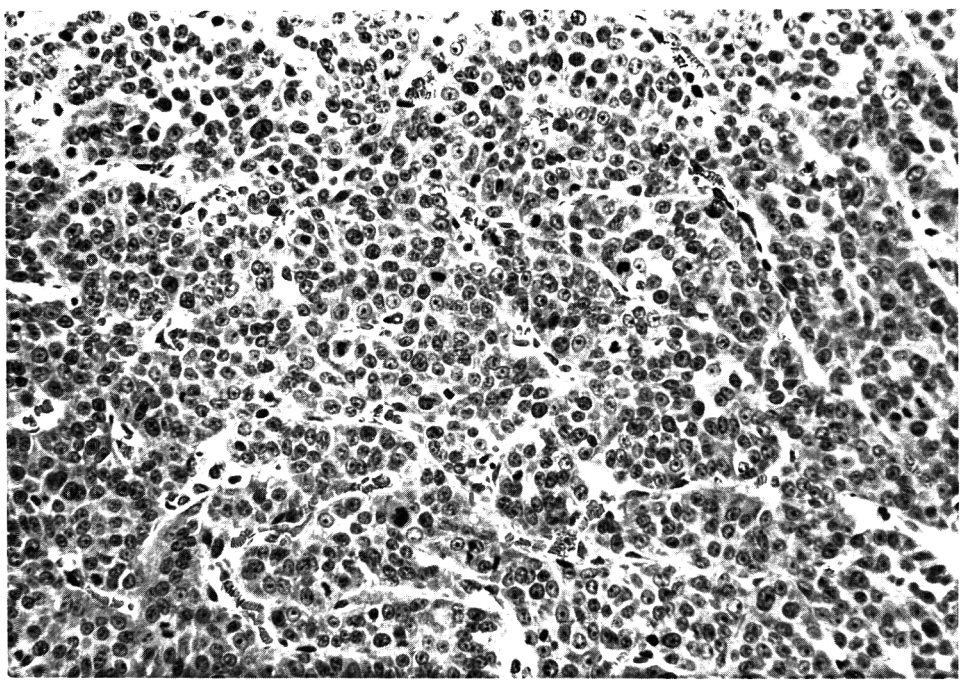

Fig. 4. Transitional cell carcinoma of the urinary bladder of a male rat fed sodium *o*-phenylphenate (1% in the diet) for 91 wk showing numerous mitotic figures. Haematoxylin and eosin × 200.

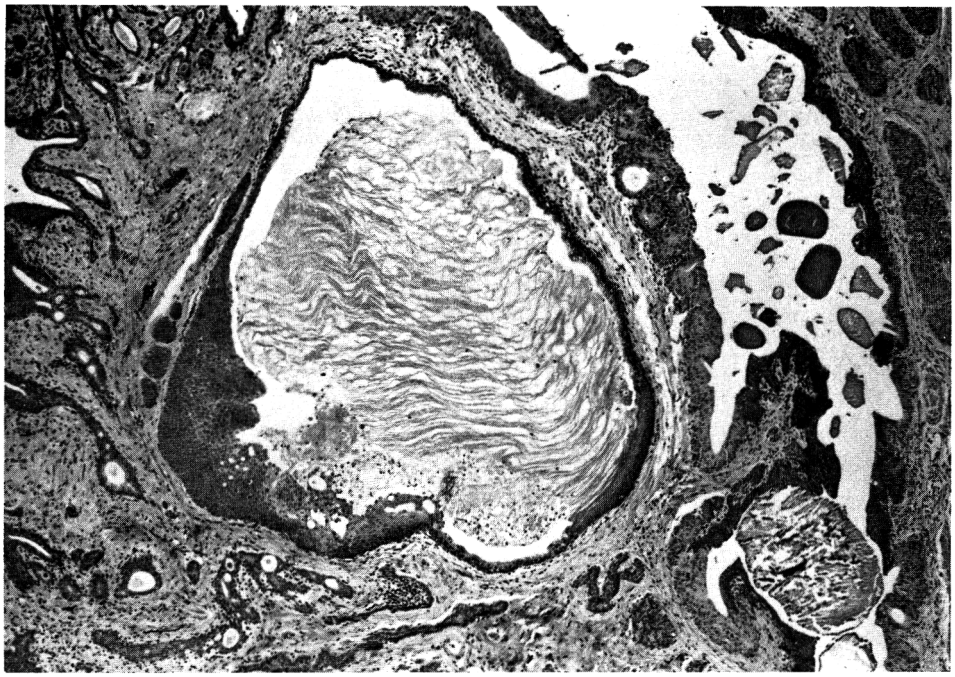

Fig. 5. Transitional cell carcinoma of the urinary bladder of a male rat fed sodium *o*-phenylphenate (2% in the diet) for 91 wk, showing squamous metaplasia with keratinization. Haematoxylin and eosin × 40.

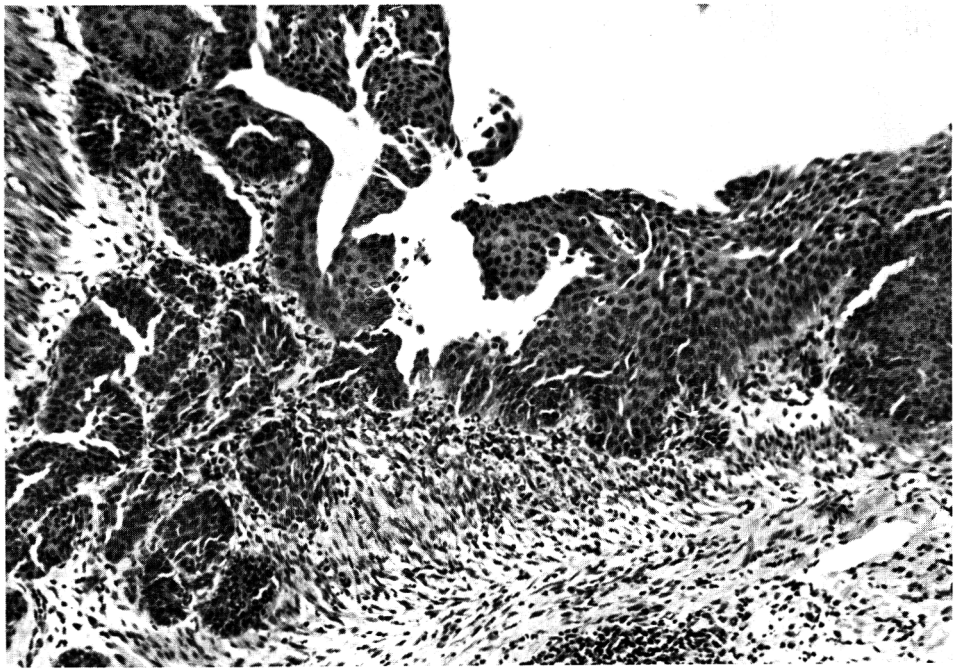

Fig. 6. Transitional cell carcinoma of the renal pelvis of a male rat fed sodium *o*-phenylphenate at 4% in the diet for 91 wk. Haematoxylin and eosin × 100.

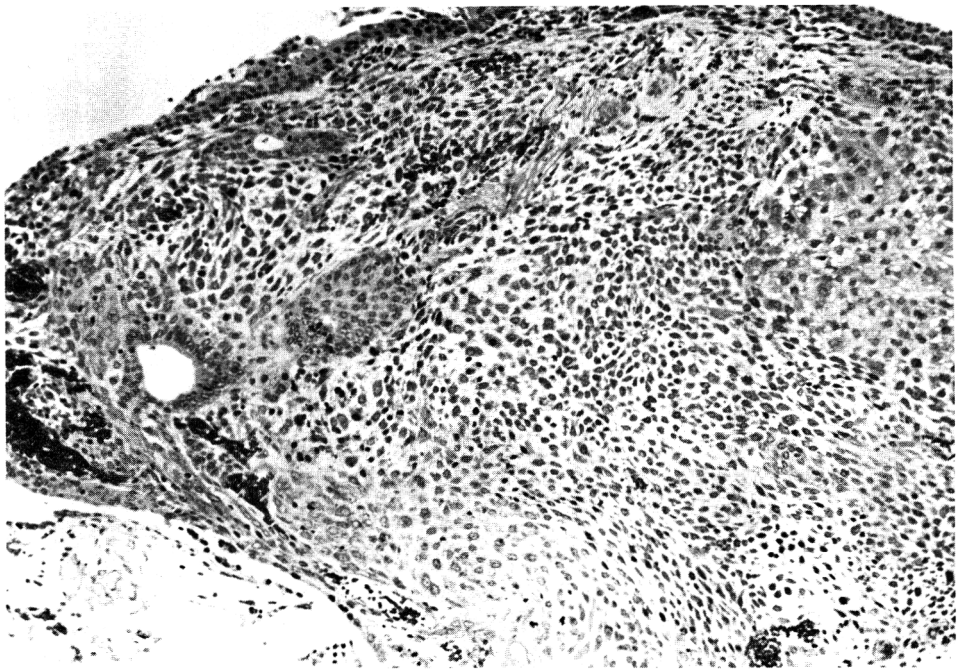

Fig. 7. Transitional cell carcinoma of the renal papilla of a male rat fed sodium *o*-phenylphenate (4% in the diet) for 91 wk. Haematoxylin and eosin × 100.

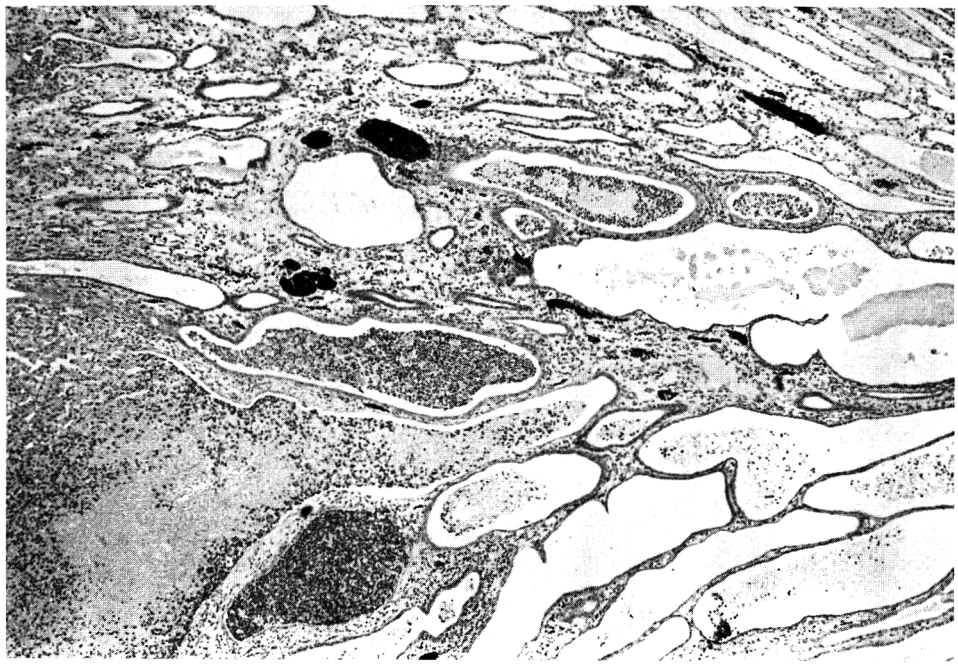

Fig. 8. Pyelonephritis with cystic dilation of the tubules in a male rat fed sodium *o*-phenylphenate at 4% in the diet for 91 wk. Haematoxylin and eosin × 40.

found in dead rats 79 and 89 wk after the start of experiment, respectively. There was a dose-related increase in the incidence of tumours of the urinary system at dietary levels of 0·5 to 2%.

Grossly, urinary bladder tumours were papillary or polyploid, growing into the lumen and having a cauliflower-like appearance (Fig. 1). Many of these tumours were multiple and the masses were about 1–30 mm in diameter. Bladder calculi, dark brown and having rough spiny surfaces were observed grossly in eight out of 34 rats with bladder tumours in the 91-wk study, but were not observed in the 13-wk study.

Histologically, benign tumours of the urinary bladder were papillomas, showing outgrowing epithelium with blood vessels and connective tissue. The papillomas were covered by a relatively normal looking epithelium without invasion into the muscle layer. Most of the malignant tumours of the urinary bladder (transitional cell carcinomas and one carcinosarcoma) grew out into the bladder lumen (Fig. 2), but some also grew back into the wall of the bladder. The transitional cell carcinomas contained relatively little connective tissue which was covered by transitional epithelium, and they showed various degrees of anaplasia (Fig. 3). Mitotic figures and focal areas of squamous or glandular metaplasia were often seen in the tumours (Figs 4 & 5). The one carcinosarcoma that was observed grew out into the bladder lumen and showed both connective tissue and epithelial differentiation.

Histological examination showed that all of the malignant tumours of the renal pelvis and renal papilla were transitional cell carcinomas. Tumours of the renal pelvis showed a slight exophytic growth pattern, an increase in the number of cells and pleomorphism in cell size and cell divisions (Fig. 6). Carcinomas of the renal papilla were composed of transitional epithelium with various degrees of anaplasia, and were poorly demarcated from the surrounded renal parenchyma (Fig. 7). No metastases to lymph nodes or to distant organs from these tumours of the urinary system were found. Neoplastic or non-neoplastic hyperplasia of the transitional epithelium in the renal papilla were observed in nine rats fed 4% OPP-Na.

No tumours at sites other than the urinary system were found in the 13-wk study. In the 91-wk study, interstitial cell tumours of the testes were the tumours most frequently observed at other sites. Tumours of the adrenal and pituitary glands were also found frequently. These tumours occurred with approximately equal frequency in test and control groups.

Non-neoplastic lesions attributed to the administration of OPP-Na were seen in the kidney. Histological examination showed these to be severe pyelonephritis. The lesion was contained in a wedge-shaped area, and showed a marked dilation of tubules, a flattening of the tubular epithelium, an increase of stroma and a moderate to marked infiltration of leucocytes (Fig. 8). In the 13-wk study, moderate pyelonephritis was observed in six out of ten male rats fed 4% OPP-Na and slight lesions were seen in one out of ten female rats fed 4% OPP-Na. In the 91-wk study, severe pyelonephritis, with papillary destruction and/or marked cystic dilation of the tubules, was found in 19 out of 20 of the rats fed 4% OPP-Na and in 1 out of 21 (5%) of the rats fed 2% OPP-Na. The incidence and severity of pyelonephritis in rats fed 4% OPP-Na was markedly greater than in other test and control groups.

DISCUSSION

The results demonstrate that, under the test conditions used, OPP-Na caused tumours of the urinary system in male and female rats. The majority of these tumours were malignant. In the 91-wk study, a dose-related increase in the incidence of the tumours occurred at dietary levels of 0·5 to 2% OPP-Na. Besides the results of the present studies, many tumours of the urinary tract have been observed in treated animals in a long-term (2-yr) study in which OPP-Na has been fed to male and female F344/Du rats (K. Hiraga, T. Fujii, S. Iguchi, K. Tayama and N. Yano, unpublished data, 1980). The reasons why the incidence of tumours of the urinary tract in male rats fed 4% OPP-Na in the 13- and the 91-wk study was lower than that in male rats fed 2% OPP-Na is a matter for speculation. No inflammatory or necrotic changes were observed in the bladder wall of rats fed 4% OPP-Na. The incidence of urinary bladder tumours in male rats fed 4% OPP-Na was lower than that of rats fed 2% OPP-Na. However, the incidence and severity of pyelonephritis in rats at the 4% dose level was markedly greater than that at the 2% level. It is possible that urinary bladder tumours cannot develop in male rats fed 4% OPP-Na because of the severe lesions in the kidney. The findings of a greater incidence and severity of pyelonephritis in rats fed 4% OPP-Na is similar to the observations of this lesion in rats fed diets containing 2% *o*-phenylphenol for 2 yr (Hodge, Maynard, Blachet, Spencer & Row, 1952). No tumours of the urinary tract were seen in the study by Hodge *et al.* (1952). A greater incidence of urinary bladder tumours occurred in male rats than in female rats in the present 13-wk study. Similar findings have been reported in feeding studies on sodium saccharin (Arnold, Moodie, Grice, Charbonneau, Stavric, Collins, McGuire, Zawidzka & Munro, 1980; Munro, Moodie, Krewski & Grice, 1975; Taylor, Weinberger & Friedman, 1980).

Factors that have been reported to play a role in bladder carcinogenesis include interaction between the test compound and other components of the diet, impurities within the compound, the alkalinity of urine and the presence of bladder calculi (Arnold *et al.* 1980; Clayson, 1974; Weil, Carpenter & Smith, 1965 & 1967). It is possible that one or more of these factors may have contributed to the results observed in the present studies and there seems to be an urgent need for further studies of the mechanisms by which sodium *o*-phenylphenate produces tumours of the urinary tract.

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REFERENCES

Arnold, D. L., Moodie, C. A., Grice, H. C., Charbonneau, S. M., Stavric, B., Collins, B. T., McGuire, P. F., Zawidzka, Z. Z. & Munro, I. C. (1980). Long-term toxicity of *ortho*-toluenesulfonamide and sodium saccharin in the rat. *Toxic. appl. Pharmac.* **52,** 113.

Clayson, D. B. (1974) Bladder carcinogenesis in rats and mice: Possibility of artifacts. *J. natn. Cancer Inst.* **52,** 1685.

Hicks, R. M., Wakefield, J. St. J., Vlasov, N. N. & Pliss, G. B. (1976). *Pathology of Tumors in Laboratory Animals.* Vol. 1—*Tumors of the Rat.* Part 2. Edited by V. S. Turusov. p. 103. International Agency for Research on Cancer, Lyon, France.

Hodge, H. C., Maynard, E. A., Blanchet, H. J., Jr, Spencer, H. C. & Row, V. K. (1952). Toxicological studies of orthophenylphenol (Dowicide 1). *J. Pharmac. exp. Ther.* **104,** 202.

Iguchi, S., Tayama, K. & Hiraga, K. (1979). Subacute Toxicity of Sodium o-Phenylphenate by Food Administration to Rats. Annual Report of the Tokyo Metropolitan Research Laboratory of Public Health **30**(2), 67.

Mostofi, F. K. (1973). *International Histological Classification of Tumors.* No. 10. *Histological Typing of Urinary Bladder Tumors.* p. 13. World Health Organization, Geneva, Switzerland.

Munro, I. C., Moodie, C. A., Krewski, D. & Grice, H. C. (1975). A carcinogenicity study of commercial saccharin in the rat. *Toxic. appl. Pharmac.* **32,** 513.

Nawai, S., Yoshida, S., Nakao, T. & Hiraga, K. (1979). Examination of Mutagens by Induced Sister Chromatid Exchange (SCE) (II): Test of Two Fungicides by Induced SCE, *in vitro.* Annual Report of the Tokyo Metropolitan Research Laboratory of Public Health **30**(2), 57.

Ogata, A., Ando, H., Kubo, Y. & Hiraga, K. (1979). Acute Oral Toxicity Tests of *o*-Phenylphenate (OOP-Na) in Mice. Annual Report of the Tokyo Metropolitan Research Laboratory of Public Health **30**(2), 54.

Ogata, A., Ando, H., Kubo, Y. & Hiraga, K. (1978a). Teratological Tests of o-Phenylphenol (OPP) and Sodium o-Phenylphenol (OPP-Na) in Mice. Annual Report of the Tokyo Metropolitan Research Laboratory of Public Health, **29**(2), 89.

Ogata, A., Yoshida, S., Nawai, S., Ando, H., Kubo, Y., Hiraga, K. & Masubuchi, M. (1978b). Dominant Lethal Tests of Long Term Administration with Sodium o-Phenylphenol (OPP-Na) in Mice. Annual Report of the Tokyo Metropolitan Research Laboratory of Public Health **29**(2), 99.

Sasaki, M. & Nakao, T. (1968). Effect of o-Phenylphenol on Immune Response *in vitro.* Annual Report of the Tokyo Metropolitan Research Laboratory of Public Health **29**(2), 109.

Tayama, K., Iguchi, S. & Hiraga, K. (1979). Acute Oral Toxicity of Sodium o-Phenylphenate (OPP-Na) in Rats. Annual Report of the Tokyo Metropolitan Research Laboratory of Public Health **30**(2), 57.

Taylor, J. M., Weinberger, M. A. & Friedman, L. (1980). Chronic toxicity and carcinogenicity to the urinary bladder of sodium saccharin in the *in utero*-exposed rat. *Toxic. appl. Pharmac.* **54,** 57.

Weil, C. S., Carpenter, C. P. & Smyth, H. F. (1965). Urinary bladder response to diethylene glycol: Calculi and tumors following repeated feeding and implants. *Archs envir. Hlth* **11,** 569.

Weil, C. S., Carpenter, C. P. & Smyth, H. F. (1967). Urinary bladder calculus and tumor response following either repeated feeding of diethylene glycol or calcium oxalate stone implantation. *Ind. Med. Surg.* **35,** 55.

Yoshida, S., Nawai, S. & Hiraga, K. (1979). Cytogenic Studies of Sodium o-Phenylphenate. Annual Report of the Tokyo Metropolitan Research Laboratory of Public Health **30**(2), 44.

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TOXICITY OF *PENICILLIUM ITALICUM* TO LABORATORY ANIMALS

N. P. J. KRIEK* and F. C. WEHNER

National Research Institute for Nutritional Diseases of the South African Medical Research Council, P.O. Box 70, Tygerberg 7505, Republic of South Africa

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Abstract—The toxicity to ducklings and rats of a strain of *Penicillium italicum* isolated from an orange is described. Apart from a reduction in live-weight gain, feeding *P. italicum* culture material to ducklings at a dietary level of 50% (w/w) had no apparent detrimental effect over a period of 14 days. When the culture material was fed to rats at a dietary level of 8 or 32% (w/w) five out of six rats given the higher dose level died. The mean time to death was 78 days. A prominent cirrhosis of the liver was the predominant pathological change. This cirrhosis was characterized histologically by pseudolobulation, nodular hyperplasia, scant bile-duct proliferation and the presence of various numbers of anaplastic cells.

INTRODUCTION

Penicillium italicum Wehmer is the causative agent of blue rot of citrus fruit, a post-harvest disease of widespread occurrence (Raper & Thom, 1968). Although the fungus is primarily found on citrus, it has also been isolated from meat products (Fiedler, 1973) and rice (Saito, Ohtsuba, Umeda, Enomoto, Kurata, Udagawa, Sakabe & Ichinoe, 1971).

Information on the toxicity of *P. italicum* is scarce. As far as can be established, the report by Saito *et al.* (1971) on the acute toxicity of culture extracts of a strain of *P. italicum* isolated from rice to HeLa cells and mice, is the only one indicating the production of a toxin by this fungus. Other non-citrus isolates proved to be non-toxic (Fieldler, 1973). These isolates did not produce patulin (Borkowska Opacka & Escoula, 1977) or tremorgens like some of the other species of the section Asymmetrica–Fasciculata (Ciegler & Pitt, 1970) to which *P. italicum* belongs. *P. italicum* has furthermore been implicated as one of the causative agents of extrinsic allergic alveolitis amongst workers in a molasses fermentation plant (Hořejší, Šach, Tomšíková & Mecl, 1960).

In view of the absence of information on mycotoxin production by *P. italicum* and the importance of this fungus as a post-harvest pathogen on citrus fruit, an investigation was undertaken to determine its toxicity to experimental animals. In this paper the results are presented of short-term toxicity trials on ducklings and rats using a strain of *P. italicum* isolated from an orange.

EXPERIMENTAL

Isolation of fungi. An orange showing typical signs of blue mould infection was obtained at a local supermarket. *P. italicum* was readily isolated in pure culture from the lesions. The isolate, designated MRC 1360, proved to be capable of causing typical blue rot symptoms when reinoculated onto oranges.

Preparation of bulk cultures. Yellow maize kernels in 2-litre glass jars (400 g maize kernels and 400 ml tap water/jar) were autoclaved at 121°C for 1 hr on each of 2 consecutive days. The autoclaved maize was inoculated with a conidial suspension of *P. italicum* (MRC 1360) and incubated at 25 ± 2°C in the dark for 21 days. After incubation, the contents of the jars were dried at 45°C for 2 hr and ground in a laboratory mill. The meal was stored at 5°C until the start of the experiment and during the course of the toxicity trials.

Toxicity trial in ducklings. A diet was prepared by mixing the ground culture material with an equal weight of commercial chicken mash. The control diet contained 50% ground, autoclaved, uninoculated maize in commercial chicken mash. The diets were fed *ad lib.* to groups of eight 1-day-old Pekin ducklings (average weight, 50 g). The ducklings were weighed at the start of the experiment and at weekly intervals thereafter. The mean feed intake was estimated from the amount of feed remaining at the conclusion of the trial after 14 days.

Toxicity trial in rats. Weanling male BD IX rats (average weight, 76 g) were randomly divided into four groups of six rats each and caged two to a cage. The rats were housed in a controlled environment room (25°C and 50% relative humidity) with a 12 hr light/12 hr dark cycle. Ground culture material of MRC 1360 was incorporated into commercial rat mash at concentrations of 8 and 32% (w/w) and fed *ad lib.* to two groups of rats. Control diets consisted of 8 or 32% (w/w) of ground, autoclaved, uninoculated maize in commercial rat mash. All of the animals were weighed at the start of the experiment and at weekly intervals thereafter. The rats were observed daily throughout the course of the experiment and all clinical signs were recorded. All animals that died and the survivors that were killed on day 117 with an intraperitoneal overdose of pentobarbital-sodium, were autopsied and specimens of all the organs and tissues were collected in 10% buffered formalin.

*Present address: Department of Pathology, Faculty of Veterinary Science, University of Pretoria, P.O. Box 12580, Onderstepoort 0110, Republic of South Africa.

Table 1. *Toxicity of* Penicillium italicum *(MRC 1360) culture material to ducklings*

Treatment*	Mean weight gain† after 7 days	Mean weight gain† after 14 days	No. of deaths	Feed consumed (%)†
P. italicum (MRC 1360)	30·5	75·0	0	948
Control	85·5	218·1	0	2400

**P. italicum* culture material was mixed with commercial chicken mash (50:50, w/w) and fed *ad lib.* for 14 days. The control diet consisted of ground, autoclaved uninoculated maize meal mixed with commercial chicken mash (50:50, w/w).

†Determined from the start of treatment. The mean weight of the ducklings at the start of treatment was 50 g.

Values are means for groups of eight ducklings.

Haematoxylin and eosin-stained sections were examined with a light microscope.

RESULTS

Toxicity trial in ducklings

Compared with the ducklings given the control diet, there were only marked reductions in weight gain and feed consumption in the experimental group after 14 days (Table 1). No histological examination was carried out on these animals.

Toxicity trial in rats

The rats given the culture material at the 32% dietary level were in a poor condition and showed slight abdominal distention before death. The mean weight at death, mean number of days to death and the number of animals that died during the experiment are recorded in Table 2. The weight gain of the rats in the high-dose group was markedly depressed and five of the six rats in this group died. No reduction in weight gain was observed in any of the experimental groups at a stage comparable to the time at which it was observed in the ducklings. No deaths occurred in the other experimental group or in the control groups. The final weights of the rats given the culture material at the 8% dietary level were only slightly reduced when compared to the two control groups.

Gross pathology. In all of the rats that died and in the one survivor in the high-dose group, the liver was the main organ affected. It showed various degrees of micronodular cirrhosis which was accompanied by signs of marked portal hypertension. Extensive varices were present medial to the left kidney and peri-oesophageal veins. Splenomegaly, ascites and hydrothorax were present in all of the rats that were affected.

No macroscopic changes were observed in the rats in the low-dose group or in those given the control diets.

Histopathology. The changes observed in the livers of different rats in the 32% dose group were basically similar but varied in the extent of development according to the time to death. Cirrhosis was extensive and the entire lobular structure was replaced by pseudolobules and regenerative nodules (Fig. 1). The hepatocytes within the nodules were generally the same size but showed various intensities of basophilic cytoplasmic staining. The nuclei varied in size; some were enlarged, oval shaped and contained prominent nucleoli and a reticular chromatin arrangement.

The connective tissue septae contained numerous single hepatocytes or clusters of these cells. Scattered single-cell hepatocellular necrosis and necrosis of hepatocyte cell clusters attended by a slight neutrophil infiltrate were evident. A large proportion of the hepatocytes in the connective tissue septae were anaplastic. The nuclear:cytoplasmic ratio was either markedly increased or reduced while the most prominent changes occurred in the nuclei. They were usually grossly enlarged, of an uneven shape and outline, occasionally lobulated and contained numerous large nucleoli and a reticular chromatin pattern. Large pseudo-inclusions were seen in some of the nuclei. Prominent megalocytosis was characteristic and numerous bi- and multinucleated hepatocytes were observed (Fig. 2). The extent of bile-duct proliferation varied from rat to rat but was never very extensive. The cells of the proliferating bile ducts were flat, contained flattened, elongated and hyperchromatic nuclei, and the ducts had attenuated lumens. These ducts were often branched thus imparting a stellate appearance to the structures. Abundant lipofuscin deposits occurred within the connective tissue septae.

No specific changes were seen in any of the other organs. Neither the liver, nor any other organ in the rats given the low-dose diet or either of the control diets, was affected.

Table 2. *Toxicity of* Penicillium italicum *(MRC 1360) culture material to rats*

Diet*	No. of deaths	Mean day of death (range)†	Final weight (mean ± 1 SD)
8% *P. italicum*	0	—	250 ± 10·1
32% *P. italicum*	5	78 (63–117)	113 ± 14·9
Control (8% maize meal)	0	—	288 ± 37·5
Control (32% maize meal)	0	—	293 ± 13·7

**P. italicum* culture material was incorporated into rat mash at a level of 8 or 32% (w/w). The control diets contained uninoculated ground autoclaved maize in rat mash at a level of 8 or 32%.

†From the start of the experiment. The experiment was terminated after 117 days.

Values are for groups of six rats, housed two to a cage.

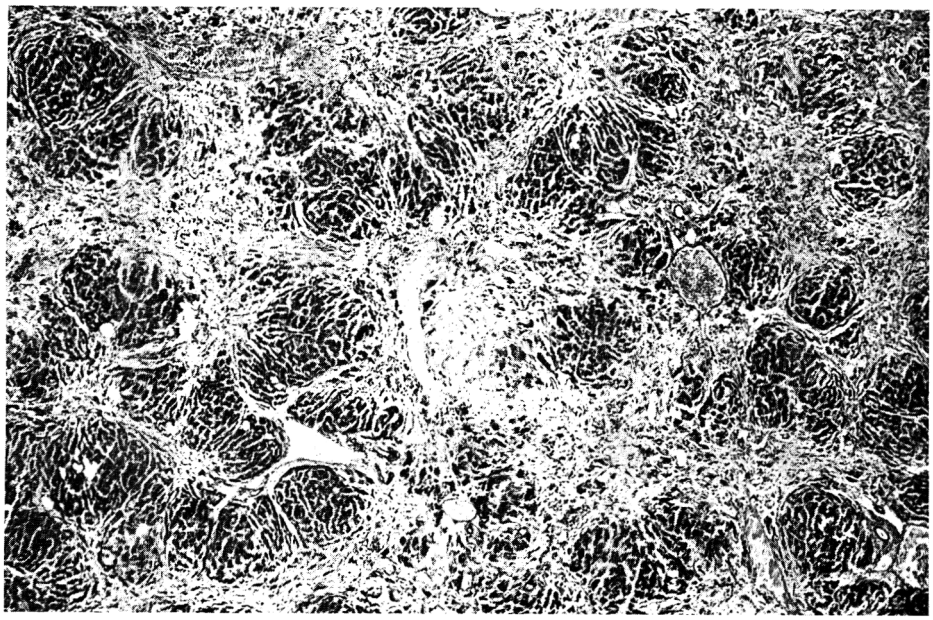

Fig. 1. Marked cirrhosis and nodular hyperplasia of the liver of a rat fed *Penicillium italicum* culture material at a dietary level of 32%. Haematoxylin and eosin × 60.

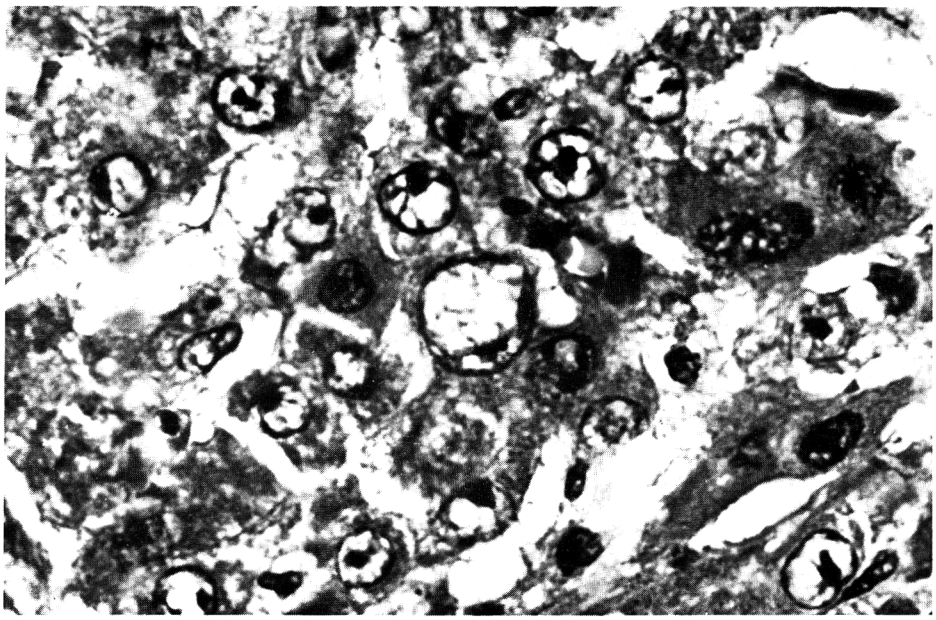

Fig. 2. Megalocytosis and a multinucleated hepatocyte in the connective tissue septae of the liver of a rat fed *Penicillium italicum* culture material at a dietary level of 32%. Haematoxylin and eosin × 500.

DISCUSSION

This is the first report of a toxic strain of *P. italicum* isolated from citrus. The nature of its toxicity at the levels fed was not acute and it resulted in the insidious development over an average of 11 wk of a marked and almost invariably fatal liver cirrhosis in the group consuming a high concentration of culture material in the diet. The fact that lesions only occurred at this high dietary level may be ascribed to a relative low level of toxicity or to a low concentration of the toxic component or components in the culture material.

A very important aspect arising from this investigation is the fact that in spite of the negative results of acute, short-term toxicity screening in ducklings, feeding the culture material to rats over a longer period resulted in the deaths of five out of six rats at the 32% dietary level after a mean exposure period of 78 days. This brings into question the value of acute, short-term experiments commonly used as initial biological screening procedures. The severity of the lesions observed emphasizes the need to employ more critical screening procedures since low-level, long-term exposure is considered to be of more importance in respect of mycotoxin intake.

The nature of the lesions observed in rats in this study bears a close similarity to that caused by *Penicillium islandicum* and in particular to the toxin cyclochlorotine (Carlton & Szczech, 1978). Certain hepatocellular changes, particularly the marked pleomorphism and anaplasia which were observed, raise the possibility that the associated toxin(s) may be carcinogenic.

The results of this investigation indicate a possible health hazard associated with the consumption of citrus products infected with *P. italicum*. However, many aspects of the toxicity of this fungus still need to be clarified. These include: (a) the nature of the toxin or toxins concerned; (b) the incidence of toxicity amongst isolates of *P. italicum*; (c) toxin production by *P. italicum* in citrus; (d) environmental factors influencing the production of the toxin; (e) chronic effects in laboratory animals feeding on rations containing low concentrations of *P. italicum*. These aspects are currently being investigated.

REFERENCES

Borkowska Opacka, B. & Escoula, L. (1977). Production de la patuline en milieu liquide par des moisissures appartenant aux genres: *Aspergillus* et *Penicillium*. *Annls Rech. Vét.* **8**, 129.

Carlton, W. W. & Szczech, G. M. (1978). Mycotoxicoses in laboratory animals. In *Mycotoxic Fungi, Mycotoxins, Mycotoxicoses: An Encyclopedic Handbook*. Edited by T. D. Wyllie & L. G. Moorhouse. Vol. 1. p. 377. Marcel Dekker, New York.

Ciegler, A. & Pitt, J. I. (1970). Survey of the genus *Penicillium* for tremorgenic toxin production. *Mycopath. Mycol. appl.* **42**, 119.

Fiedler, H. (1973). Schimmelpilzdiagnostik und Mykotoxinnachweis an Fleischprodukten. *Arch. Lebensmittelhyg.* **24**, 180.

Hořejší, D., Šach, J., Tomšíková, M. & Mecl, A. (1960). A syndrome resembling farmer's lung in workers inhaling spores of *Aspergillus* and Penicillia moulds. *Thorax* **15**, 212.

Raper, K. B. & Thom, C. (1968). *A Manual of the Penicillia*. p. 529. Hafner Publishing Company, New York & London.

Saito, M., Ohtsubo, K., Umeda, M., Enomoto, M., Kurata, H., Udagawa, S., Sakabe, F. & Ichinoe, M. (1971). Screening tests using HeLa cells and mice for detection of mycotoxin-producing fungi isolated from foodstuffs. *Jap. J. exp. Med.* **41**, 1.

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LIFESPAN ORAL TOXICITY STUDY OF VINYL CHLORIDE IN RATS*

V. J. FERON, C. F. M. HENDRIKSEN, A. J. SPEEK,
H. P. TIL and B. J. SPIT

Institute CIVO—Toxicology and Nutrition TNO, PO Box 360, 3700 AJ, Zeist, The Netherlands

(*Received 8 July 1980*)

Abstract—A lifespan oral toxicity study of vinyl chloride monomer (VCM) was carried out in Wistar rats, using five groups each of 60–80 males and 60–80 females. VCM was administered by incorporating polyvinyl chloride (PVC) powder with a high VCM content into the diet or by gastric intubation of a 10% VCM solution in soya-bean oil. The VCM doses (actual exposures) were 0 (control), 1·7, 5·0 and 14·1 mg/kg body weight/day provided by diets containing PVC powder, and 300 mg/kg body weight given by stomach tube as a solution of VCM in oil on 5 days/wk. The death rate was higher in all VCM-treated groups than in the controls and increased with increasing VCM doses. The 14·1- and 300-mg/kg treatments were associated with shortened blood-clotting times, slightly increased α-foetoprotein levels in the blood serum, liver enlargement and an increased haematopoietic activity in the spleen. A variety of neoplastic and non-neoplastic treatment-related liver lesions was found at each of the VCM levels. The changes varied from swollen and irregularly-shaped mitochondria in hepatocytes to hepatocellular carcinomas and hepatic angiosarcomas. The tumour response of the liver appeared to shift from a predominance of angiosarcomas at the highest dose level *via* a mixture of angiosarcomas and hepatocellular tumours at the intermediate levels to the exclusive development of hepatocellular tumours at the lowest VCM level. Tumours attributable to VCM exposure and found at other sites included pulmonary angiosarcomas, extrahepatic abdominal angiosarcomas and tumours of the Zymbal glands; these neoplasms occurred at VCM levels of 5·0 mg/kg and above. In addition, there was some evidence that VCM exposure enhanced the development of abdominal mesotheliomas and of adenocarcinomas of the mammary glands. Thus, the present study showed that orally administered VCM is a carcinogen in rats, and that the "no-observed-adverse-effect level" in rats was lower than 1·7 mg/kg body weight/day under the rigorous conditions of continuous oral VCM exposure resulting from the release of VCM from PVC powder present in the gastro-intestinal tract.

INTRODUCTION

Industrial exposure to vinyl chloride monomer (VCM) has been associated with disorders such as acro-osteolysis, non-malignant liver disease, angiosarcoma and carcinoma of the liver, and tumours of the brain and lungs and of the lymphatic and haematopoietic systems (Haley, 1975; Hopkins, 1980; Monson, Peters & Johnson, 1974; Rawls, 1980; Thomas & Popper, 1975; Vale, Kipling & Walker, 1976; Waxweiler, Stringer, Wagoner, Jones, Falk & Carter, 1976). In addition, various types of tumours, such as hepatic and extrahepatic angiosarcomas, hepatocellular tumours, mammary-gland carcinomas, Zymbal-gland tumours, nephroblastomas, brain tumours, pulmonary adenomas and nasal olfactory carcinomas, as well as a series of non-neoplastic lesions in several organs, have been found in a number of animal species after prolonged exposure to atmospheres containing VCM at sufficiently high concentrations (Basalaev, Vazin & Kotchetkov, 1972; Feron & Kroes, 1979; Lee, Bhandari, Winston, House, Dixon & Woods, 1978; Maltoni & Lefemine, 1975; Suzuki, 1978; Torkelson, Oyen & Rowe, 1961; Williamson, 1976; Winell, Holmberg & Kronevi, 1976).

Residual VCM present in extruded polyvinyl chloride (PVC) has been shown to be liable to migrate into PVC-packed foods and drinks (Daniels & Proctor, 1975; Fuchs, Gawell, Albanus & Slorach, 1975; Potter, 1976; Randolph, 1973; Williams & Miles, 1975). There is still only a small amount of data on the oral toxicity of VCM. In a 13-wk toxicity study, in which the monomer was dissolved in soya-bean oil and administered to rats at levels of 0, 30, 100 or 300 mg/kg body weight, once daily on 6 days/wk, the no-effect-level was conservatively placed at 30 mg/kg body weight, but was probably higher since the effects at the higher doses were of doubtful toxicological significance (Feron, Speek, Willems, van Battum & de Groot, 1975). From preliminary observations it appeared that a more practical method for chronic oral exposure of rats to VCM is the feeding of diets containing PVC powder with a high VCM content (Feron *et al.* 1975). Therefore, in the present lifespan study, PVC powder containing VCM was incorporated in the diet at levels to give planned daily intakes of 1, 3 or 10 mg VCM/kg body weight. This method does not allow dietary VCM intakes much higher than 10 mg/kg body weight/day, a dose that is, however, very high in comparison with a recent estimate of the maximum likely oral daily intake by man, i.e. 0·0017 μg VCM/kg body weight/day or 0·1 μg VCM/

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man/day (Ministry of Agriculture, Fisheries and Food, 1978). On the other hand, 10 mg VCM/kg body weight/day is low when compared with the doses used in the aforementioned 13-wk study (Feron *et al.* 1975). Since a chronic toxicity study should include at least one effect-producing level, a group of rats given VCM in soya-bean oil by gavage at a level as high as 300 mg/kg body weight/day (on 5 days/wk) was included in the present experiment, despite the disadvantages of gastric intubation.

EXPERIMENTAL

Test materials. Vinyl chloride monomer (VCM) was obtained from Akzo Zout Chemie, Rotterdam, in pressurized stainless-steel cylinders. The product had the following standard specification: vinyl chloride monomer $\geqslant 99{\cdot}97\%$ w/w; acetylene $\leqslant 2$, monovinylacetylene $\leqslant 15$, 1,3-butadiene $\leqslant 10$, methyl chloride $\leqslant 75$, ethyl chloride $\leqslant 50$, chloroprene $\leqslant 1$, 1,1-dichloroethane $\leqslant 1$ and 1,2-dichloroethane $\leqslant 20$ μl/litre (gas); acetaldehyde $\leqslant 5$, hydrochloric acid $\leqslant 1$, iron $\leqslant 0{\cdot}5$, water $\leqslant 100$ and evaporation residue $\leqslant 10$ mg/kg.

PVC powder (Carina S 65-02) was supplied by Shell Nederland Chemie, Pernis. The particle-size distribution (by weight), was max $0{\cdot}1\% > 300$, max $4\% > 200$, max $90\% > 88$ and max $95\% > 40$ μm. The VCM content of a portion of the PVC powder was raised to approximately 4000 ppm by mixing the powder with a calculated amount of liquid VCM in a closed steel barrel. This PVC powder was stored in tightly closed steel containers in a refrigerator at 4°C until a few minutes before being mixed with the diet. Part of the PVC powder obtained from Shell was freed from VCM by being kept in thin layers in a vacuum oven at 60°C for a period of 3–4 days. After this treatment the VCM content of the PVC powder was found to be less than 0·3 ppm.

A 10% solution of VCM in soya-bean oil was prepared by injecting liquid VCM into the oil. The VCM concentration was checked by gas–liquid chromatography according to the method described previously (Feron *et al.* 1975). The solution was stored at 4°C for a maximum period of 4 wk.

Preparation and administration of PVC-containing diets. Each diet contained 10% PVC powder, with varying proportions of VCM-containing and 'VCM-free' (i.e. <0·3 ppm VCM) powders. Thus the control diet contained 10% VCM-free PVC powder and the high-dose diet 10% PVC powder containing 4000 ppm VCM, while the diets for the low- and mid-dose groups contained, respectively, 1 and 3% VCM-containing PVC powder with the balance of 9 and 7% PVC made up with powder without VCM. The various diets were prepared daily by mixing appropriate amounts of PVC powder (with or without VCM) with the Institute's rat stock diet just prior to their being offered to the rats. The diets were available to the rats each day for a period of four consecutive hours (generally between 09.00 and 15.00 hr); thereafter, the rats had no food until the 4-hr feeding period on the next day. The rats had constant access to bottled, unfluoridated tap-water.

Calculation of the oral VCM exposure levels. To calculate the actual oral exposure levels of VCM provided by VCM-containing PVC powder in the diets, the following information was needed: (a) the amount of VCM evaporating from the diets during the 4-hr feeding period; (b) the speed with which the animals consumed the food during this period; and (c) the amount of VCM excreted in the faeces.

The rate of evaporation of VCM from the diets was determined by measuring the VCM content of the diets at the beginning of the feeding period and after 1, 2 and 4 hr. Samples to be analysed were taken at random from the feeders in the cages, without homogenization of the diets in the feeders. Each test diet was sampled in this way on 11 or 12 different days and analysed for VCM content by gas–liquid chromatography (Feron *et al.* 1975). The average VCM contents of the various diets at the different times are depicted in Fig. 1.

The eating-speed was determined by measuring the amount of residual feed in the feeders after periods of 1, 2 and 4 hr. Since no appreciable variations were encountered in the rate of food consumption by the animals in the various groups, average rates of food consumption were calculated for males and for females (Fig. 1).

The VCM intake (mg/kg body weight/day) by male and female rats of each group was calculated from the graphs representing the rate of evaporation of VCM from the diets (Fig. 1) and the rate of food consumption over the 4-hr feeding period. Both graphs were

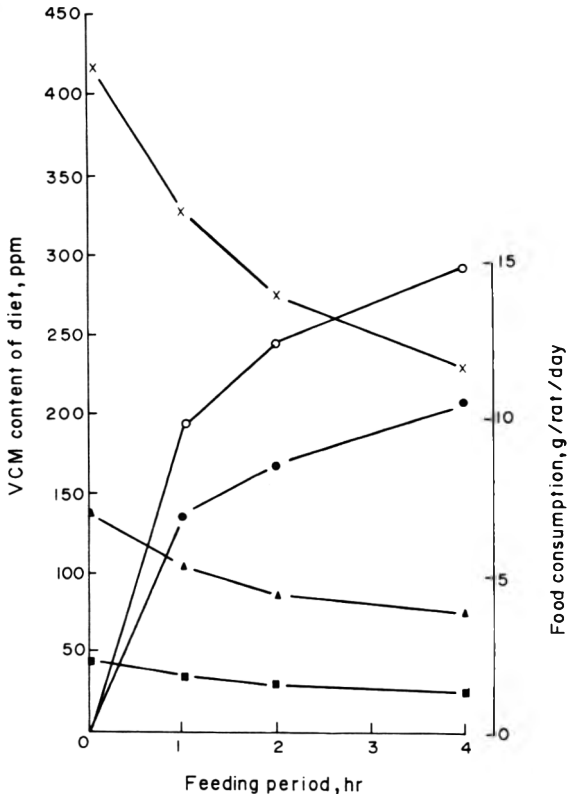

Fig. 1. Average VCM levels of the high-dose (×), mid-dose (▲) and low-dose (■) diets and average amounts of food consumed by males (○) and females (●) at different times during the 4-hr feeding period.

assumed to consist of three straight lines, one line for the first hour of the feeding period, one for the second hour and one for the last two hours. The VCM intake during each of these three periods was calculated by multiplying the amount of food eaten during a certain period with the average VCM content of the food in that period. The total VCM intake, the sum of the VCM intakes during these three periods, was subsequently expressed as a percentage of the theoretical intake, calculated from the average total amount of food consumed and the VCM content of the diet at the initiation of the feeding period. This figure was found to be 82·4, 79·1 and 79·1% for males, and 81·3, 79·3 and 79·0% for females of the low-, mid- and high-dose groups, respectively. The overall average for males and females of the various test groups was calculated to be 80%.

To measure faecal excretion of VCM, freshly produced faeces from a representative number of rats of each test group were collected at 09.00 hr (1 hr before the start of the feeding period), at 14.00 hr (at the end of the feeding period) and at 18.00 and 23.00 hr (4 and 9 hr after termination of feeding). Fresh faecal samples were obtained by squeezing the lower part of the rat's abdomen. The droppings were weighed, submerged in 10 ml ethyl acetate, and stored at 4°C in a closed vessel prior to analysis of the supernatant liquid by gas chromatography. Within a particular test group there were no appreciable differences in the VCM levels of faeces collected at the various times, although the optimum value was invariably obtained from faeces collected 9 hr after termination of feeding (at 23.00 hr). The average amount of VCM found in the faeces, expressed as a percentage of the VCM intake, was found to be 8, 10 and 17% for the low-, mid- and high-dose groups, respectively.

The VCM content of freshly prepared test diets was determined regularly, some twenty determinations for each dosage level being carried out during the study. Daily determinations were not considered necessary because the VCM content of the PVC powder stored at 4°C in a closed barrel was found to remain constant, and the VCM content of the PVC powder used appeared to correspond very well with the VCM content of the various diets. From these VCM determinations, the average VCM contents of the various diets were calculated as 46, 139 and 424 ppm for the low-, mid- and high-dose groups, respectively. Since both the loss of VCM from the diets before consumption and the VCM content of the faeces were known, the actual oral exposure levels of VCM could be calculated. They were found to be 1·7, 5·0 and 14·1 mg/kg body weight/day for the low-, mid- and high-dose groups (Table 1).

VCM in oil administered by gavage. One group of rats received VCM in oil by gavage. The approximate dose was 300 mg/kg body weight, administered daily on 5 days/wk for 83 wk, in calculated volumes of a 10% VCM solution in soya-bean oil. The volumes were adapted to the mean body weights once every week if necessary. These rats were offered the Institute's stock diet for rats and bottled unfluoridated tap-water *ad lib.*

Animals and housing. Newly weaned albino Wistar rats (Cpb: WU; Wistar random), obtained from the SPF colony of the Central Institute for the Breeding of Laboratory Animals TNO, Zeist, were allocated randomly over five groups of males and five groups of females in such a way that the mean body weights were virtually the same. The control group and the two highest dosage groups each consisted of 80 males and 80 females. The two lowest dosage groups each consisted of 60 males and 60 females. During the 5 days before the start of the experiment, the rats that were to be fed the PVC-containing diets received stock diet without PVC for 4–6 hr each day, to adapt them to the daily feeding period of 4 hr. At the start of the study the rats were 5 wk old. The study was terminated when about 75% of the control rats were dead, a point reached for males in wk 135 and for females in wk 144.

The rats receiving PVC powder in their diet were housed under conventional conditions, in groups of five in suspended stainless-steel cages with a wire-screen bottom in a well-ventilated room maintained at 24 ± 1°C. The rats given VCM in oil by gavage were housed in groups of two in suspended, tinned wire-screen cages in a well-ventilated cabinet maintained at 25–28°C. Animals in bad condition were housed individually in separate cages until they died or were killed *in extremis.*

Conduct of the experiment. The rats were individually weighed, initially, at wk 1, 2, 4, 6, 8, 10 and 12, and at 4-wk intervals thereafter. Food consumption of

Table 1. *Designed and actual dosage of VCM in rats maintained on diets containing PVC powder*

Designed VCM treatment		Actual initial dietary VCM level (ppm)*	Oral intake of VCM (mg/kg body weight/day)		Actual oral exposure level of VCM‡ (mg/kg body weight/day)
Dietary level (ppm)	Intake (mg/kg body weight/day)		Theoretical†	Actual	
0	0	0	0	0	0
20	1	46	2·3	1·8	1·7
60	3	139	7·0	5·6	5·0
200	10	424	21·2	17·0	14·1

*Average dietary VCM contents determined immediately after preparation of the diets.
†Assuming no loss of VCM by evaporation from the diets (see Fig. 1).
‡Oral intake of VCM diminished by the faecal VCM, which was found to be 8, 10 and 17% of the actual oral VCM intake for the low-, mid- and high-dose groups, respectively. The VCM excreted in the faeces was considered to be still enclosed in the PVC granules and thus not to have been in contact with the body.

20 rats/sex/group was measured during wk 1–4, 10–11, 24–25, 36–37, 60–61, 72–73 and 84–85.

Blood samples were collected from the tip of the tail of ten rats/sex/group in wk 13, 26, 52, 78 and 94. All samples were used for determinations of haemoglobin concentration (cyanmethaemoglobin method of van Kampen & Zijlstra, 1961) and packed cell volume (microhaematocrit), and for thrombocyte and red and white blood cell counts (Coulter Counter), and differential white cell counts by direct visual count of smears after Pappenheim staining (Gorter & De Graaff, 1955). Fasting blood glucose (Technicon AutoAnalyzer method N-9a) and blood-urea nitrogen (Ceriotti & Spandrio, 1965) were determined in wk 13, 26, 52 and 106. The analyses were conducted upon blood from the tip of the tail of ten rats/sex/group after the animals had been fasted overnight. During wk 13, 26, 52 and 106, samples of blood were collected from the orbital sinus of ten rats/sex/group. The following measurements were made in the serum after centrifugation at 3000 rpm for 20 min: alkaline phosphatase (Bessey, Lowry & Brock, 1946), glutamic-oxalacetic transaminase and glutamic-pyruvic transaminase (Reitman & Frankel, 1957), total protein (biuret reaction), albumin (De Leeuw-Israël, Arp-Neefjes & Hollander, 1967) and serum-protein pattern (Wieme, 1965).

Individual urine samples, collected from ten rats/sex/group in wk 13, 26, 52, 78 and 94, in each case during the last 16 hr of a 24-hr period of deprivation of food and water, were measured for volume (in calibrated tubes), specific gravity (by an Abbe-type refractometer), uric acid (Gorter & De Graaff, 1955) and glutamic-oxalacetic transaminase activity (Reitman & Frankel, 1957). Semi-quantitative measurements were made of pH, protein, sugar, occult blood and ketones in pooled urine samples, using Labstix from Ames Laboratories. Deposits obtained by centrifugation at 3000 rpm for 3 min were examined microscopically for erythrocytes, leucocytes, epithelial cells, amorphous substances, phosphate crystals, casts, bacteria, worm eggs and sperm cells.

All males still alive in wk 135 and all females surviving to wk 144 were killed by decapitation, autopsied and subjected to a careful gross examination. A thorough autopsy was also performed on rats found dead or killed *in extremis*. Samples of the following organs were fixed in 10% neutral formalin: heart, kidneys, liver, spleen, brain, testes, ovaries, pituitary, thyroid, adrenals, thymus, pancreas, epididymides, prostate, coagulating glands, seminal vesicles, preputial glands, mammary glands, lungs, skeletal muscle, spinal cord, sciatic nerve, urinary bladder, parotid, sublingual and submaxillary salivary glands, axillary and mesenteric lymph nodes, nose, oesophagus, stomach, duodenum, jejunum, ileum, caecum, colon, skin, femur with joint and bone marrow, trachea, aorta, exorbital lachrymal glands, Zymbal glands, cervix and uterus. The organs to be examined microscopically were processed through paraffin wax, sectioned at 5 μm and stained with haematoxylin and eosin. Microscopic examination of all organs preserved was carried out in 20 males and 20 females of the control group and of each of the two highest dosage groups. For the control groups these 20 males and 20 females comprised all animals killed at the end of the experimental period, supplemented by the animals that had lived longest before they had to be killed in a moribund condition. In the two highest dosage groups the rats subjected to detailed histopathology comprised the 20 males and 20 females that had lived longest before being killed in a moribund condition. Histopathological examination of all other rats was restricted to the liver, Zymbal glands, lungs, kidneys, spleen, pituitary, thyroid, adrenals, grossly visible tumours and organs containing gross lesions suspected of being tumours.

Batches of ten males and ten females from the control group and the two highest dosage groups were killed by decapitation and subjected to a thorough autopsy after 26 and 52 wk (interim kills). At these times, the following were recorded: blood-clotting time (Normotest reagents from Nyogaard and Co., Oslo, Norway), serum electrolytes Na, K, Ca, Mg (Paschen & Fuchs, 1971) and Cl (coulometric method), serum alkaline phosphatase, serum glutamic-oxalacetic transaminase, serum glutamic-pyruvic transaminase, total serum protein, albumin and serum-protein patterns (determined by the methods mentioned before), lactic dehydrogenase (Wróblewski & LaDue, 1955), serum α-foetoprotein* (Boekestein-Tjahjadi & Kroes, 1976), liver function (bromosulphophthalein-extinction test and barbiturate sleeping-time method), kidney function (phenol-red excretion test; Sharratt & Frazer, 1963), activities of aminopyrine demethylase (Gram, Wilson & Fouts, 1968) and aniline hydroxylase (Gilbert & Golberg, 1965) in liver preparations, and weights of liver and kidneys. Histopathology of the liver, kidneys and Zymbal glands was studied and the liver was also examined by light microscopy of semi-thin Paragon-stained plastic sections and by electron microscopy. For the latter the liver was fixed by perfusion with 1·5% glutaraldehyde buffered with 0·067 M-sodium cacodylate containing 1% sucrose (pH 7·4) at 4°C for 17 hr and this was followed by post-fixation in cacodylate-buffered 1% OsO_4, dehydration in graded acetone/water mixtures, and embedding in Epon 812.

Additional control group. One extra control group of 60 male and 60 female rats was housed in a room separate from that used for the other rats fed PVC-containing diets, thus preventing any possible contact of these control rats with VCM by inhalation. The diet containing 10% PVC containing no VCM was fed to the animals in this group *ad lib.* instead of for the restricted daily feeding period of 4 hr used for the real control group. Body weights and mortality were recorded, and all the animals were autopsied. The organs and tissues were preserved, but no slides were prepared.

RESULTS

Behaviour and appearance

During yr 2 of the test the general condition of the rats in the 300-mg/kg group gradually declined and administration of VCM by gavage became increasingly difficult. Many rats grew lethargic and filthy

*The α-foetoprotein determinations were carried out in the Laboratory of Pathology of the National Institute of Public Health, Bilthoven, under the supervision of Dr R. Kroes.

before they died or were killed in a moribund condition. Since most of these rats had severe lesions, including tumours, of the liver and lungs, the VCM treatment of this group was discontinued at wk 84.

After month 18 the number of unthrifty rats in the 5·0 and 14·1-mg/kg groups gradually increased, more rapidly in the latter (high-dose) group and more rapidly in females than in males. The poor condition started with a humpbacked position and slight emaciation followed by pale eyes, lethargy, filthiness and often severe emaciation. Liver masses could be detected in many of these rats by abdominal palpation. Swollen abdomens were occasionally seen, generally associated with multiple intra-abdominal tissue masses detectable by abdominal palpation. In addition, animals with breathing difficulties were fairly common in these groups; their lungs were invariably found to contain multiple nodules, which appeared microscopically to be primary angiosarcomas or metastases from either hepatic angiosarcomas or hepatocellular carcinomas. External tissue masses occurred fairly often, mainly after month 18; most appeared to be mammary-gland tumours, but there were also other types of tumours originating from the skin, subcutis or dermal adnexa.

Randomly distributed major abnormalities, not attributable to VCM, included staring coats, a bloody discharge from the nose, wet stools, focal alopecia, focal dermatitis, blood around the muzzle and eyes, paresis of the hind legs, loss of one or both eyes, and a white opaque cornea.

Body weights and food consumption

Body weights (Fig. 2) and food intakes of the rats fed the VCM-treated diets were very similar to those of the controls. The rats of the additional control group were much heavier than those of the other groups receiving diets containing PVC powder. This difference was undoubtedly due to the fact that the extra controls had constant access to their food, whereas diet was available to the other rats only during a period of four consecutive hours each day. The rats of the 300-mg/kg group had constant access to stock diet and had much higher body weights than the animals fed PVC-containing diets for a limited period each day, but their body weights were lower than those of the additional control group.

Mortality

Mortality was low in males and females of the 4-hr-fed control group during the first 2 yr of the test, amounting to 10% after 2 yr (Table 2). At this time the mortality among rats of the additional control group was some three times higher (about 30%).

About 40% of the males and females of the 300-mg/kg group had already died by month 18 of treatment. Thereafter, mortality rapidly increased, and by just after month 24 all the animals in this group had died, mainly from pulmonary or hepatic insufficiency resulting from neoplastic or non-neoplastic lesions in these organs. A striking and dose-related increase in death-rate was also found in the 5·0- and 14·1-mg/kg groups, with females dying earlier than males. In the low-dose group (1·7 mg VCM/kg body weight), the mortality of males was comparable to that of the male controls, and the death rate of females was only slightly higher than that of female controls.

Haematology, biochemistry, urine analysis and organ function

No relevant changes were found in any of the parameters studied, except for a shorter blood-clotting time and an increased content of α-foetoprotein in the blood serum of animals in the group fed the highest VCM dose and in the intubated group (Table 3).

Gross pathology

Liver-to-body weight ratios were higher in the 14·1- and 300-mg/kg groups than in the control group after both 26 and 52 wk (Table 3). Many rats exposed to VCM had severe liver lesions. Pronounced swelling, discoloration and altered consistency of one or more lobes, often with varying numbers of cysts, were common findings, as were nodules and nodule-like processes, varying widely in size (up to 4 cm in diameter), appearance and consistency. Many of the nodules were solid and pale; others were cystic and haemorrhagic. The larger firm and pale nodules with central necrosis appeared, upon microscopy, to be carcinomas. These were found mainly in completely distorted livers. Angiosarcomas were most often seen as multiple soft dark cystic nodules, containing blood and granular necrotic material. Nodules, which were later classified as 'neoplastic nodules', were relatively small, firm and compact; they were either pale or had the same colour as the adjacent normal liver tissue, and never contained necrotic material. These liver changes were most pronounced and occurred earliest

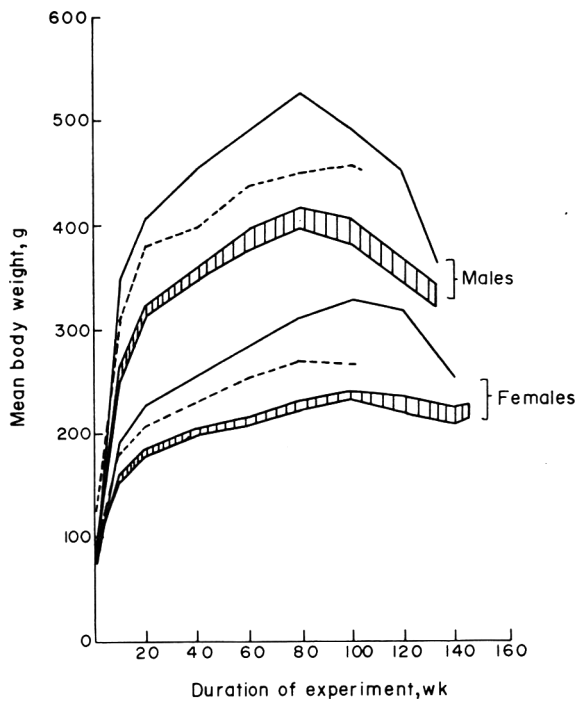

Fig. 2. Average body weights of the extra controls fed the 10%-PVC diet *ad lib.* (—) and of the rats given 300 mg VCM/kg body weight in oil by gavage (– – –). The weight curves of the rats receiving 0, 1·7, 5·0 or 14·1 mg VCM/kg body weight/day from the 10%-PVC diets fed for 4 hr each day all lie within the shaded area.

Table 2. *Cumulative mortality of rats exposed orally to VCM for up to 2·7 yr*

Treatment group (mg VCM/kg body weight/day)	Number of deaths† by end of wk:									
	12	36	52	80	92	105	120	128	134‡	143‡
Males										
0	0	0	0	0	2	6	18	40	46	
1·7	0	0	1	1	3	6	13	37	40	
5·0	0	0	0	2	7	12	30*	49	60**	
14·1	0	1	2	8**	22***	40***	56***	60***	60**	
300§	0	6	6	23	47	53	60	60	60	
0‖	0	0	0	1	3	19	28	46	46	
Females										
0	0	0	0	1	5	6	22	27	32	41
1·7	0	0	1	2	4	13	26	32	34	55**
5·0	0	1	2	7*	16**	31***	55***	60***	60***	60***
14·1	0	0	1	7*	43***	60***	60***	60***	60***	60***
300§	0	3	7	24	47	58	60	60	60	60
0‖	0	0	1	4	10	17	27	42	43	52

†Initial number of rats: 60/sex/group.
‡Surviving males were killed in wk 135 and surviving females in wk 144.
§The figures for this group were not evaluated statistically, because no corresponding control group was included in the study.
‖Additional control group housed in a separate room and having constant access to the diet containing 10% PVC without VCM.
Values marked with asterisks differ significantly from those of the controls according to the chi-square test: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

and most frequently in the 300- and 14·1-mg/kg groups. Angiosarcomatous nodules were not seen at all in the low-dose group.

Pulmonary alterations that could be ascribed to VCM treatment consisted of small haemorrhagic or greyish nodules, often located at the edge of a pulmonary lobe. These changes were not observed in controls or low-dose animals.

Rats with a swollen abdomen due to ascites and to numerous pale firm nodules (diameter 2–15 mm) in the peritoneum were encountered slightly more frequently in each of the three lowest dosage groups (maximum incidence 15%) than in the control or 300-mg/kg groups (maximum incidence 5%). Microscopically the nodules appeared to be mesotheliomas.

Many other types of gross changes, both neoplastic and non-neoplastic, were seen either frequently or just in a few animals, but there was no indication that any of these changes were related to VCM.

Light microscopy of the liver

The type and incidence of treatment-related histopathological changes found in the liver are given in Table 4. The incidence of foci of cellular alteration

Table 3. *Mean prothrombin times, α-foetoprotein contents of the blood serum, and liver weights of rats exposed orally to VCM for 26 or 52 wk*

Treatment group (mg VCM/kg body weight/day)	Prothrombin time (sec) at wk		α-Foetoprotein (μg/ml) at wk		Liver weight (g/100 g body weight) at wk	
	26	52	26	52	26	52
Males						
0	41·3	41·6	75	30	2·54	2·63
14·1	38·9*	38·8*	74	51*	2·91*	3·00
300†	35·2	37·3	84	55	3·31	3·38
Females						
0	37·2	32·8	99	53	2·60	2·57
14·1	34·1***	30·7	91	109**	3·08**	3·31*
300†	31·7	30·5	93	56	3·86	3·44

†The figures for this group were not evaluated statistically, because no corresponding control group was included in the study.
Values marked with asterisks differ significantly from those of the controls according to the test of Wilcoxon (prothrombin time and α-foetoprotein) or Student's *t* test (liver weight): $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Table 4. *Type and incidence of treatment-related histopathological changes in the liver of rats exposed orally to VCM*

Type of change†	Treatment group (mg VCM/kg/day)...	Incidence of change									
		Males					Females				
		0	1·7	5·0	14·1	300‡	0	1·7	5·0	14·1	300‡
Animals killed after 26 wk											
	No. of rats examined...	10	—§	—	10	9	10	—	—	10	10
Clear-cell foci		0	—	—	1	1	0	—	—	5**	2
Animals killed after 52 wk											
	No. of rats examined...	9	—	—	10	9	9	—	—	10	8
Clear-cell foci		1	—	—	8**	0	0	—	—	8**	0
Basophilic foci		0	—	—	0	0	0	—	—	4	1
Eosinophilic foci		0	—	—	2	0	0	—	—	5**	0
Neoplastic nodule		0	—	—	1	0	0	—	—	2	0
Hepatocellular carcinoma		0	—	—	1	0	0	—	—	1	0
Cystic proliferation of bile ducts		0	—	—	0	0	0	—	—	4*	0
Animals found dead or killed *in extremis* or terminally											
	No. of rats examined...	55	58	56	59	55	57	58	59	57	54
Clear-cell foci		0	9**	16***	21***	9	4	24***	22***	36***	10
Basophilic foci		8	18	21*	22**	12	0	33***	17	28***	19
Eosinophilic foci		3	23***	27***	33***	11	8	35***	20*	29***	6
Neoplastic nodule		0	1	7**	23***	3	2	26**	39***	44***	2
Hepatocellular carcinoma		0	1	2	8**	1	0	4	19***	29***	0
Angiosarcoma		0	0	6*	27***	27	0	0	2	9**	29
Proliferation of atypical sinusoidal cells only		2	0	4	7	6	4	6	3	4	7
Extensive necrosis		4	4	8	23***	21	5	6	19***	27***	24
Cysts		2	3	4	16***	3	9	30***	41***	49***	3
Liver-cell polymorphism		4	16*	28***	42***	36	34	51*	38	41	41
Centrilobular degeneration		0	0	0	1	1	1	2	3	1	18
Focal haematopoiesis		0	1	0	10**	8	1	3	1	6	12

†Specific hepatocellular lesions were classified according to Squire & Levitt (1975).
‡The figures of this group were not evaluated statistically, because no corresponding control group was included in the study.
§Not examined.
¶The initial number of animals was 60/sex/group. A number of rats could not be examined because of cannibalism or advanced autolysis.
Values marked with asterisks differ significantly from those of the controls according to the chi-square test: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

was much higher in each of the three test groups receiving VCM-containing PVC powder (the 1·7-, 5·0- and 14·1-mg/kg groups) than in the control group, and in addition, was nearly always higher than in the group receiving VCM in oil by gavage (300-mg/kg group). Similar differences also existed for neoplastic nodules and hepatocellular carcinomas. In the test groups receiving VCM-containing diets, the incidence of both neoplastic nodules and hepatocellular carcinomas was positively related to the VCM dose, and was much higher in females than in males.

Angiosarcomas of the liver (Figs 3 and 4) were found in males and females of the three highest dosage groups, but did not occur at all in controls and low-dose animals (Table 4). In both the 5·0- and 14·1-mg/kg groups the incidence of angiosarcomas was three times higher in males than in females. This difference between the sexes did not exist in the 300-mg/kg group, in which an angiosarcoma incidence of about 50% was found in both males and females.

In the 300-mg/kg group, the average latent period for the detection of liver tumours (almost exclusively angiosarcomas) at death was found to be 84 wk for males and 83 wk for females. In the 14·1-mg/kg group these average latent periods appeared to be 104 and 88 wk for males and females, respectively, clearly indicating an earlier appearance of liver tumours in the former group especially in males.

Several rats bearing angiosarcomas or liver-cell tumours also showed focal proliferation of atypical sinusoidal cells, often accompanied by distension of sinusoids. In addition, similar changes were observed in several rats not bearing a tumour in the liver. Large areas of necrosis were found in the livers of a fairly large number of rats of the three highest dosage groups. Cysts, very probably lined by proliferated bile-duct epithelium, were seen in a relatively large number of males of the 14·1-mg/kg group, and in females of the 1·7-, 5·0- and 14·1-mg/kg groups. The size and multiplicity of the cysts varied widely in individual animals. Cellular and nuclear polymorphism of hepatocytes was much more common in the test groups than in controls. Centrilobular liver degeneration was a frequent finding in females, but not in males, of the 300-mg/kg group. Focal haematopoiesis was encountered more often in males and females in the 14·1- and 300-mg/kg groups than in those of the other groups.

Electron microscopy of hepatic parenchyma

In semi-thin sections, foci of hepatocytes and also isolated hepatocytes with a finely 'vacuolized' cytoplasm were found in rats of the 14·1- and 300-mg/kg groups after both 26 and 52 wk. Ultrastructurally the 'vacuoles' appeared to represent swollen mitochondria with a pale matrix and short cristae (Fig. 5). The abnormal mitochondria closely resembled those found in rats following inhalation exposure to 5000 ppm VCM for periods of 4–52 wk and already described by Feron, Spit, Immel & Kroes (1979b).

Foci of hepatocytes, each containing numerous highly swollen, irregularly-shaped mitochondria without cristae and a matrix widely varying in density, were encountered after 26 wk. An increased amount of tubular smooth endoplasmic reticulum (SER) was invariably present among the swollen mitochondria. Occasionally scattered individual hepatocytes were found to contain a few extremely swollen mitochondria but were otherwise normal, as were their other mitochondria. After 52 wk, large areas of hepatocytes containing numerous swollen mitochondria were found, mainly in rats of the 14·1-mg/kg group. These hepatocytes had large nuclei with pronounced nucleoli. Tubular SER and whorls of SER were occasionally found together with mitochondria containing lucent areas in which a membranous or flocky material was often encountered. Single membranes of rough endoplasmic reticulum surrounding these mitochondria had partially lost their ribosomes.

Light microscopy of organs other than the liver

A wide variety of alterations was found, nearly all apparently related to the normal ageing process. An exception may have been the very marked haematopoietic activity found in the spleen of six out of 40 males and ten out of 40 females from the two highest dosage groups, while in controls only slight to moderate splenic haematopoiesis was observed.

The site and type of tumours observed in organs other than the liver and their incidence in the different groups are presented in Table 5. Angiosarcomas were frequently found in the lungs at the two highest dose levels, and also occurred in a few rats of the 5·0-mg/kg group. They were most often seen as multiple small foci of tumour cells with an angiomatous growth pattern. Their appearance was highly suggestive of metastases. On the other hand, in several cases, the histological appearance of the neoplasms did not permit the exclusion of their being diagnosed as primary pulmonary angiosarcomas. In addition, in three rats with a pulmonary angiosarcoma, no angiosarcoma was encountered outside the lungs. In four rats from the 300- and 14·1-mg/kg groups an angiosarcoma was found in the abdominal cavity outside the liver, while the liver showed no signs of angiosarcoma formation. Therefore, these tumours were considered to be primary extrahepatic angiosarcomas.

Pulmonary metastases of hepatocellular carcinomas were not uncommon. A total of five tumours of the Zymbal glands (ceruminous glands) were found—two squamous-cell carcinomas in the 5·0-mg/kg group, and two squamous-cell carcinomas and one adenoma in the 300-mg/kg group. Abdominal mesotheliomas were observed in each of the groups, including the control group. In several groups their incidence was higher than in controls, but a positive dose–response relationship with respect to incidence was absent. On the other hand, the latent period for detection of abdominal mesothelioma at death was found to decrease with increasing dose levels for both males and females. The histological appearance of the peritoneal mesotheliomas varied, but two main types could be distinguished, a fibrous type in which sarcomatous areas predominated and an epithelial type consisting mainly of tubulo-papillary formations. In several tumours both the sarcomatous areas and the tubulo-papillary structures occurred to the same extent. Mitotic figures were never abundant.

Fibroadenomas of the mammary glands were much less frequent in females of the 5·0-, 14·1- and

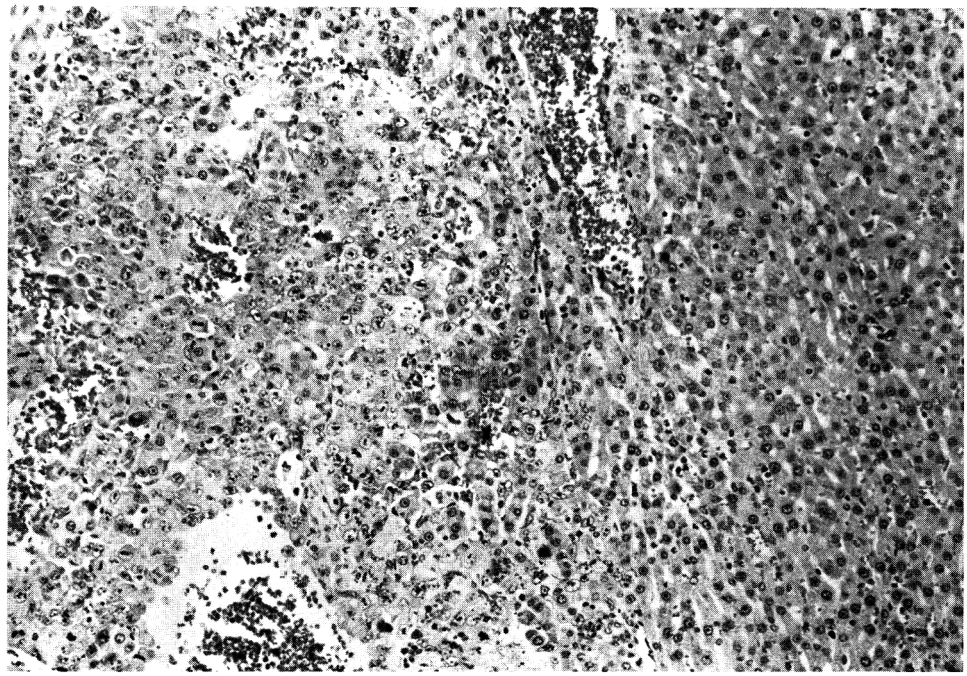

Fig. 3. Hepatic angiosarcoma of a male rat exposed orally to 14·1 mg VCM/kg body weight/day for a period of 101 wk. Haematoxylin and eosin ×160.

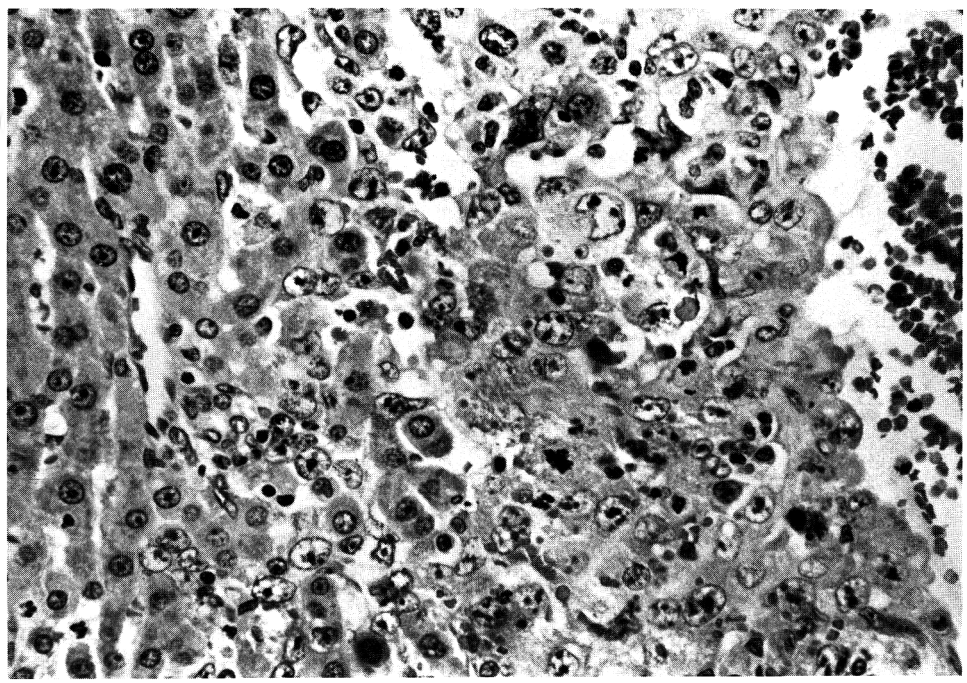

Fig. 4. Higher magnification of the hepatic angiosarcoma depicted in Fig. 3, showing large irregularly-shaped cells with big pale nuclei often containing multiple conspicuous nucleoli. Haematoxylin and eosin ×400.

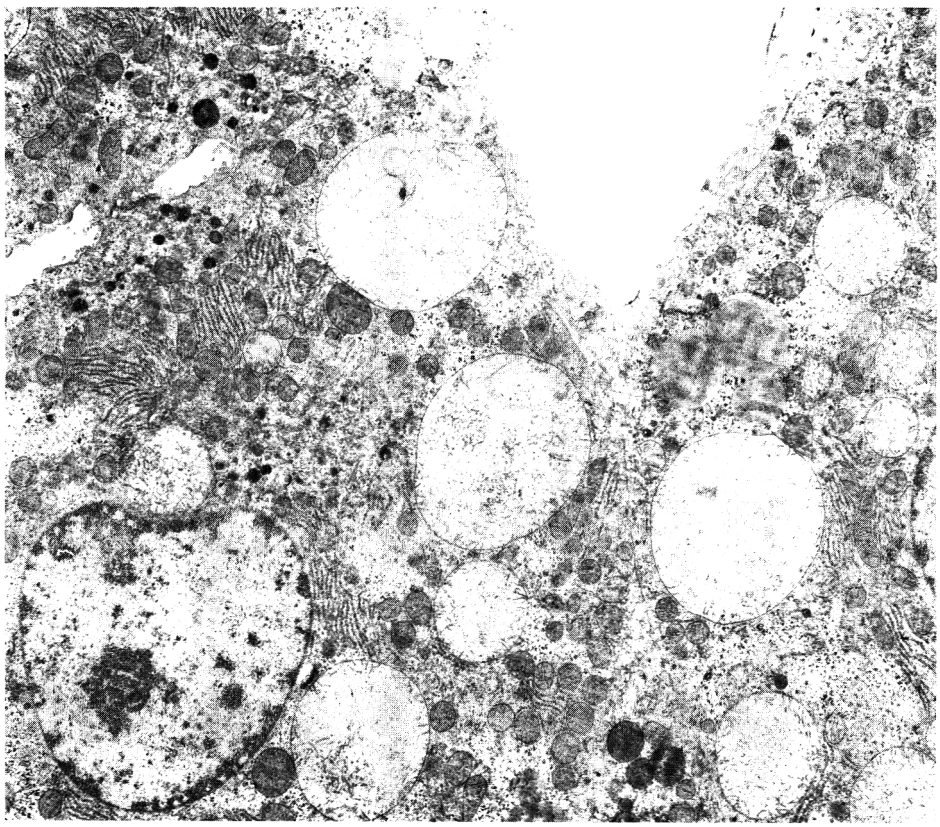

Fig. 5. Hepatocytes containing normal and highly swollen mitochondria with a pale matrix and short cristae from a male rat exposed orally to 300 mg VCM/kg body weight on 5 days/wk for 26 wk. Uranyl acetate, lead citrate ×6400.

Table 5. *Site, type and incidence of tumours in organs other than the liver† in rats exposed orally to VCM for over 2·5 yr*

Site and type of tumour	Treatment group (mg VCM/kg/day)...	Incidence of tumours									
		Males					Females				
		0	1·7	5·0	14·1	300‡	0	1·7	5·0	14·1	300‡
	Effective no. of rats...	55	58	56	59	55	57	58	59	57	54
	No. of rats with *primary* tumours...	38	50	49	52	44	54	56	55	57	47
Lungs											
Angiosarcoma		0	0	4*	19***	19	0	0	1	5*	23
Adenoma		0	0	0	0	1	0	0	0	0	0
Zymbal glands											
Squamous-cell carcinoma		0	0	2	0	1	0	0	0	0	1
Adenoma		0	0	0	0	0	0	0	0	0	1
Abdomen											
Mesothelioma		3	1	7	8	1	1	6*	3	3	0
Angiosarcoma		0	0	0	0	1	0	0	0	2	1
Fibrosarcoma		0	0	0	3	0	1	2	0	0	0
Osteosarcoma		0	0	0	0	1	0	0	0	1	0
Sarcoma		0	0	3	1	0	0	0	1	0	0
Reticulum-cell sarcoma		0	1	0	0	1	0	0	0	0	0
'Schwann-cell tumour'		0	0	0	0	0	0	1	0	0	0
Unclassified		0	0	0	0	0	2	0	0	0	0
Spleen											
Haemangioendotheliosarcoma		0	1	0	0	0	0	0	0	0	0
Lymphosarcoma		0	0	0	0	0	0	0	0	0	1
Nose											
Squamous-cell carcinoma		0	0	0	1	0	0	0	0	0	0
Brain											
Granular-cell myoblastoma		1	1	0	0	0	0	1	0	0	0
Oligodendroglioma		1	0	0	0	0	0	0	0	0	0
Plexus papilloma		0	0	0	0	0	0	1	0	0	0
Glial-cell tumour		0	0	2	0	1	0	0	0	0	0
Ependymoma		0	0	0	1	0	0	0	0	0	0
Mesodermal tumour		0	0	1	0	0	0	0	0	0	0
Pancreas											
Adenocarcinoma		0	0	0	0	0	1	2	1	0	0
Thorax											
Mesothelioma		1	0	0	0	0	0	0	0	0	0
Thyroid											
Parafollicular-cell adenoma		4	12*	10	3	3	7	10	3	2	0
Parafollicular-cell carcinoma		1	0	1	0	0	0	0	0	0	0
Follicular-cell adenoma		0	0	0	1	0	1	0	0	1	0

Table 5—*continued*

Site and type of tumour	Treatment group (mg VCM/kg/day)...	Incidence of tumours									
		Males					Females				
		0	1·7	5·0	14·1	300‡	0	1·7	5·0	14·1	300‡
Adrenals											
Cortical adenoma		18	25	17	10	9	26	30	20	17	14
Phaeochromocytoma		11	21	8	4	6	2	1	2	0	2
Pituitary											
Adenoma		12	25**	6	2**	0	14	16	10	5*	3
Carcinoma		1	0	1	0	0	3	0	2	0	0
Blood											
Leukaemia		1	0	1	1	3	1	2	1	0	1
Heart											
'Endocardial disease'§		2	0	2	2	1	1	0	0	0	1
Haemangioendotheliosarcoma		1	0	0	0	0	0	0	0	0	0
Kidneys											
Nephroblastoma		1	0	0	1	0	0	0	0	0	0
Clear-cell tumour		0	0	0	0	0	0	0	0	1	0
Lipomatous tumour		0	0	1	0	0	0	0	0	0	0
Unclassified epithelial tumour		0	0	0	0	0	1	0	0	0	0
Thymus											
Fibrosarcoma		0	1	0	0	0	0	0	0	0	0
Reticulum-cell sarcoma		0	0	0	0	1	0	1	1	0	0
Mesenteric lymph nodes											
Reticulum-cell sarcoma		0	0	0	0	1	0	0	0	0	0
Skin											
Squamous-cell carcinoma		2	3	3	1	0	0	0	0	1	0
Subcutis											
Fibroma		2	1	1	1	1	3	3	1	0	0
Fibrosarcoma		0	1	1	0	0	1	1	0	0	0
Mesenchymal tumour		0	0	0	0	0	1	0	0	0	0
Skeletal muscle											
Rhabdomyosarcoma		0	0	0	1	1	1	0	0	0	0
Skull											
Osteoma		1	0	0	0	0	0	0	0	0	0
Mesenchymal tumour		0	0	0	0	0	0	1	0	0	0
Ear region											
Adenocarcinoma of unknown origin		0	0	1	0	0	0	0	0	0	0
Urinary bladder											
Unclassified epithelial tumour		0	0	1	0	0	0	0	0	0	0
Preputial glands											
Squamous-cell carcinoma		0	0	1	0	0	0	0	0	0	0

Mammary glands										
Adenoma	0	0	0	0	0	0	0	0	2	0
Fibroadenoma	0	0	0	0	0	21	25	12**	4***	7
Adenocarcinoma	0	1	0	2	0	3	2	4	7	7
Anaplastic carcinoma	0	0	0	0	0	0	0	1	0	0
Testes										
Interstitial-cell tumour	3	0	0	1	1					
Uterus										
Adenocarcinoma						6	3	1	1	0
Malignant fibroadenomatous tumour						0	1	0	0	0
Leiomyoma						0	0	1	0	0
Cervix										
Mesenchymal type of tumour						2	0	1	0	0
Adenocarcinoma						0	1	0	0	0
Ovaries										
Theca-cell tumour						0	0	1	0	0

†A small number of primary liver tumours unrelated to treatment were found in several groups. These tumours were one Kupffer-cell sarcoma, three reticulum-cell sarcomas, two fibrosarcomas, one haemangioendothelioma and one mesenchymal tumour.

‡The figures for this group were not evaluated statistically, because no corresponding control group was included in the study.

§In several cases the neoplastic character of the lesion was doubtful.

Values marked with asterisks differ significantly from those of the controls according to the chi-square test: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

300-mg/kg groups than in controls or low-dose animals (Table 5). The low incidence of this common type of 'spontaneous' tumour, which is associated with old age, at the three highest dose levels is undoubtedly connected with the much shorter survival time of the animals in these groups compared with that of the controls or low-dose animals. Despite the relatively short survival time of females in the 14·1- and 300-mg/kg groups, the incidence of adenocarcinomas of the mammary glands in each of these groups was twice as high as in the control group. This may indicate that VCM enhances the development of mammary-gland carcinomas.

The incidence of several other common types of tumour known to be associated with old age was found to decrease with increasing dose levels, as was to be expected from the dose-related reduction in survival time. Examples of these were cortical adenomas and phaeochromocytomas of the adrenals, pituitary-gland adenomas, parafollicular-cell adenomas of the thyroid (the incidence of this tumour was exceptionally low in males of the control group), and adenocarcinomas of the uterus.

DISCUSSION

The limited daily feeding period of 4 hr in the day-time appeared to be associated with lower body weights but also with a higher survival rate. Apparently, reduced body-weight gain caused by a restricted feeding period is a favourable rather than an unfavourable effect. The much higher survival rate also points to the rats being healthy and having an adequate nutritional status unaffected by the relatively short feeding period in the day-time.

Only slight and often inconsistent differences were found between controls and test animals in several of the haematological and biochemical parameters studied. These slight deviations were regarded as toxicologically insignificant, with two possible exceptions, the shortening of the blood-clotting time and the increase in α-foetoprotein levels in the blood serum of VCM-exposed rats. Failure of the blood to clot was noted in guinea-pigs that died during exposure to an atmosphere containing 40% VCM (Mastromatteo, Fischer, Christie & Danziger, 1960) and in two workers in a PVC factory who died from acute VCM poisoning (Danziger, 1960). However, rats exposed to 5 or 2% VCM for 19 or 92 days, respectively, had normal blood-clotting times (Lester, Greenberg & Adams, 1963). In contrast, both in the present study and in a previous long-term inhalation study with VCM in rats (Feron, Kruysse & Til, 1979a), prothrombin times were shorter in VCM-exposed rats than in controls. Because of the conflicting observations, it is difficult to assess the toxicological importance of the slight hypercoagulability of the blood seen in the rats exposed to VCM in our studies. Müller, Buchter, Gross & Bolt (1976) recently reported a disturbed thrombocytic function in nine of 17 patients suffering from 'vinyl chloride disease'. Thrombocytopenia, which has been reported to be one of the signs of VCM intoxication in man (Jühe, Lange, Stein & Veltman, 1973; Lange, Jühe, Stein & Veltman, 1974; Müller *et al.* 1976), was observed neither in the present oral experiment nor in the previous 1-yr inhalation study (Feron *et al.* 1979a). In future studies, it would be desirable to pay special attention to the possible effects of VCM on thrombocytes.

Slight, though statistically significant, increases in the α-foetoprotein content of the blood serum were found in males and females of the 14·1-mg/kg group at wk 52. At wk 26 α-foetoprotein levels were normal in animals of this group. Very similar results have been obtained in rats exposed to 5000 ppm VCM in air for periods varying from 4 to 52 wk (Feron *et al.* 1979a). These findings may indicate the presence of foetoglobulin-producing neoplastic or preneoplastic cells in the liver of rats exposed to VCM for a prolonged period. However, since the increase in α-foetoprotein level was only slight, aspecific liver damage, such as necrosis or degeneration of hepatocytes, may have been responsible for this slight effect.

Although hepatocellular tumours have been reported in rats treated with VCM (Maltoni, 1977; Williamson, 1976) and some evidence exists as to the occurrence of hepatocellular carcinomas in persons exposed to VCM (Berk, Martin, Young, Creech, Selikoff, Falk, Watanabe, Popper & Thomas, 1976; Popper, Thomas, Telles, Falk & Selikoff, 1978), the high incidence of neoplastic liver-cell nodules and hepatocellular carcinomas in test animals (especially females) receiving VCM orally in PVC powder was an unexpected finding in the present study. Even at the lowest level (1·7 mg VCM/kg body weight/day), VCM-related liver-cell tumours and an increased incidence of foci of cellular alteration were noticeable in both males and females. It was remarkable that only a few hepatocellular neoplasms occurred in animals receiving VCM in oil by gavage at the very high dose of 300-mg/kg body weight, whereas nearly 50% of these rats had a hepatic angiosarcoma. The nature of the tumour response of the liver in the various test groups suggests a shift from an almost exclusive development of angiosarcomas at the very high dose level, *via* both angiosarcoma and hepatocellular tumours at the intermediate dose levels, to the exclusive occurrence of liver-cell tumours at the lowest level. On the other hand, not only the dose but also the way of administering VCM may have been of significance for the difference in tumour response between the 300-mg/kg group and the lower dosage groups, because gastric intubation of VCM in oil (daily on 5 days/wk) implies that the body (the liver) has to deal with a large amount of VCM in a short time, whereas in the case of oral intake of VCM-containing PVC powder the body (the liver) is continuously exposed (most probably for 24 hr/day on 7 days/wk) to 'fresh' VCM released gradually and without interruption from the PVC powder during its transport through the gastro-intestinal tract. One may also speculate that the unexpectedly high incidence of liver-cell tumours in the groups receiving VCM in PVC powder could be due to an unusual sensitivity of the liver cells to VCM, developing as a consequence of an altered metabolic state caused by the reduction of the daily period of food intake to 4 hr. This restricted period of food intake may have caused a daily, transitory depression of hepatic glutathione, which has been demonstrated to be of significance for the detoxification of VCM or its reactive metabolites

(Watanabe, Hefner & Gehring, 1976; Watanabe, McGowan & Gehring, 1976; Watanabe, McGowan, Madrid & Gehring, 1976).

The lesions of the hepatic parenchyma that could be attributed to VCM included not only 'foci of cellular alterations', neoplastic nodules and carcinomas, but also necrosis, centrilobular degeneration and mitochondrial damage. In addition, the slight increases in the α-foetoprotein content of the blood and in relative liver weight may also have been indications of VCM injury to the hepatic parenchymal cells. Since it is known that tissue injury followed by repair (hyperplasia) may stimulate tumour formation, the possibility exists that the 'promoting' properties of this chronic process of continuous damage and repair play a major role in the development of hepatocellular tumours following VCM exposure.

The association between VCM and angiosarcoma in man was recognized some years ago (Creech & Johnson, 1974; Lloyd, 1975) and several investigators have produced angiosarcomas in experimental animals by exposure to VCM either by inhalation or oral administration (Feron *et al.* 1979b; Holmberg, Kronevi & Winell, 1976; Keplinger, Goode, Cordon & Calandra, 1975; Lee *et al.* 1978; Maltoni, 1975 & 1977). The results of the present experiment show that oral exposure of rats to VCM at levels of 5·0 mg/kg body weight/day or more caused hepatic angiosarcomas, pulmonary angiosarcomas (probably both primary tumours and metastases) and, at the higher levels, also a few primary extrahepatic abdominal angiosarcomas. No angiosarcomas were found at the lowest level of 1·7 mg VCM/kg body weight/day.

Ultrastructural studies of the liver showed a scattering of foci of parenchymal cells affected by VCM. Swelling of mitochondria was the earliest change observed and mitochondrial alterations were also characteristic of the VCM-induced damage seen at later stages. Swelling of mitochondria has been found after exposure to other carcinogens (Bannasch, 1975) and also under various pathological conditions, such as ethanol damage (Dobbins, Rollins, Brooks & Fallon, 1972) and riboflavin deficiency (Tandler, Erlandson & Wynder, 1968). In addition to mitochondrial swelling, prolonged treatment with VCM for 26 or 52 wk appeared to cause a decrease in and loss of ribosomes from the RER, and most importantly an increase in SER. These findings suggest an enhancing effect of VCM on the so-called mixed-function oxidase system (m.f.o. system) which has been shown to be involved in the detoxification of VCM (Bolt, Kappus, Bruchter & Bolt, 1976; Reynolds, Moslen, Szabo, Jaeger & Murphy, 1975). However, the determinations of aminopyrine-demethylase and aniline-hydroxylase activities in the liver did not produce evidence of m.f.o. induction by VCM.

Full development of VCM-induced angiosarcoma has been found to be preceded by nodular hypertrophy and hyperplasia of liver cells, which may proceed to hepatocellular carcinoma (Feron *et al.* 1979b; Popper, Selikoff, Maltoni, Squire & Thomas, 1977). The liver changes found in the three lowest dosage groups are well in line with this observation, but in this connection it is difficult to understand the high incidence of hepatic angiosarcomas in the 300-mg/kg group, because focal hypertrophy and hyperplasia of the parenchyma occurred in only a very limited number of rats of this group.

Several investigators (Lee *et al.* 1978; Maltoni, 1975) have found extrahepatic angiosarcomas in rats exposed to VCM. In the present oral study, extrahepatic angiosarcomas were also seen, although it should be stressed that most of the pulmonary angiosarcomas were considered to be metastases from hepatic angiosarcomas.

Only a few test animals bore a tumour of the Zymbal glands, but since spontaneous Zymbal-gland tumours are rare and tumours of this organ are known to be induced in rats by VCM, it seems justifiable to ascribe to the VCM treatment the few Zymbal-gland neoplasms found.

VCM-induced carcinomas of the mammary glands have been observed in mice (Lee *et al.* 1978; Maltoni, 1975) and presumably also in rats (Maltoni, 1975). The incidence of mammary-gland carcinomas in several of the test groups was only slightly higher than that in the controls and was not considered to be conclusive evidence that VCM can induce this type of neoplasm in rats. At most it may be regarded as an indication that VCM may enhance carcinoma formation in this organ.

To our knowledge, VCM-induced mesotheliomas have not been reported. In the present study, the number of rats with abdominal mesothelioma was higher in each of the test groups receiving PVC-powder containing VCM than in controls fed PVC powder free of VCM. This may indicate that the ingestion of VCM has a potentiating effect on the development of mesothelioma in rats.

The high incidence of liver-cell tumours in females of the low-dose group (28/58) clearly demonstrates the absence of both a 'no-observed-adverse-effect level' and a 'minimum-effect level' in our experimental system. These levels must be known before any attempt can be made to extrapolate the animal data to man and to assess the carcinogenic risk of VCM. Therefore, a similar lifespan oral carcinogenicity study with VCM in rats has been initiated, using three different dose levels (0·017, 0·17 and 1·7 mg VCM/kg body weight/day) and two control groups; this study had been running for 14 months in October 1980.

Conclusions

This study shows that the incorporation of VCM-containing PVC powder into the diets of rats is an effective method for studying the long-term effects of oral administration of VCM. VCM is a carcinogen when administered to rats by the oral route and the tumour response of the rat liver to the oral intake of VCM seems to shift from an almost exclusive development of angiosarcomas at very high levels to the exclusive induction of hepatocellular tumours at low levels of exposure. The ingestion of VCM in PVC powder may enhance in rats the development of abdominal mesotheliomas and of adenocarcinomas of the mammary glands. The 'no-observed-adverse-effect level' was lower than 1·7 mg VCM/kg body weight/day under the rigorous conditions of continuous oral VCM exposure resulting from the gradual release of VCM from PVC powder present in the gastrointestinal tract.

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REFERENCES

Ministry of Agriculture, Fisheries and Food (1978). The Surveillance of Food Contamination in the United Kingdom: Survey of Vinyl Chloride Content of Polyvinyl chloride for Food Contact and of Foods. The Second Report of the Steering Group on Food Surveillance—The Working Party on Vinyl Chloride. Food Surveillance Paper No. 2. p. 6. HMSO, London.

Bannasch, P. (1975). Die Cytoplasmaorganellen der Hepatocyten während der Carcinogenese. 1. Mitochondrien. In *Handbuch der allgemeinen Pathologie. Sechster Band, siebenter Teil, Geschwülste-Tumors III.* Edited by E. Grundmann. p. 211. Springer Verlag, Berlin.

Basalaev, A. V., Vazin, A. N. & Kotchetkov, A. G. (1972). On the pathogenesis of changes developing due to a long-term exposure to the effect of vinyl chloride. *Gig. Truda prof. Zabol.* **16**, 24.

Berk, P. D., Martin, J. F., Young, R. S., Creech, J., Selikoff, I. J., Falk, H., Watanabe, P., Popper, H. & Thomas, L. (1976). Vinyl chloride-associated liver disease. *Ann. intern. Med.* **84**, 717.

Bessey, O. A., Lowry, O. H. & Brock, M. J. (1946). A method for the rapid determination of alkaline phosphatase with five cubic millimetres of serum. *J. biol. Chem.* **164**, 321.

Boekestein-Tjahjadi, H. M. & Kroes, R. (1976). α_1-Fetoproteine (AFP): Radioimmunologische Bepaling en de Vroege Verschijning van APF na Aflatoxine B_1 Toediening. Berichten uit het RIV-1975. p. 136. Staatsuitgeverij, Den Haag.

Bolt, H. M., Kappus, A., Bruchter, A. & Bolt, W. (1976). Disposition of (1,2-^{14}C) vinyl chloride in the rat. *Archs Toxicol.* **35**, 153.

Ceriotti, G. & Spandrio, L. (1965). Catalytic acceleration of the urea-diacetylmonoxime-phenazone reaction and its application to automated analysis. *Clinica chim. Acta* **11**, 519.

Creech, J. L., Jr & Johnson, M. N. (1974). Angiosarcoma of the liver in the manufacture of polyvinyl chloride. *J. occup. Med.* **16**, 150.

Daniels, A. G. & Proctor, D. E. (1975). VCM extraction from PVC bottles. *Modern Packaging* April, p. 45.

Danziger, H. (1960). Accidental poisoning by vinyl chloride. *Can. Med. Ass. J.* **82**, 828.

De Leeuw-Israël, F. R., Arp-Neefjes, J. M. & Hollander, C. F. (1967). Quantitative determination of albumin in microliter amounts of rat serum. *Expl Geront.* **2**, 255.

Dobbins, W. O., Rollins, E. L., Brooks, S. G. & Fallon, H. J. (1972). A quantitative morphological analysis of ethanol effect upon rat liver. *Gastroenterology* **62**, 1020.

Feron, V. J. & Kroes, R. (1979). One-year time-sequence inhalation toxicity study of vinyl chloride in rats. II. Morphological changes in the respiratory tract, ceruminous glands, brain, kidneys, heart and spleen. *Toxicology* **13**, 131.

Feron, V. J., Kruysse, A. & Til, H. P. (1979a). One-year time sequence inhalation toxicity study of vinyl chloride in rats. I. Growth, mortality, haematology, clinical chemistry and organ weights. *Toxicology* **13**, 25.

Feron, V. J., Speek, A. J., Willems, M. I., van Battum, D. & de Groot, A. P. (1975). Observations on the oral administration and toxicity of vinyl chloride in rats. *Fd Cosmet. Toxicol.* **13**, 633.

Feron, V. J., Spit, B. J., Immel, H. R. & Kroes, R. (1979b). One-year time-sequence inhalation toxicity study of vinyl chloride in rats. III. Morphological changes in the liver. *Toxicology* **13**, 143.

Fuchs, G., Gawall, B. M., Albanus, L. & Slorach, S. (1975). Vinyl chloride monomer levels in edible fats. *Vår Föda* **27**, 134.

Gilbert, D. & Golberg, L. (1965). Liver response tests. III. Liver enlargement and stimulation of microsomal processing enzyme activity. *Fd Cosmet. Toxicol.* **3**, 417.

Gorter, E. & De Graaff, W. C. (1955). *Klinische Diagnostiek. I.* 7th Ed. p. 267. Edited by H. E. Stenfert, Kroese NV, Leiden.

Gram, T. E., Wilson, J. T. & Fouts, J. R. (1968). Some characteristics of hepatic microsomal systems which metabolize aminopyrine in the rat and rabbit. *J. Pharmac. exp. Ther.* **159**, 172.

Haley, T. J. (1975). Vinyl chloride: how many unknown problems? *J. Toxicol. envir. Hlth* **1**, 47.

Holmberg, B., Kronevi, T. & Winell, W. (1976). The pathology of vinyl chloride exposed mice. *Acta vet. scand.* **17**, 328.

Hopkins, J. (1980). Vinyl chloride—Part 4: Carcinogenicity in man. *Fd Cosmet. Toxicol.* **18**, 94.

Jühe, S., Lange, C. E., Stein, G. & Veltman, G. (1973). Über die sogenannte Vinylchloride-Krankheit. *Dt. med. Wschr.* **98**, 2034.

Keplinger, M. L., Goode, J. W., Cordon, D. E. & Calandra, J. C. (1975). Interim results of exposure of rats, hamsters, and mice to vinyl chloride. *Ann. N.Y. Acad. Sci.* **246**, 219.

Lange, C. E., Jühe, S., Stein, G. & Veltman, G. (1974). Die sogenannte Vinylchloride-Krankheit—eine berufsbedingte Systemsklerose? *Int. Arch. Arbeitsmed.* **32**, 1.

Lee, C. C., Bhandari, J. C., Winston, J. M., House, W. B., Dixon, R. L. & Woods, J. S. (1978). Carcinogenicity of vinyl chloride and vinylidene chloride. *J. Toxicol. envir. Hlth.* **4**, 15.

Lester, D., Greenberg, L. A. & Adams, W. A. (1963). Effects of single and repeated exposures of humans and rats to vinyl chloride. *Am. ind. Hyg. Ass. J.* **24**, 265.

Lloyd, J. W. (1975). Angiosarcoma of the liver in vinyl chloride/polyvinyl workers. *J. occup. Med.* **17**, 333.

Maltoni, C. (1975). The value of predictive experimental bioassays in occupational and environmental carcinogeneses. An example: vinyl chloride. *Ambio* **4**, 18.

Maltoni, C. & Lefemine, G. (1975). Carcinogenicity bioassays of vinyl chloride: current results. *Ann. N.Y. Acad. Sci.* **246**, 195.

Maltoni, C. (1977). Vinyl chloride carcinogenicity: an experimental model for carcinogenesis studies. In *Origins of Human Cancer. Book A.* Edited by H. H. Hiatt, J. D. Watson & J. A. Winsten. p. 119. Cold Spring Harbor Laboratory, NY.

Mastromatteo, E., Fischer, A. M., Christie, H. & Danziger, H. (1960). Acute inhalation toxicity of vinyl chloride to laboratory animals. *Am. ind. Hyg. Ass. J.* **21**, 394.

Monson, R. R., Peters, J. M. & Johnson, M. N. (1974). Proportional mortality among vinyl-chloride workers. *Lancet* **II**, 397.

Müller, K. T., Buchter, A., Gross, R. & Bolt, W. (1976). Ergebnisse einer Studie an 17 Fällen von Langzeitexposition gegenüber Vinylchlorid. *Medsche Welt. Berl.* **27**, 21.

Paschen, K. & Fuchs, C. (1971). A new micro-method for Na, K, Ca and Mg determinations in a single serum dilution by atomic absorption spectrophotometry. *Clinica chim. Acta* **35**, 401.

Popper, H., Selikoff, I. J., Maltoni, C., Squire, R. A. & Thomas, L. B. (1977). Comparison of neoplastic hepatic lesions in man and experimental animals. In *Origins of Human Cancer.* Edited by H. H. Hiatt, J. D. Watson & J. A. Winsten. p. 1359. Cold Spring Harbor Laboratory, NY.

Popper, H., Thomas, L. B., Telles, N. C., Falk, H. & Selik-

off, I. J. (1978). Development of hepatic angiosarcoma in man induced by vinyl chloride, thorotrast and arsenic: comparison with cases of unknown etiology. *Am. J. Path.* **92,** 349.

Potter, H. R. (1976). Vinyl Chloride—Part II. *Fd Cosmet. Toxicol.* **14,** 498.

Randolph, W. F. (1973). Prior-sanctioned polyvinyl chloride resin. *Federal Register* **38,** 12934.

Rawls, R. (1980). Studies update vinyl chloride hazards. *Chem. Engng News* **58** (14), 27.

Reitman, S. & Frankel, S. (1957). A colorimetric method for the determination of serum glutamic-oxalacetic and glutamic-pyruvic transaminases. *Am. J. clin. Path.* **28,** 56.

Reynolds, E. S., Moslen, M. T., Szabo, S., Jaeger, R. J. & Murphy, S. D. (1975). Hepatotoxicity of vinyl chloride and 1,1-dichloroethylene: role of mixed function oxidase system. *Am. J. Path.* **81,** 219.

Sharratt, M. & Frazer, A. C. (1963). The sensitivity of function tests in detecting renal damage in the rat. *Toxic. appl. Pharmac.* **5,** 36.

Squire, R. A. & Levitt, M. H. (1975). Report of a workshop on classification of specific hepatocellular lesions in rats. *Cancer Res.* **35,** 3214.

Suzuki, Y. (1978). Pulmonary tumors induced in mice by vinyl chloride monomer. *Envir. Res.* **16,** 285.

Tandler, B., Erlandson, R. A. & Wynder, E. L. (1968). Riboflavin and mouse hepatic cell structure and function. I. Ultrastructural alterations in simple deficiency. *Am. J. Path.* **52,** 69.

Thomas, L. B. & Popper, H. (1975). Pathology of angiosarcoma of the liver among vinyl chloride–polyvinyl chloride workers. *Ann. N.Y. Acad. Sci.* **246,** 268.

Torkelson, T. R., Oyen, F. & Rowe, V. K. (1961). The toxicity of vinyl chloride as determined by repeated exposure of laboratory animals. *Am. ind. Hyg. Ass. J.* **22,** 354.

Vale, P. T., Kipling, M. D. & Walker, A. E. (1976). Miscellaneous symptoms occurring in workers engaged in the manufacture of PVC. *J. Soc. occup. Med.* **26,** 95.

van Kampen, E. J. & Zijlstra, W. C. (1961). Standardization of hemoglobinometry. II. The hemoglobincyanide method. *Clinica chim. Acta* **6,** 538.

Watanabe, P. G., Hefner, R. E., Jr & Gehring, P. J. (1976). Vinyl chloride-induced depression of hepatic non-protein sulfhydryl content and effects on bromosulphalein (BSP) clearance in rats. *Toxicology* **6,** 1.

Watanabe, P. G., McGowan, G. R. & Gehring, P. J. (1976). Fate of [^{14}C]vinyl chloride after single oral administration in rats. *Toxic. appl. Pharmac.* **36,** 339.

Watanabe, P. G., McGowan, G. R., Madrid, E. O. & Gehring, P. J. (1976). Fate of [^{14}C]vinyl chloride following inhalation exposure in rats. *Toxic appl. Pharmac.* **37,** 49.

Waxweiler, R. J., Stringer, W., Wagoner, J. K., Jones, J., Falk, H. & Carter, C. (1976). Neoplastic risk among workers exposed to vinyl chloride. *Ann. N.Y. Acad. Sci.* **271,** 40.

Wieme, R. J. (1965). *Agar Gel Electrophoresis.* p. 425. Elsevier Publishing Corp., Amsterdam.

Williams, D. T. & Miles, W. F. (1975). Gas–liquid chromatographic determination of vinyl chloride in alcoholic beverages, vegetable oils and vinegars. *J. Ass. off. analyt. Chem.* **58,** 272.

Williamson, K. S. (1976). Review of animal studies. *Proc. R. Soc. Med.* **69,** 281.

Winell, M., Holmberg, B. & Kronevi, T. (1976). Biological effects of vinyl chloride: an experimental study. *Envir. Hlth Perspect.* **17,** 211.

Wroblewski, F. & LaDue, J. S. (1955). Lactic dehydrogenase activity in blood. *Proc. Soc. exp. Biol. Med.* **90,** 210.

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THE PRESENCE OF POLYCHLORINATED QUATERPHENYLS IN THE TISSUES OF YUSHO VICTIMS

T. KASHIMOTO, H. MIYATA and N. KUNITA

Osaka Prefectural Institute of Public Health, 3-69, 1-Chome, Nakamichi, Higashinari-ku, Osaka, 537, Japan

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Abstract—Tissue samples obtained *post mortem* from Yusho victims and from members of the general population who had died accidentally, were analysed for polychlorinated biphenyl (PCB) and polychlorinated quaterphenyl (PCQ) by gas chromatography and gas chromatography-mass spectrometry. Similar analyses were carried out on blood samples obtained from Yusho victims, from individuals considered to have ingested the rice oil which caused the outbreak of Yusho, from workers occupationally exposed to PCB and from unexposed individuals. PCQ was identified in the organs and blood of Yusho victims by gas chromatography–mass spectrometry. Some Yusho victims showed high tissue concentrations of residual PCQ, and it was also found that levels of 1·3–2·7 ppb of PCQ were present in the adipose tissue of the controls. Thus in addition to PCBs there are also trace amounts of PCQs in the general environment. PCQ levels within the range 0·05–8·92 ppb were detected in the blood of all the people who had consumed rice oil. On the other hand, at a 0·02 ppb detection limit, no PCQ was detected in the blood of other PCB-exposed individuals. Thus, even now, 12 yr after the first outbreak of Yusho, PCQs can be detected in as little as 10 ml of the Yusho patients' blood.

INTRODUCTION

In 1968, the disease 'Yusho', characterized by acne, pigmentation of the skin, and nervous and gastrointestinal disorders, affected over 1300 people in western Japan. It was eventually shown to have been caused by contamination of rice oil (used for cooking) with Kanechlor-400 (KC-400), a Japanese PCB preparation, which was used as the heat-transfer medium during the manufacturing process (Tsukamoto, Makisumi, Hirose, Kojima, Fukumoto *et al.* 1969).

The concentration of total chlorine in the toxic rice oil, which caused the outbreak of Yusho, was determined by various methods, and the PCB level in the oil was estimated to be 2000–3000 ppm (Tanabe, Takeda, Itho, Suzuki, Kanda *et al.* 1970; Yoshimura, 1971). However, we determined by gas chromatography that the toxic rice oil manufactured on 5 February 1968 contained 968 ppm PCB and 7·8 ppm polychlorodibenzofuran (Miyata & Kashimoto, 1978). This PCB value was barely one third of the PCB concentration calculated on the basis of the total chlorine level in the same oil. Further analysis of the rice oil (to investigate this discrepancy) subsequently showed that polychlorinated quaterphenyl (PCQ) amounting to 0·9–3·5 times the concentration of PCB was present in the oil (Miyata, Kashimoto & Kunita, 1978a,b). The presence of PCQ in the Yusho oil was confirmed in the USA (Kamps, Trotter, Young, Carson, Roach *et al.* 1978).

It appeared that the PCB heat-transfer medium, KC-400, in which amounts of PCQ and PCDF of the order of 10,000 ppm and 100 ppm, respectively, had been generated, had leaked out into the rice oil in the deodorizing stage of manufacture, and that the ratios of PCQ:PCB and PCDF:PCB in the oil had become very high (0·9–3·5 and 0·008–0·015, respectively) during the deodorization process at reduced pressure and high temperature because of the differences of vapour pressure (PCB > PCDF ≫ PCQ). Yusho patients had thus ingested rice oil containing high levels of PCQ, PCB and PCDF (Miyata & Kashimoto, 1979; Miyata, Murakami & Kashimoto, 1978).

To clarify further the differences between Yusho and straightforward PCB poisoning, we have analysed the PCB and PCQ concentrations in organ samples obtained *post mortem* from Yusho victims and from members of the general public who had died accidentally. Similar analyses were carried out on blood samples obtained from Yusho victims, from persons considered to have ingested toxic rice oil, from workers occupationally exposed to PCB and from unexposed individuals.

EXPERIMENTAL

Material analysed. Samples of the liver and adipose tissue or intestine of seven Yusho patients who had died were obtained from Dr I. Taki, Chief of the Yusho Therapy Study Group, Faculty of Medicine, Kyushu University. Sample no. 583 was from a 10-foetal-month stillborn baby delivered to a Yusho patient on October 14, 1968 (Kikuchi, Hashimoto, Hozumi, Koga, Oyoshi & Nagayama, 1969). Samples 15868, 16634 and AN-77-34 were considered as grades 4, 3 and 2 Yusho, respectively, on the basis of the severity of dermal lesions (Kikuchi & Masuda, 1974; Kikuchi, Mikagi, Hashimoto & Kojima, 1971; Kikuchi, Shigematsu & Umeda, 1979). Although samples no. 651 and AN-77-100 were classified as grade 1 Yusho (slight skin change), the clinical manifestations

and PCB gas chromatogram patterns were not those peculiar to Yusho (Kikuchi, 1972; Kikuchi *et al.* 1979). The normal individuals studied all died accidentally in September 1978. The worker occupationally exposed to PCB handled Kanechlor-300 and -500 at a condenser factory from 1959 to 1966. The milk of a female worker occupationally exposed to PCB was collected for analysis in October 1977.

Blood samples were collected in 1979 from 52 people who had consumed the contaminated rice oil (13 of them were not considered to be Yusho victims at that time on the basis of clinical examination), six people who were engaged in charging transformers with PCB, and 29 normal individuals. The blood samples of Yusho victims were supplied by Dr M. Takamatsu, Faculty of Medicine, Kurume University.

Chemicals. The PCQ standard was prepared as described previously (Miyata *et al.* 1978c). Florisil (60–100 mesh) was activated overnight at 130°C. Alumina was obtained from Merck Co. Ltd (Darmstadt, Germany) Art. no. 1077. Ethanol, diethyl ether, methylene dichloride, antimony pentachloride and anhydrous sodium sulphate of reagent grade for PCB analysis were obtained from Wako Pure Chemical Co. Ltd, Osaka, Japan. Potassium hydroxide, hydrochloric acid and sodium bicarbonate of reagent grade were also obtained from Wako Pure Chemical Co. Ltd.

Analytical procedure. The tissue sample (5 g) was saponified with 1·5 N-KOH–ethanol solution, extracted with *n*-hexane, washed and concentrated to 5 ml by the method previously described (Miyata, Kashimoto & Kunita, 1977). The hexane extract was placed on a Florisil column (20 g, 1·8 cm ID) and eluted successively with 130 ml *n*-hexane, 50 ml 2% diethyl ether in *n*-hexane and 100 ml 5% diethyl ether in *n*-hexane, at a rate of approximately 2 ml/min. The first and second eluates were combined, concentrated and analysed for PCB by gas chromatography (GC) on a Varian Aerograph 2800 machine equipped with ^{63}Ni-electron capture detector (ECD) under the conditions described elsewhere (Miyata *et al.* 1977). PCB was estimated quantitatively by the method of Ugawa, Nakamura & Kashimoto (1973). To analyse for PCQ, the third fraction was concentrated and injected first into a 1·8 × 2 mm glass column containing 2% OV-210 on 100/120 mesh Gas Chrom Q in a Varian Aerograph 2100 gas chromatograph with a ^{63}Ni-ECD, then into a 1 m × 3 mm glass column containing 2% OV-210 on 100/120 mesh Gas Chrom Q in a Shimadzu-LKB 9000 gas chromatograph–mass spectrometer under the conditions described previously (Miyata *et al.* 1978b). Furthermore, an aliquot of the eluate was put into a Pyrex glass ampoule (8 cm × 1·5 cm ID) and evaporated to dryness under a stream of nitrogen. After addition of antimony pentachloride (0·5 ml), the ampoule was sealed and heated at 200°C for 2 hr in a GC oven in order to achieve exhaustive chlorination. This provided an easy method for quantitating and identifying PCQ residues with high sensitivity. After careful addition of 6 N-HCl (10 ml), the product was transferred to a 100-ml separating funnel and the extract was washed successively with 20 ml each of water (twice) and 5% sodium bicarbonate solution and water, and then dried over anhydrous sodium sulphate and concentrated to c. 5 ml. To remove impurities, the perchlorinated sample was applied to an alumina column (5 g aluminium oxide, 1·0 cm ID) and eluted with 30 ml of 2% methylene dichloride in *n*-hexane. The eluate containing perchlorinated PCQ (octadecachloroquaterphenyl, $C_{24}Cl_{18}$) was evaporated to dryness and the residue was redissolved in *n*-hexane and subjected first to GC in a Shimadzu GC-6A fitted with ^{63}Ni-ECD and using a glass column (50 cm × 2·2 mm) containing Gas Chrom Q (100/120 mesh) coated with 2% OV-210 (column, injector and detector temps., 260, 285 and 285°C, respectively; carrier gas, N_2 at 60 ml/min) and then to gas chromatography–mass spectrometry (GC–MS) using a JEOL-20KP gas chromatography–JMS-300 mass spectrometer with a computer system. PCQ was estimated quantitatively by comparing the total areas of the GC peaks with those of the perchlorinated PCQ standard.

The blood (10 ml) was saponified with 20 ml of 2 N-KOH ethanol solution for 1 hr under reflux. Water (20 ml) was added in the saponified sample, the aqueous sample was then extracted with 30 ml *n*-hexane, washed with 50 ml water and dried over anhydrous sodium sulphate. After concentration, the extract was chromatographed on a Florisil column (6 g, 1 cm ID) eluted successively with 60 ml *n*-hexane and 45 ml 4% diethyl ether in *n*-hexane. The first eluate was concentrated and analysed for PCB by ECD–GC. The second eluate containing PCQ was concentrated, perchlorinated with antimony penta-

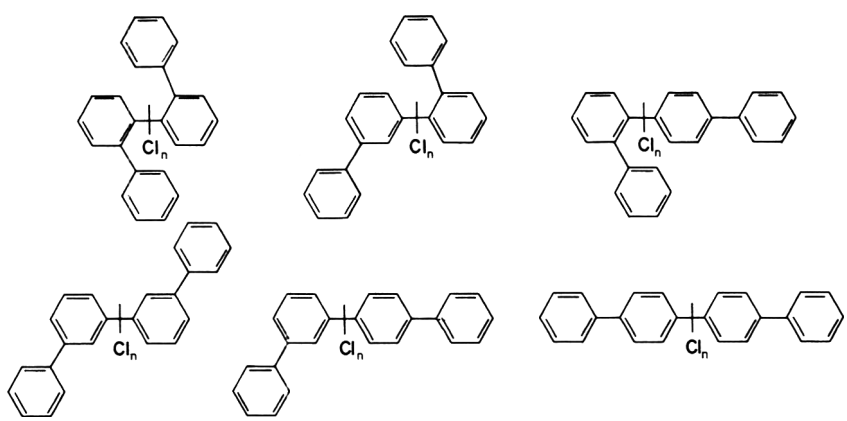

Fig. 1. Possible structures of PCQ (n = 1–18).

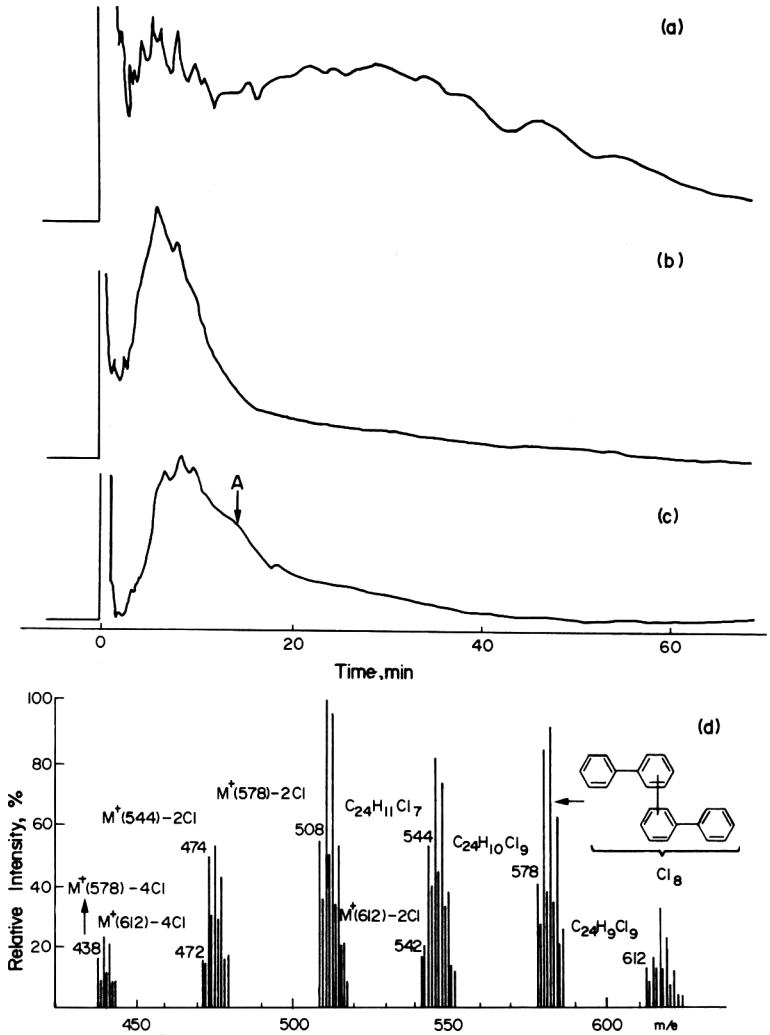

Fig. 2. Gas chromatograms of PCQ in the liver (a) and adipose tissues (b) of a Yusho victim (no. 15868 in Table 1) and in the Yusho oil (c), and mass spectrum of a position A(d).

chloride and cleaned-up on an alumina column by the method described above. Octadecachloroquaterphenyl was determined by ECD–GC and confirmed by GC–MS fragmentography because of the low levels involved.

RESULTS

Figure 1 shows the six possible types of PCQ skeleton. When positional isomers are considered, the number of individual compounds that may exist is more than 100,000. Clearly it is not feasible to isolate the individual members of the PCQ class.

PCQ in samples was separated from PCB and other impurities by gas chromatography. As shown in Fig. 2, the gas chromatogram of PCQ obtained from the organs of Yusho patients shows the same broad peak as a standard PCQ. It was confirmed to be PCQ by GC–MS. A position 'A' in the gas chromatogram was identified as hepta, octa- and nona-chlorinated quaterphenyls. For instance, heptachlorinated quaterphenyl gave a molecular ion (M^+) at *m/e* 578, while loss of 2 Cl yielded the peak at *m/e* 508, and loss of a further 2 Cl gave the peak at *m/e* 438.

Proceeding to quantitative analysis, we chlorinated the PCQ fractions from the Florisil column with antimony pentachloride and they were confirmed as fully

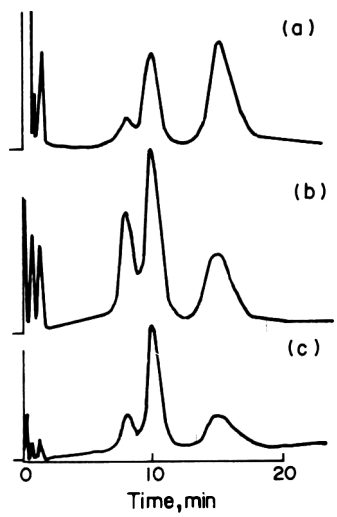

Fig. 3. Gas chromatograms of PCQ in the liver (a) and adipose tissues (b) of a Yusho patient (no. 16634 in the PCQ standard (c) after perchlorination.

Table 1. *Individual PCB and PCQ levels in the tissues of Yusho victims, unexposed individuals and in the milk fat of a worker occupationally exposed to PCB*

Sample no.	Age	Sex	Date of death	Severity of Yusho*	Tissue (or fluid)	GC pattern of PCB†	PCB level (ppb)	PCQ level (ppb)
					Yusho victims			
583	0	M	14.10.68		Intestine	C	29·7	83·6
					Liver	C	50·5	393·0
15868	25	M	9.7.69	4	Adipose tissue	A	5090·7	2400·0
					Liver	A	225·9	217·5
651	48	F	29.12.70	1	Adipose tissue	C	270·5	2·2
					Liver	C	7·4	1·2
16634	46	M	16.5.72	3	Adipose tissue	A	6091·3	1444·0
					Liver	A	68·5	143·5
8	72	M	30.4.75		Intestine	A	3471·8	1770·0
					Liver	A	114·4	51·7
AN-77-34	59	M	17.3.77	2	Intestine	A	3630·3	1125·0
					Liver	A	68·4	27·0
AN-77-100	69	M	4.9.77	1	Intestine	B	1273·4	24·5
					Liver	C	17·7	1·0
					Unexposed individuals			
1	46	M	19.9.78		Adipose tissue		1477·8	2·7
					Liver		71·1	0·8
2	70	M	28.9.78		Adipose tissue		530·1	2·7
					Liver		17·9	0·6
3	54	F	21.9.78		Liver		22·0	0·7
4	35	F	24.9.78		Adipose tissue		248·0	1·3
					PCB worker			
	37	F			Mother's milk fat		6241·1	0·3

*Grade of severity of skin lesions; grades increase with increasing severity.
†The PCB GC patterns are classified according to Masuda *et al.* (1974). A is peculiar to Yusho patients, B is similar to A and C is that of unexposed individuals.

chlorinated PCQ (octadeca-chlorinated quaterphenyl, $C_{24}Cl_{18}$) by GC–MS. These PCQ chromatograms show that the residual patterns in the intestines, adipose tissues and livers are all different although the samples were taken from the same person (Fig. 3).

The amounts of PCB and PCQ obtained from the organs of Yusho patients, from unexposed individuals and from a worker occupationally exposed to PCB are presented in Table 1. It was found that extremely low levels of PCQ (1·3–2·7 ppb) were present in the adipose tissues and livers of unexposed individuals. PCQ was also present in a worker in the PCB industry, who had high residual PCB levels. It appears that in addition to PCB there is also a trace amount of PCQ in the general environment. However, it was clear that the tissue PCQ levels in Yusho victims (apart from two (no. 651 and AN-77-100) whose clinical manifestions and PCB gas chromatogram patterns had not been peculiar to Yusho) were extremely high in comparison with those of unexposed individuals or a worker. Like PCB levels, PCQ levels were much higher in the fatty tissues than in livers. Table 1 also indicates that there is a correlation between the PCQ level and the severity of Yusho disease in the victims, but there appears to be no relation between the concentrations of PCB and PCQ. A baby (sample no. 583 in Table 1) stillborn immediately after the first outbreak of Yusho had a higher PCQ concentration in the liver than in the fatty intestine and the GC pattern of PCB in the tissues appeared to be strongly influenced by PCB transfer into his body; namely, this case did not show pattern 'A', which is peculiar to Yusho, but showed pattern 'C' which is characteristic of unexposed individuals.

Since some recent Yusho victims showed high tissue concentrations of residual PCQ, the PCQ concentration in the blood of living Yusho patients and others was measured by the chlorination method. PCQ was confirmed in the blood by GC–MS fragmentography (Fig. 4). As shown in Table 2, 0·05–8·92 ppb of PCQ was detected in all the people who had consumed the rice oil. On the other hand, at a 0·02 ppb detection limit, no PCQ was found in the blood of workers occupationally exposed to PCB (who had PCB levels more than 20 times those of

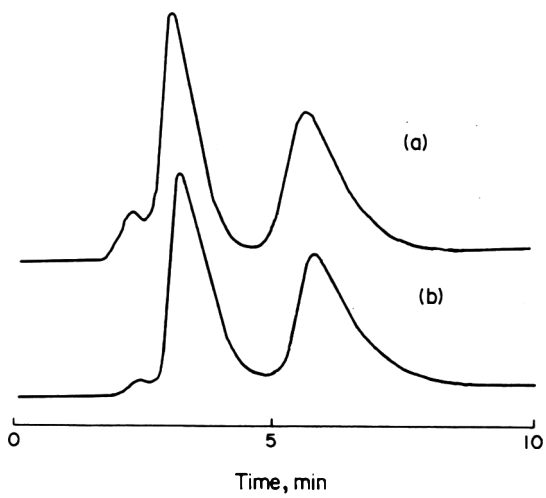

Fig. 4. Single ion monitorings at *m/e* 926 ($M^+ + 8$) of the PCQ in a Yusho patient's blood (a) and in the PCQ standard (b) after perchlorination.

Table 2. *PCB and PCQ levels in the blood of individuals who consumed Yusho oil, workers exposed to PCB and unexposed individuals*

Group	No. of people in sample	GC pattern of PCB	PCB level (ppb)*	PCQ level (ppb)*
Individuals who had consumed Yusho oil†	29 (4)‡	A	7·3 ± 4·5 (1·8–22·6)	3·04 ± 2·11 (0·30–8·92)
	15 (6)‡	B	5·4 ± 3·6 (0·6–10·7)	1·39 ± 1·34 (0·05–4·30)
	8 (3)‡	C	2·7 ± 1·2 (1·3–4·6)	0·28 ± 0·19 (0·05–0·65)
Workers exposed to PCB	6		55·6 ± 20·7 (19·9–88·9)	<0·02
Unexposed individuals	29		2·3 ± 1·5 (0·8–8·4)	<0·02

*Values are means ± SD and the ranges are given in parentheses.
†Yusho patients, their families and others who insist that they had ingested Yusho oil.
‡The numbers of individuals who were not diagnosed as Yusho patients are given in parentheses.

unexposed individuals), or in the blood of unexposed individuals. PCQ was not detected in the fresh PCB to which the workers were occupationally exposed (Miyata *et al.* 1978c).

DISCUSSION

It has previously been reported that the amounts of PCB, PCDF and PCQ in the toxic rice oil ingested by Yusho patients varied depending on the date of manufacture (Miyata *et al.* 1978b). It also seems that amounts of residual PCB and PCQ in the disease victims depend on the length of the period between ingestion of the rice oil and death.

Comparing the level of PCQ in fatty tissue such as the intestine or adipose tissue with that in the liver of a given person (with the exception of victim no. 651), however, PCQ tends to accumulate increasingly in the fatty tissue as the time since ingestion of the rice oil increases. The ratio of the PCQ level in the fatty tissue to that in the liver of victims who died in 1968, 69, 72, 75 and 77 were 0·2, 11·0, 10·1, 34·2, and 41·7 and 24·5 respectively (Table 1). A similar tendency was also observed with PCB, and it has been confirmed in experiments using rats that PCQ undergoes rather slower transfer than PCB (Hori, Kashimoto & Kunita, 1980a; Hori, Obana, Kashimoto & Kunita, 1980b).

Since even workers with high tissue concentrations of PCB have tissue levels of PCQ similar to those of the general population, we conclude that high tissue concentrations of PCQ are peculiar to Yusho. It is not clear whether the individual who was the source of sample no. 651 was actually a Yusho victim, since the PCQ/PCB residue concentration ratios are as low as those of the general population. This is consistent with the results of clinical study and *post mortem* examination (which did not allow a clear diagnosis of Yusho).

As previously mentioned, there are theoretically six types of PCQ each with different basic skeletons. The gas chromatograms of perchlorinated PCQ from Yusho patients and the toxic rice oil showed three peaks, but it was not possible to confirm the corresponding structures in this experiment. The PCQ residue gas chromatograms differed depending on the tissue although the samples were taken from the same Yusho patient. The gas chromatograms of PCQ in the patients' blood were similar to those of PCQ in the liver but less like those from the adipose tissue. This result suggests that PCQs with different structures may be distributed in different ways in the human body. However, further studies in this area are needed.

It was found that 0·05–8·92 ppb of PCQ was present in the blood of those who had consumed the rice oil, while no PCQ was detected in the blood of other people. Thus, Yusho patients appear to have higher PCQ levels than other PCB-exposed people. Even today, 12 yr after the first outbreak of Yusho, PCQ can be detected in as little as 10 ml of the Yusho patients' blood.

There appears to be a trace amount of PCQ in the general environment, and this may account for the PCQ found in the tissues of unexposed individuals. In addition, workers who had handled PCB used as a heat-transfer medium may have been heavily exposed to PCQ in the used PCB (Miyata *et al.* 1978c). Further investigation of these possibilities should be given priority.

In the past, there has been a tendency to treat other PCB poisoning cases as identical to Yusho (Jensen, 1972). However, analysis of many kinds of samples has shown that the composition of residual PCBs in Yusho patients, in workers occupationally exposed to PCB, and in healthy unexposed people are all different (Kuwabara, Yakushiji, Watanabe, Yoshida, Koyama, Kunita & Hara, 1978; Nakamura & Kashimoto, 1977; Watanabe, Yakushiji, Kuwabara, Yoshida, Koyama, Hara & Kunita, 1977). The progress of the clinical symptoms observed also differ; workers occupationally exposed to PCB were rapidly cured of chloracne on changing their job (Geoffrey & Davinder, 1976; Hara, Hirata, Watanabe, Yakushiji, Takahashi & Nishitani, 1979), whereas Yusho patients continue to suffer from chloracne despite having lower blood-PCB levels (Yoshimura, 1971; Watanabe *et al.* 1977). Thus, it seems likely that

Yusho is not simply due to PCB toxicity. Both our results and those of Nagayama, Masuda & Kuratsune (1975), who first reported the presence of PCDF in the organs of Yusho patients lend support to this view.

It is now clear that Yusho is quite different from ordinary PCB toxicity, being characterized by the presence of PCQ in the patients' blood, and by the presence of PCQ and PCDF in the organs. The presence of PCQ in the blood may offer a straightforward method of distinguishing Yusho from simple PCB poisoning. Further studies on the effects of PCQ as well as PCB and PCDF in the human body are desirable.

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REFERENCES

Geoffrey, R. S. & Davinder, S. S. (1976). Use and health effects of Arochlor 1242, polychlorinated biphenyl in an electric industry. *Archs envir. Hlth* **31,** 189.

Hara, I., Hirata, M., Watanabe, I., Yakushiji, T., Takahashi, M. & Nishitani, N. (1979). Health survey of PCB-workers in a paint factory. Osaka Prefectural Institute of Public Health, Edition of Industrial Health **17,** 11 (In Japanese).

Hori, S., Kashimoto, T. & Kunita, N. (1980a). Distribution and excretion of polychlorinated quaterphenyl (PCQ) in rats. *J. Fd Hyg. Soc., Japan.* In press (in Japanese).

Hori, S., Obana, H., Kashimoto, T. & Kunita, N. (1980b). Difference of transfer among PCB, PCT and PCQ from mother to suckling-rats. *J. Fd Hyg. Soc., Japan.* In press (in Japanese).

Jensen, S. (1972). The PCB story. *Ambio* **1,** 123.

Kamps, L. R., Trotter, W. J., Young, S. G., Carson, L. L., Roach, J. A. G. *et al.* (1978). Polychlorinated quaterphenyls identified in rice oil associated with Japanese "Yusho" poisoning. *Bull. envir. Contam. Toxicol.* **20,** 589.

Kikuchi, M. (1972). An autopsy case of PCB poisoning with liver cirrhoses and liver cell carcinoma. *Fukuoka Acta med.* **63,** 387 (In Japanese).

Kikuchi, M., Hashimoto, M., Hozumi, M., Koga, K., Oyoshi, S. & Nagayama, M. (1969). An autopsy case of stillborn of chlorobiphenyls poisoning. *Fukuoka Acta med.* **60,** 489 (In Japanese).

Kikuchi, M. & Masuda, Y. (1974). Studies on autopsy cases of PCB poisoning ("Yusho" Patients)-pathological findings and chemical analysis. *Clin. Path.* **21,** 422 (In Japanese).

Kikuchi, M., Mikagi, Y., Hashimoto, M. & Kojima, T. (1971). Two autopsy cases of chronic chlorobiphenyls poisoning. *Fukuoka Acta med.* **62,** 89 (In Japanese).

Kikuchi, M., Shigematsu, N. & Umeda, G. (1979). Autopsy report of two "Yusho" patients who died nine years after onset. *Fukuoka Acta med.* **70,** 215 (In Japanese).

Kuwabara, K., Yakushiji, T., Watanabe, I., Yoshida, S., Koyama, K., Kunita, N. & Hara, I. (1978). Relationship between breast feeding and PCB residues in blood of the children whose mothers were occupationally exposed to PCBs. *Int. Archs occup. envir. Hlth* **41,** 189.

Masuda, Y., Kagawa, R., Shimamura, K., Takada, M. & Kuratsune, M. (1974). Polychlorinated biphenyls in the blood of "Yusho" patients and ordinary persons. *Fukuoka Acta med.* **65,** 25 (In Japanese).

Miyata, H. & Kashimoto, T. (1978). Investigation on polychlorodibenzofuran formation. *J. Fd Hyg. Soc., Japan* **19,** 78 (In Japanese).

Miyata, H. & Kashimoto, T. (1979). Investigation on organochlorinated compounds formed in Kanemi rice oil that caused the "Yusho". *J. Fd Hyg. Soc., Japan* **20,** 1.

Miyata, H., Kashimoto, T. & Kunita, N. (1977). Detection and determination of polychlorodibenzofurans in normal human tissues and Kanemi rice oils caused "Kanemi Yusho". *J. Fd Hyg. Soc., Japan* **18,** 260.

Miyata, H., Kashimoto, T. & Kunita, N. (1978a). Detection of unknown organochlorinated compounds in Kanemi rice oils which caused "Kanemi Yusho". *J. Fd Hyg. Soc., Japan* **19,** 126 (In Japanese).

Miyata, T., Kashimoto, T. & Kunita, N. (1978b). Detection and determination of unknown organochlorinated compounds in Kanemi rice oil caused the "Yusho". *J. Fd Hyg. Soc., Japan* **19,** 364.

Miyata, H., Murakami, Y. & Kashimoto, T. (1978c). Determination of polychlorinated quaterphenyl (PCQ) in Kanemi rice oil caused the "Yusho" and investigation on the PCQ formation. *J. Fd Hyg. Soc., Japan* **19,** 417.

Nagayama, J., Masuda, Y. & Kuratsune, M. (1975). Chlorinated dibenzofurans in Kanechlors and rice oil used by patients with "Yusho". *Fukuoka Acta med.* **66,** 593.

Nakamura, A. & Kashimoto, T. (1977). Studies on a calculation method for polychlorinated biphenyl (PCB) isomers. *J. Fd Hyg. Soc., Japan* **18,** 1 (In Japanese).

Tanabe, H., Takeda, M., Itho, K., Suzuki, T., Kanda, H. *et al.* (1970). Special report on the prevention, diagnosis and treatment for the "Yusho". *Science and Technology Agency of Japan.* p. 19 (In Japanese).

Tsukamoto, H., Makisumi, S., Hirose H., Kojima, T., Fukumoto, K. *et al.* (1969). The chemical studies on detection of toxic compounds in the rice bran oils used by the patients of Yusho. *Fukuoka Acta med.* **60,** 496 (In Japanese).

Ugawa, M., Nakamura, A. & Kashimoto, T. (1973). *New Methods in Environmental Chemistry and Toxicology.* p. 223. International Academic Printing Co., Totsuka, Tokyo.

Ugawa, M., Nakamura, A. & Kashimoto, T. (1973). Studies on a calculation method for polychlorinated biphenyl (PCB) isomers. *J. Fd Hyg. Soc., Japan* **14,** 415 (In Japanese).

Watanabe, I., Yakushiji, T., Kuwabara, K., Yoshida, S., Koyama, K., Hara, I. & Kunita, N. (1977). Polychlorinated biphenyls in the blood of Yusho patients and ordinary persons. *J. publ. Hlth Ass. Japan* **24,** 749 (In Japanese).

Yoshimura, T. (1971). Epidemiological analysis of "Yusho" patients with special reference to sex, age, clinical grades and oil consumption. *Fukuoka Acta med.* **62,** 104 (In Japanese).

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STUDIES OF THE ARYLHYDROXYLATION OF MONOCHLOROPHENYLUREAS IN THE ISOLATED PERFUSED RAT LIVER

D. WESTPHAL, K. LUCAS and V. HILBIG*

Max von Pettenkofer-Institut des Bundesgesundheitsamtes, Thielallee 88–92, D-1000 Berlin 33, Bundesrepublik Deutschland

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Abstract—Biotransformation studies with phenylurea and the three isomers of monochlorophenylurea were performed using an isolated perfused rat-liver preparation. In the perfusate and the bile, ring-hydroxylation was detected only with phenylurea and with *o*- and *m*-chlorophenylurea, and yielded the corresponding *p*-hydroxylated compounds in each case, while with *p*-chlorophenylurea no ring hydroxylation was observed at the ortho or meta position. Similar results were obtained with the arylhydroxylation of the herbicide monolinuron (3-(4-chlorophenyl)-1-methoxy-1-methylurea) in the perfused rat liver. In contrast, studies of monolinuron biotransformation *in vivo* in rats, pigs and hens showed that ring hydroxylation at the ortho or meta position was the main degradation step. The possible reasons for the difference in biotransformation *in vivo* and *in vitro* are discussed.

INTRODUCTION

Comparison of the results of metabolism studies on monolinuron (3-(4-chlorophenyl)-1-methoxy-1-methylurea) in rats (Böhme & Ernst, 1965; Ernst & Böhme, 1965; V. Hilbig, K. Lucas and D. Westphal unpublished data, 1976), pigs (Hilbig, Lucas & Sebek, 1977) and hens (Hilbig, Lucas, Westphal & Münchow, 1979) with those obtained from isolated perfused rat livers (Westphal, 1977) and hen livers (D. Westphal, K. Lucas, V. Hilbig and U. Mechler unpublished data, 1981) shows marked differences between the biotransformation products formed *in vivo* and *in vitro*. Although the metabolites generated from the aliphatic moiety of monolinuron were found to be identical both in intact animals and in perfused livers, no arylhydroxylated products could be identified in any of the perfusion experiments, although 2- and 3-hydroxy-4-chlorophenylurea isomers have been determined as the principal end-products in rat and pig urine and in the excreta of hens. In connection with these conflicting results, earlier studies by Daly, Guroff, Udenfriend & Witkop (1968) and Daly (1970) on rat-liver microsomal biotransformation appear to be significant. These authors were able to show that ring hydroxylation of anilines, acetanilides and anisoles was impaired by the presence of alkyl and halogen substituents. It thus seemed appropriate to investigate the influence of the chlorine atom and its position in the phenyl ring on the arylhydroxylation of monochlorophenylureas in the isolated perfused rat liver.

EXPERIMENTAL

Animals. Male Wistar rats (weight 300–320 g), bred at the Federal Health Office laboratories, were maintained on a standard laboratory diet (Altromin GmbH, Lage, Lippe) and water *ad lib.* prior to the commencement of the perfusion experiments.

Chemicals. Three perfusions were carried out with each of the following substances: phenylurea (≥99% purity, from Riedel de Haen, Seelze), and 2-, 3- and 4-chlorophenylureas synthesized by addition of ammonia to a benzene solution of the corresponding chlorosubstituted phenylisocyanate and purified by several recrystallizations from acetone/water.

The following standards were used for the thin-layer chromatographic identification of metabolites: 2-, 3- and 4-hydroxyphenylureas prepared by reaction of the corresponding aminophenols with potassium cyanate in hydrochloric acid solution, and 2- and 3-chloro-4-hydroxyphenylureas, 3-chloro-6-hydroxyphenylurea, and 2- and 3-hydroxy-4-chlorophenylureas. The last five compounds were synthesized by preparation of the corresponding substituted chloronitrophenols and reduction of these with sodium dithionite in alkaline solution to yield the required aminochlorophenols, which were then converted to the urea derivatives as described above.

The other reagents and solvents used were of Merck analytical grade (Merck AG, Darmstadt). Mass spectrometry and NMR spectroscopy were used for the unambiguous identification of the compounds synthesized.

Liver perfusion technique

Apparatus. The perfusion apparatus was based essentially on that described by Miller, Bly, Watson & Bale (1951) and by Schimassek (1963), using continuous recirculation of the perfusate. The perfusing medium was oxygenated in a thermostatically controlled (37°C) bubble oxygenator by a counterflow of an O_2–CO_2 (19:1, v/v) gas mixture. The portal pressure was set at 18–20 cm water, giving a perfusate flow rate of 16–18 ml/min.

Liver preparation. After induction of anaesthesia by ip injection of sodium pentobarbital (Nembutal; Abbot, Saint Remy), the livers of rats heparinized with Liquemin (Hoffmann-La Roche, Grenzach) were

*To whom requests for reprints should be sent.

cannulated and perfused *in situ via* the hepatic portal vein, mainly by the technique of Hems, Ross, Berry & Krebs (1966) and Mortimore (1961). A detailed description of this procedure has been given by Westphal (1977). Experience has shown that in a successful operation, the liver immediately clears of endogenous blood at the start of the perfusion, attaining an even pale-brown colour, and remains unswollen and moist throughout the perfusion, a sufficient flow of perfusate is obtained without excessive input pressure, and the bile flow appears to be unimpeded (1·5–1·7 ml/2 hr of perfusion).

Perfusion medium. On the basis of data given by Schimassek (1963 & 1968), a modified tyrode solution was used, with the electrolyte concentration adjusted to the rat plasma values. Hydroxyethyl starch (Fresenius, Bad Homburg) served as the oncotic substance (Franke, Sobotta, Witzki & Unsicker, 1975). In contrast to the perfusion medium selected by Westphal (1977), that used in the present study contained no bovine erythrocytes, since preliminary studies in our laboratory had shown the biotransformation products of monolinuron, a chlorophenylurea, to be identical, whether or not erythrocytes had been used as oxygen carriers. This implies that the oxygen supply was adequate in spite of the fairly high perfusion temperature. The volume of perfusate used for each perfusion was 200 ml. The substrate (30 mg per experiment) was dissolved completely in 0·5 ml polyethylene glycol 400 and then added to the pre-warmed perfusion medium. The duration of each perfusion was 2 hr.

Analytical procedures

Bile. For the determination of hydroxylated metabolites in the bile an enzymatic hydrolysis was carried out as follows: the bile was mixed with 10 ml sodium acetate buffer (pH 4·5) and with 0·5 ml β-glucuronidase/arylsulphatase solution from *Helix pomatia* (Boehringer AG, Mannheim) and incubated for 24 hr at 37°C. At the end of this period, the pH was adjusted to 6·2 by addition of 5 N-NaOH and the mixture was re-incubated for a further 24 hr. The incubation mixture was extracted three times with 2 vols ethyl acetate and the organic phase was dried over anhydrous sodium sulphate, filtered, and evaporated to dryness *in vacuo*. The residue was taken up in 2 ml ethyl acetate, and 20 µl portions of this solution were used for thin-layer chromatographic analysis (TLC).

Perfusate. Unconjugated metabolites were isolated from the perfusate by a two-stage extraction with 2 vols ethyl acetate. The conjugated compounds, in the aqueous phase, were decomposed enzymatically as described above and the products were isolated by extraction with ethyl acetate. The ethyl acetate phase was worked up as described for bile.

Thin-layer chromatography. TLC of the ethyl acetate extracts was carried out on precoated F 254 silicagel plates (Merck AG; layer thickness 0·25 mm), eluted with the solvent systems (A) chloroform–ethanol, 9:1 (v/v), (B) chloroform–ethanol–acetic acid, 18:1:1 (by vol.) or (C) chloroform–ethanol–33% ammonia, 18:2:1 (by vol.).

The metabolites were identified by co-chromatography with solutions of the standards. The compounds under study were detected by their quenching effect on the fluorescent indicator with which the plates had been pretreated, and appeared under UV light (254 nm) as bright blue-violet spots. Colour reactions of the metabolites with *N*-[naphthyl-(1)]ethylenediammonium dichloride (Bratton & Marshall, 1939), carried out according to the procedure described by Schütz & Schütz (1972), indicated the presence of aromatic amines formed from phenylurea derivatives. Reaction with chloroimide 2,6-dichloroquinone/ammonia was used for the qualitative detection of ring-hydroxylated compounds and for distinguishing the 2-, 3- or 4-hydroxyphenylurea derivatives. The detection limit of the compounds tested, using either fluorescence quenching or the colour reaction, was within the range 0·5–1·0 µg.

RESULTS

The results of the liver perfusions are presented in Table 1. It is seen that metabolism of the class of compound under review proceeds *via* aromatic hydroxylation. Molecules containing a chlorine substituent in the para position do not yield hydroxylated products, whereas those containing a chlorine substituent at the ortho or meta position are transformed, like the unsubstituted molecule, only to the para-hydroxylated product, which is found alongside the unchanged parent substance.

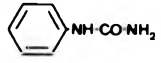
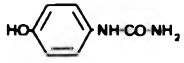
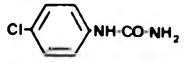
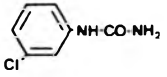
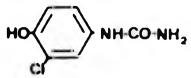
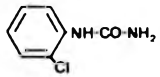
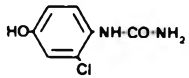
DISCUSSION

It has been demonstrated that arylhydroxylation only takes place in the isolated perfused rat liver if certain conditions are fulfilled. Apparently, the isolated liver merely retains the ability to hydroxylate the aromatic ring in the para position, which is the preferred site for oxidative attack in monosubstituted benzenes, whereas the enzyme complex responsible for ortho and meta hydroxylation is inhibited. This could explain why hydroxylated metabolites of phenylureas containing a para substituent are not produced in the isolated liver. However, despite the predominant role of the liver in the biotransformation of foreign compounds, one cannot exclude completely the possibility that other organs may be responsible for hydroxylation at the 2- and 3- positions in particular, while the liver function is restricted to ring hydroxylation at the para position.

The results of the present study show partial agreement with the findings of Daly *et al.* (1968) and Daly (1970), in their comprehensive studies of the influence of alkyl and halogen substituents on the hydroxylation of anilines, acetanilides and anisoles using rat-liver microsomes. These authors noted a marked reduction in aromatic hydroxylation of compounds containing a halogen atom or a methyl group para to the functional group. Although in these cases some degree of hydroxylation at free ortho and meta positions was observed, the overall proportion of ring-hydroxylated products was less than 10% of that found in the absence of substituents. For 4-trifluoromethylacetanilide, no ring hydroxylation could be demonstrated.

Similarly, the inhibition of microsomal arylhydroxylation by the presence of halogen substituents at the preferred hydroxylation sites of 6-fluoro-α-methyl-

Table 1. *Ring hydroxylation of phenylurea and chlorine-substituted phenylureas in the isolated perfused rat liver*

Substrates				Ring-hydroxylated biotransformation products*					
				para-Hydroxylation					
	R_f value in solvent system†:				R_f value in solvent system†:				*ortho-* or *meta-*
Compound	A	B	C	Compound	A	B	C	Conversion‡	Hydroxylation
	0·32	0·49	0·30		0·12	0·19	0·06	Major (*c.* 50%)	ND
	0·29	0·40	0·25	ND					ND
	0·43	0·42	0·27		0·19	0·25	0·03	Major (*c.* 50%)	ND
	0·59	0·56	0·45		0·28	0·24	0·08	Trace (*c.* 1%)	ND

ND = Not detectable (<0·5–1·0 μg)

*Perfusions, three for each compound, were carried out as described under Experimental, and biotransformation products were isolated from perfusate and bile and identified by thin-layer chromatography.

†Solvent systems A, B and C are identified under Experimental.

‡Estimated tentatively from colour intensity under UV light. The approximate amount of the product as a percentage of the added substrate is given in brackets.

tryptamine (microsomal test) and 4-chloroamphetamine (*in vivo*) has been described (Daly *et al.* 1968; Ellison, Gutzait & Van Loon, 1966; Jepson, Zaltzman & Udenfriend, 1962). No ring-hydroxylated products could be identified for any of these substances, whereas the related compounds, tryptamine and 5-fluorotryptamine, yielded 6-hydroxy derivates under the influence of liver microsomes, and the principal route of *in vivo* degradation of amphetamine was found to be 4-hydroxylation.

Furthermore it is important to mention the observation made by Bauer & Kiese (1964) who noted an almost complete loss of 2-hydroxylase activity towards aniline after the preparation and storage of rabbit-liver microsomes, whereas the 4-hydroxylase activity remained nearly constant.

In accounting for the observed phenomena, methodological errors in the isolation and supply of the liver can be disregarded. The use of the medium described, containing no erythrocytes, at 37°C can also be excluded from consideration as a possible cause of the lack of ring hydroxylation at the 2- and 3- positions, since earlier experiments (Westphal, 1977) using a perfusion medium containing erythrocytes also failed to produce the expected hydroxylated products. The use of a medium containing no erythrocytes was preferred for technical reasons, and a comparison of the two media showed identical degradation products of the monochlorophenylureas in both cases. Detection of the hydroxylated products listed in the table must be viewed as evidence of the liver functions retained. The possibility that the enzyme complex that catalyses hydroxylation at the 2- and 3-positions is inhibited when the para position is occupied has already been referred to. This explanation, however, also appears unsatisfactory, if the situation *in vivo* is considered, since it must be assumed that intact liver cells that are adequately supplied with oxygen and nutrients should show an identical metabolic activity both *in vivo* and *in vitro*. This presupposes of course that the metabolic steps involved actually take place in the liver.

The results of the present study suggest rather that the manipulations carried out during the isolation or perfusion of the liver represent a severe and unavoidable disturbance of the supply system, which results in a restriction of the metabolic activity. It has been suggested by Möhr (1965) that to an increasing extent from the beginning of perfusion onwards, the liver cells lose their ability to maintain the integrity of the membranes. Even in perfusions with media containing erythrocytes and highly saturated with oxygen at physiological pressures, the cell enzymes of the energy-supply metabolism show an increase of up to 10 times the initial values, whereby the measured initial values themselves must also be interpreted as a result of the damage caused. Möhr (1965) suggests that this damage, which manifests itself as a loss of energy from the cells, can certainly not be ascribed to an inadequate oxygen supply to the cells. The true reasons remain obscure, but it is possible that in the few minutes during which the circulation in the liver stagnates, as a result of the manipulation, such a severe energy deficiency occurs that the surviving cells can no longer adequately compensate for it.

On the basis of our findings we conclude that the isolated perfused rat liver is of only limited suitability for studies of the biotransformation of foreign materials, at least with respect to arylhydroxylation when halogen substituents are involved.

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REFERENCES

Bauer, S. & Kiese, M. (1964). Heterogeneousness of the microsomal enzymes effecting the o- and p-hydroxylation of aniline. *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **247,** 144.

Böhme, C. & Ernst, W. (1965). Über den Stoffwechsel von Harnstoff-Herbiciden in der Ratte. 2.Mitteilung. Diuron und Afalon. *Fd Cosmet. Toxicol.* **3,** 797.

Bratton, A. C. & Marshall, E. K., Jr (1939). A new coupling component for sulfanilamide determination. *J. biol. Chem.* **128,** 537.

Daly, J. (1970). Metabolism of acetanilides and anisoles with rat liver microsomes. *Biochem. Pharmac.* **19,** 2979.

Daly, J. W., Guroff, G., Udenfriend, S. & Witkop, B. (1968). Hydroxylation of alkyl and halogen substituted anilines and acetanilides by microsomal hydroxylases. *Biochem. Pharmac.* **17,** 31.

Ellison, T., Gutzait, L. & Van Loon, E. J. (1966). The comparative metabolism of *d*-amphetamine-C^{14} in the rat, dog and monkey. *J. Pharmac. exp. Ther.* **152,** 383.

Ernst, W. & Böhme, C. (1965). Über den Stoffwechsel von Harnstoff-Herbiciden in der Ratte. 1.Mitteilung. Monuron und Aresin. *Fd Cosmet. Toxicol.* **3,** 789.

Franke, H., Sobotta, E. E., Witzki, G. & Unsicker, K. (1975). Funktion und Morphologie der isolierten Rattenniere nach zellfreier Perfusion mit verschiedenen Plasmaexpandern. *Anaesthesist* **24,** 231.

Hems, R., Ross, B. D., Berry, M. N. & Krebs, H. A. (1966). Gluconeogenesis in the perfused rat liver. *Biochem. J.* **101,** 284.

Hilbig, V., Lucas, K. & Sebek, V. (1977). Untersuchungen zur Elimination und Biotransformation von 3-(4-Chlorphenyl)1-methoxy-1-methylharnstoff(Monolinuron)beim Schwein. *Zbl. VetMed. A* **24,** 311.

Hilbig, V., Lucas, K., Westphal, D. & Münchow, H. (1979). Biokinetische Untersuchungen mit dem Herbizid Monolinuron bei der Legehenne. *Arch. Geflügelk.* **43,** 86.

Jepson, J. B., Zaltzman, P. & Udenfriend, S. (1962). Microsomal hydroxylation of tryptamine, indoleacetic acid and related compounds to 6-hydroxy derivatives. *Biochim. biophys. Acta* **62,** 91.

Miller, L. L., Bly, C. G., Watson, M. L. & Bale, W. F. (1951). The dominant role of the liver in plasma protein synthesis. A direct study of the isolated perfused rat liver with the acid of lysine-ϵ-C^{14}. *J. exp. Med.* **94,** 431.

Möhr, J. R. (1965). Untersuchungen über den Austritt von Zell-Enzymen unter der Einwirkung verschiedener Gifte am Modell der isolierten perfundierten Rattenleber. Dissertation Med. Fak. Marburg.

Mortimore, G. E. (1961). Effect of insulin on potassium transfer in isolated rat liver. *Am. J. Physiol.* **200,** 1315.

Schimassek, H. (1963). Metabolite des Kohlenhydratstoffwechsels der isoliert perfundierten Rattenleber. *Biochem. Z.* **336,** 460.

Schimassek, H. (1968). Moglichkeiten und Grenzen der Methodik. In Stoffwechsel der isoliert perfundierten Leber, W. Staib & R. Scholz. p. 1. Spinger–Verlag, Berlin.

Schütz, C. & Schütz, H. (1972). Zur Unterscheidung der Benzodiazepine Lorazepam und Oxazepam mit Hilfe der dünnschichtchromatographischen Trennung-Reaktion-Trennung-Technik. *Z. klin. Chem. klin. Biochem.* **10**, 528.

Westphal, D. (1977). Untersuchungen zur Biotransformation des Herbizids Monolinuron (N-(4-Chlorphenyl)-N'-methoxy-N'-methylharnstoff) im System der isoliert perfundierten Rattenleber. Naturwissenschaftliche Dissertation Freie Universität Berlin.

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A METHOD FOR DETERMINING THE MAXIMUM TOLERATED DOSE FOR *IN VIVO* CYTOGENETIC ANALYSIS

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

and

R. A. HILES
Springborn Institute for Bioresearch, Spencerville, OH 45887, USA

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Abstract—Data from acute studies of methyl methanesulphonate, glycidol and dimethylsulphoxide were used to determine maximum tolerated doses (MTD) for subchronic studies. The acute LD_{50} values were determined for each compound, and the LD_{10} and LD_1 values were calculated by probit analysis. The amounts represented by these values, along with those equivalent to the 0·5 LD_1 and 0·1 LD_1 values were dosed daily for 5 days to rats. The groups dosed with the 0·5 LD_1 level generally had few deaths and the body weights were within 80% of the solvent-control group. Moreover, bone-marrow cells from these animals produced acceptable mitotic figures. Thus, the 0·5 LD_1 value derived from acute studies was determined to be a generally acceptable MTD for subchronic cytogenetic studies.

INTRODUCTION

In 1971 the Ad Hoc Committee of the Environmental Mutagen Society and the Institute for Medical Research proposed a standardized protocol for *in vivo* cytogenetic studies (Nichols, Moorhead & Brewen, 1972). The protocol consisted of acute and subchronic dosing regimes, with six groups in each. The groups were (1) the maximum tolerated dose (MTD), (2) an intermediate level, (3) the usage level, (4) a direct-acting positive control, (5) a positive control requiring metabolic activation, and (6) a negative control. Most protocols today omit one of the positive control groups, and frequently, on account of the added burden of demonstrating a dose response in mutagenicity assays, the dose range is narrowed.

Performance of the acute dosing regime is straightforward, but it is not so easy to establish the MTD for the subchronic study. The mutagenesis testing is often done before detailed metabolism or pharmacokinetic data are available, and the investigator must either use an arbitrary value or run a preliminary subchronic study to determine toxicity.

The importance of determining the appropriate MTD cannot be overemphasized. If the value chosen is too high, the animals show signs of toxicity, such as weight loss or lethargy. Quite frequently the bone-marrow cells stop dividing, and the absence of mitotic figures in the bone-marrow cells precludes cytogenetic analysis. But if the dose is too low, the data may not be acceptable.

In this report we present a method for determining the MTD for a subchronic dosing regime from the results of acute studies, both oral and ip. The first step was to determine the acute LD_{50} values of three model compounds (methyl methanesulphonate, dimethylsulphoxide and glycidol). The LD_{10}, LD_1, 0·5 LD_1 and 0·1 LD_1 values were calculated from the LD_{50} data, and administered on a g/kg-body weight basis for five consecutive days to rats, either by ip injection or by gavage. It was anticipated that one of the above dose levels would approximate to the MTD, as determined by the following criteria: (1) the group would have less than 30% deaths, (2) the group's average body weight would be no less than 80% of the control-group body weight and (3) the surviving animals would have enough mitotic figures for cytogenetic analysis.

EXPERIMENTAL

Test materials. Dimethylsulphoxide (ACS grade; DMSO) was obtained from Fisher Scientific Co., Fairlawn, NJ, and methyl methanesulphonate (minimum purity 98%; MMS) and glycidol were supplied by Eastman Chemical Co., Rochester, NY.

Determination of LD_{50} values. Acute LD_{50} values for the oral and ip routes of administration were determined in four groups of ten female Sprague–Dawley rats (weighing 190–270 g). The rats were fasted overnight and given a single dose of the test compound the next morning. The doses were in a geometric progression of 1·4, the estimated LD_{50} value being between the middle two doses. Occasionally these data did not encompass the LD_{50} value and a fifth dose group had to be added. The LD_{50} value and the slope of the dose-response curve were calculated by the probit method (Finney, 1971).

Determination of subchronic MTD. For each test compound and route of administration, groups of ten female Sprague–Dawley rats were dosed orally or ip on five consecutive days with doses equivalent to the LD_{10}, LD_1, 0·5 LD_1 and 0·1 LD_1. After the fifth dos-

ing, six rats from each selected group were used for cytogenetic analyses.

Preparation of bone-marrow cells for chromosome analysis. Selected animals were given 4 mg colchicine/kg after receiving the five doses of the test material and were then killed with an overdose of barbiturate. Bone-marrow cells were aspirated from the femurs of both legs with an 18-gauge needle attached to a 10-cc syringe containing 5 ml Eagle's minimal medium. The cells were gently suspended in the medium, collected by centrifugation at 160 g, resuspended in 5 ml 0·075 M-KCl at 37°C, and incubated for 10 min at room temperature. The cells were again centrifuged (160 g) and resuspended in freshly prepared methanol–acetic acid (3:1, v/v) fixative. After a 30-min incubation, they were collected by centrifugation and resuspended in fresh fixative. After storage overnight at 4°C, the cells were dropped on to wet slides that had been stored in cold water. The slides were air-dried and stained for 10 min with Giemsa. The slides were destained by rinsing in distilled water for 1 min, and were then given successive 10–20-sec rinses in acetone and acetone–xylene (1:1, v/v), and a final 5-min rinse in xylene. A drop of Permount was added to each slide and a coverslip was put in place.

RESULTS

The results of the single-dose (acute) studies are shown in Table 1. Probit analyses for the ip and oral dosing regimes of the three compounds are shown in Fig. 1. Of particular importance in these figures are the slopes of the dose-response curves and the range of the doses. All these compounds have relatively steep dose-response curves, but their LD_{50} values differ by two orders of magnitude. MMS is the most toxic (LD_{50} 0·1–0·2 g/kg) and DMSO is the least toxic (LD_{50} 10–20 g/kg). Thus the three compounds provide a range of acute toxic responses.

The subchronic MTD was determined by dosing groups of ten rats on five consecutive days with different levels of the test materials to determine which dosage groups met our criteria for a suitable cytogenetic study. After the fifth dosing, the group receiving the highest dose for each test compound and route of administration and still fitting the criteria for number of deaths and weight loss was selected, and six of the ten animals that had received this dosage were subjected to cytogenetic analyses.

The results of the subchronic dosing of MMS, glycidol and DMSO are shown in Tables 2–4. At least one dose level for each compound and each route of administration yielded animals suitable for cytogenetic evaluation. The acceptable dose varied with both the compound and the route of administration.

Cytogenetic analyses are reported in Table 5. Only MMS showed significant ($P < 0·05$) levels of chromosome damage (Hollander & Wolfe, 1973). Neither glycidol nor DMSO showed significant clastogenic action in these assays.

DISCUSSION

When pharmacokinetic data are not available, the use of both the LD_{50} value and the slope of the dose *v.* response curve from an acute toxicity study to determine the MTD for a subchronic dosing regime should better ensure the acquisition of usable subchronic data than would the use of an arbitrary percentage of the LD_{50} value (e.g. 20% of the LD_{50}). In the experimental approach to this problem assessed here, a cytogenetic evaluation using bone-marrow cells was used as the eventual end point. MMS was selected as a model compound because it had been

Table 1. *Results of acute LD_{50} studies in rats given either methyl methanesulphonate, glycidol or dimethylsulphoxide*

	Oral administration			Intraperitoneal administration		
Compound	Dose level (g/kg)	Lethal value*	LD_{50} (g/kg)	Dose level (g/kg)	Lethal value*	LD_{50} (g/kg)
MMS†	0·15	0/10		0·106	0/10	
	0·21	4/10		0·134	2/10	
			0·225			0·14
	0·265	8/10		0·148	7/10	
	0·29	10/10		0·207	10/10	
Glycidol‡	0·23	0/10		0·13	0/10	
	0·32	5/10		0·18	3/10	
			0·42			0·20
	0·45	6/10		0·21	6/10	
	0·63	8/10		0·25	10/10	
DMSO§	12·7	0/10		4·6	0/10	
	15·0	2/10		6·4	1/10	
			17·5			
	17·7	5/10		9·0	5/10	
						9·8
	24·8	10/10		12·6	8/10	

*Lethal value = no. of dead animals/no. of animals dosed.
†Methyl methanesulphonate, administered as a 1% solution in water.
‡Administered as a 10% solution in water.
§Dimethylsulphoxide, administered undiluted.

Table 2. *The effects of subchronic (5-day) oral and ip dosing of Sprague–Dawley rats with methyl methanesulphonate*

Dosage group	Dose (g/kg)	No. of survivors*	No. of rats used for cytogenetic analysis	Mean terminal body weight† (g)	Group body weight (% of negative control value)
LD_{10} (oral)	0·183	2	—	—	—
LD_1 (oral)	0·156	7	6	177	86
0·5 LD_1 (oral)	0·078	10	—	—	—
0·1 LD_1 (oral)	0·016	10	—	—	—
LD_{10} (ip)	0·134	0	—	—	—
LD_1 (ip)	0·126	1	—	—	—
0·5 LD_1 (ip)	0·063	10	6	187	91
0·1 LD_1 (ip)	0·013	10	—	—	—
Water (oral)	20	10	6	205	—

*Ten animals were dosed in each group.
†The mean initial body weight for each of the four test groups was within 4 g of the mean for all four groups. The six animals in the control group had an initial mean body weight that was 11 g below the mean for the other four groups.

Table 3. *The effects of subchronic (5-day) oral and ip dosing of Sprague–Dawley rats with glycidol*

Dosage group	Dose (g/kg)	No. of survivors*	No. of rats used for cytogenetic analysis	Mean terminal body weight† (g)	Group body weight (% of negative control value)
LD_{10} (oral)	0·226	8	6	184	80
LD_1 (oral)	0·143	10	—	—	—
0·5 LD_1 (oral)	0·072	10	—	—	—
0·1 LD_1 (oral)	0·014	10	—	—	—
LD_{10} (ip)	0·167	5	—	—	—
LD_1 (ip)	0·145	8	6	192	84
0·5 LD_1 (ip)	0·072	10	—	—	—
0·1 LD_1 (ip)	0·014	10	—	—	—
Water (oral)	20	10	6	229	—

*Ten animals were dosed in each group.
†The mean initial body weight for each of the groups was within 4 g of the mean for all five groups.

Table 4. *The effects of subchronic (5-day) oral and ip dosing of Sprague–Dawley rats with dimethylsulphoxide*

Dosage group	Dose (g/kg)	No. of survivors*	No. of rats used for cytogenetic analysis	Mean terminal body weight† (g)	Group body weight (% of negative control value)
LD_{10} (oral)	14·5	0	—	—	—
LD_1 (oral)	12·5	1	—	—	—
0·5 LD_1 (oral)	6·2	4	—	—	—
0·1 LD_1 (oral)	1·2	10	6	214	91
LD_{10} (ip)	6·4	7	6	221	94
LD_1 (ip)	4·6	10	—	—	—
0·5 LD_1 (ip)	2·3	10	—	—	—
0·1 LD_1 (ip)	0·5	10	—	—	—
Water (oral)	15	9	6	234	—

*Ten animals were dosed in each group.
†The mean initial body weight for each of the groups was within 4 g of the mean for all four groups.

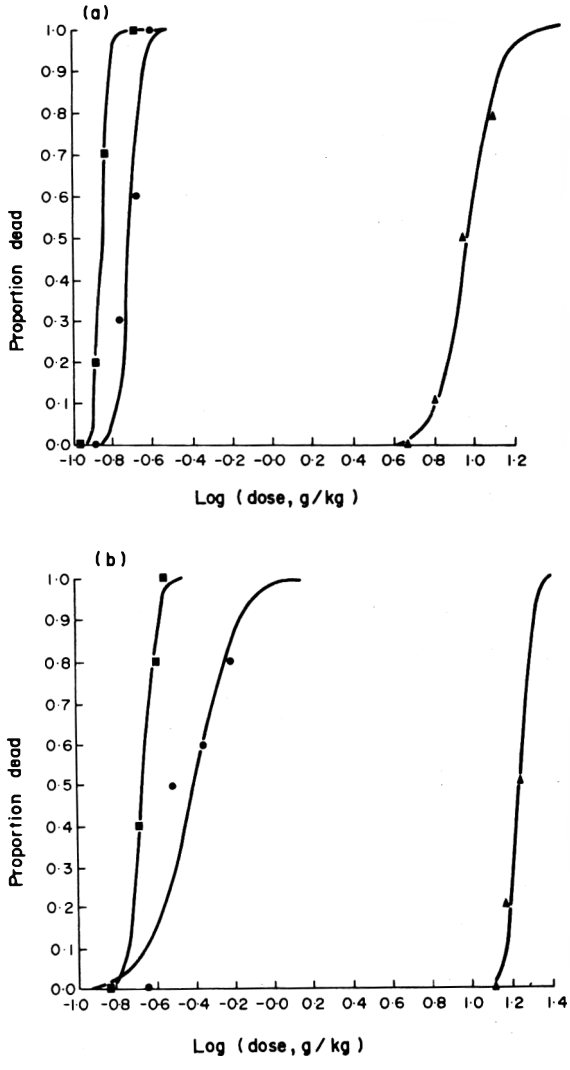

Fig. 1. Probit analysis of the acute ip dosing (a) and acute oral dosing (b) of methyl methanesulphonate (■), glycidol (●) and dimethylsulphoxide (▲).

reported as a clastogen in both *in vitro* and *in vivo* systems (Broegger, 1974; Frei & Venitt, 1975). Glycidol was selected because other aliphatic epoxides have been shown to be clastogenic when dosed ip (Hendry, Homer, Rose & Walpole, 1951), but not when dosed orally (Bootman, Lodge & Whalley, 1979). DMSO is used as a solvent in a number of mutagenicity assays and, although it is not generally considered clastogenic, there is a recent report to the contrary (Kapp & Eventott, 1979).

The three compounds in this study had LD_{50} values differing by as much as 100-fold. However, they all had steeply sloping dose *v.* response curves. When they were dosed in the series LD_{10}, LD_1, 0·5 LD_1 and 0·1 LD_1, each compound yielded an acceptable group for the cytogenetic evaluation (70% survival with mean weights no more than 20% below that of the negative controls). However, the acceptable dose levels varied with the route of administra-

Table 5. *Results of cytogenetic analyses of bone-marrow cells from rats dosed for five consecutive days with either methyl methanesulphonate (MMS), glycidol or dimethylsulphoxide (DMSO)*

Test group		Dose (g/kg)	No. of cells		Incidence of aberrations* (%)	No. of aberrations of specific type				
			Evaluated	With aberrations*		Gap	Break†	Exchange	Fragments	Rings
Water			898	1	0·1	7	0	0	1	0
MMS:	oral	0·156	176	9	5·1	5	2	2	4	1
	ip	0·063	175	4	2·3	7	3	0	1	0
Glycidol:	oral	0·226	285	2	0·7	3	1	1	0	0
	ip	0·145	300	3	1·0	8	0	1	2	0
DMSO:	oral	1·2	300	2	0·7	5	0	0	2	0
	ip	6·4	300	0	0·0	1	0	0	0	0

*Not including gaps.
†Both chromatid and isochromatid breaks.

tion (for DMSO, 0·1 LD_1 for oral dosing and LD_{10} for ip dosing) and with the compound (0·1 LD_1 for orally administered DMSO and LD_{10} for ip administered DMSO and orally administered glycidol). Such variations probably reflect differences in the elevation of blood levels with repeated exposure or in cumulative toxic effects.

The data in Table 5 show that in the rats in these groups a sufficient number of mitotic figures were present for the cytogenetic analysis. As expected, MMS produced an increase in chromosome aberrations; glycidol and DMSO did not, although glycidol has been shown to be a moderately potent mutagen in the Ames assay (McCann, Choi, Yamasaki & Ames, 1975). Although the number of acceptable mitotic figures for MMS was lower than expected, subsequent studies with these dose levels have produced anticipated mitotic data.

In all cases except that of orally administered DMSO, a 0·5 LD_1 dosing level would have provided acceptable animals for a cytogenetic evaluation. This value has been used in three subsequent cytogenetic studies using MMS as the positive control, and all have produced excellent results. In addition, we are now using 80% as the required survival rate. Thus, the procedure that we have described offers a method for determining the MTD when other data are not available.

REFERENCES

Bootman, J., Lodge, D. C. & Whalley, H. E. (1979). Mutagenic activity of propylene oxide in bacterial and mammalian systems. *Mutation Res.* **67,** 101.

Broegger, A (1974). Caffeine-induced enhancement of chromosome damage in human lymphocytes treated with methyl methanesulfonate, mitomycin C, and x-ray. *Mutation Res.* **23,** 353.

Finney, D. J. (1971). *Probit Analysis.* 3rd Ed. p. 50. Cambridge University Press, Cambridge.

Frei, J. V. & Venitt, S. (1975). Chromosome damage in the bone marrow of mice treated with methylating agents methyl methanesulfonate and N-methyl-N-nitrosourea in the presence or absence of caffeine and its relation with thymoma induction. *Mutation Res.* **30,** 89.

Hendry, J. A., Homer, R. F., Rose, F. L. & Walpole, A. L. (1951). Cytotoxic agents—II. Bis-epoxides and related compounds. *Br. J. Pharmac.* **6,** 235.

Hollander, M. & Wolfe, D. A. (1973). *Nonparametric Statistical Methods.* p. 68. John Wiley & Sons, New York.

Kapp, R. W., Jr & Eventott, B. E. (1979). *In vivo* cytogenetic effects of DMSO in rats. *Envir. Mutagen.* **1,** 178.

McCann, J., Choi, E., Yamasaki, E. & Ames, B. N. (1975). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: Assay of 300 chemicals. *Proc. natn. Acad. Sci. U.S.A.* **72,** 5135.

Nichols, W. W., Moorhead, P. & Brewen, G. (Co-chairman) (1972). Chromosome methodologies in mutation testing. Report of the Ad Hoc Committee of The Environmental Mutagen Society and The Institute for Medical Research. *Toxic. appl. Pharmac.* **22,** 269.

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SHORT PAPERS

NON-CARCINOGENIC RESPONSE TO COUMARIN IN SYRIAN GOLDEN HAMSTERS

I. UENO and I. HIRONO

Department of Carcinogenesis and Cancer Susceptibility, Institute of Medical Science, University of Tokyo, Minato-ku , Tokyo 108, Japan

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Abstract—Groups of 11–12 male and 10–13 female Syrian golden hamsters received diets containing 0·0 (controls), 0·1 or 0·5% coumarin for up to 2 yr. In these three groups, survivors of the initial totals of 24, 24 and 21 hamsters, respectively, numbered 17, 9, and 18 at 18 months, 12, 1 and 12 at 22 months and 10, 1 and 5 at 24 months. There was no evidence that coumarin had induced fatty change or cysts in the liver, bile-duct proliferation, cholangiofibrosis or cholangiocarcinoma in animals dying during the study or in those killed terminally. Tumours observed at extra-hepatic sites were randomly distributed between the control and the test groups. On the basis of this study, coumarin does not appear to be hepatotoxic or hepatocarcinogenic in the Syrian golden hamster.

Introduction

Coumarin occurs naturally in various plant products and is widely used as a flavouring ingredient, although its usage as a food additive is restricted. Although its hepatotoxicity has been demonstrated unequivocally in the rat (Hagen, Hansen, Fitzhugh, Jenner, Jones, Taylor, Long, Nelson & Brouwer, 1967; Hazleton, Tusing, Zeitlin, Thiessen & Murer, 1956), conflicting evidence has been obtained on the carcinogenicity of coumarin in that species. Bär & Griepentrog (1967) and Griepentrog (1973) reported the occurrence of bile-duct carcinoma in rats fed coumarin at 5000 or 6000 ppm in the diet for 2 yr. In contrast, Hagan *et al.* (1967) detected neither benign nor malignant tumours in the liver or any other organs of rats fed dietary levels up to 5000 ppm for 2 yr, although they did observe slight fatty change, bile-duct proliferation and minimal focal necrosis at 2500 and 5000 ppm and cholangiofibrosis at the 5000-ppm level. In a mouse-skin bioassay, coumarin showed no tumour-initiating activity (Roe & Salaman, 1955).

Metabolic studies on coumarin in the rat (Booth, Masri, Robbins, Emerson, Jones & DeEds, 1959; Kaighen & Williams, 1961; Van Sumere & Teuchy, 1971), the hamster (Gangolli, Shilling, Grasso & Gaunt, 1974), the baboon (Gangolli *et al.* 1974) and man (Shilling, Crampton & Longland, 1969) indicated considerable species differences in its metabolic fate (Cohen, 1979). This suggests that different species may have different levels of susceptibility to the chronic hepatotoxicity of coumarin.

The Syrian golden hamster has been reported to develop bile-duct proliferation and liver cysts spontaneously, together with other lesions and malignant tumours (Della Porta, Shubik & Scortessi, 1959; Fortner, 1957). A continuum of lesions from bile-duct hyperplasia to cholangioadenoma and cholangiocarcinoma was induced in the hamster by a chemical carcinogen (2-acetamidofluorene) in the study reported by Della Porta *et al.* (1959). The subject of chemical carcinogenesis in hamsters has been comprehensively reviewed by Homburger (1979a,b). In view of the susceptibility of the hamster to known liver carcinogens, a study on coumarin was undertaken in this species.

Experimental

Crystalline coumarin (99% pure), from Tokyo Kasei Kogyo Co. Ltd, Tokyo, was administered at 0·1 and 0·5% in the diet to Syrian golden hamsters (from Hoshino Co., Tokyo). The composition of the basal diet (CE-2 powder; Nihon CLEA Co. Ltd, Tokyo) has been described by Hirono, Mori, Yamada, Hirata, Haga, Tatematsu & Kanie (1977).

When 8 wk old, the hamsters were divided into three groups and housed five or six per cage, separated by sex and treatment. The basal (control) diet was fed to 12 males and 12 females, 0·1% coumarin to 11 males and 13 females and 0·5% to 11 males and 10 females, for up to 2 yr. Food and water was available *ad lib.* Food intake was measured weekly for the first 6 months of treatment and body weight was recorded monthly. All animals that died during the experiment or that were killed when moribund or at termination were autopsied. After macroscopic examination, tissues were fixed in 10% buffered formalin, sectioned and stained with haematoxylin and eosin for histopathological study.

The chi-square test was used for statistical analysis of the incidence of tumours, cystic liver lesions and fatty degeneration.

Results and Discussion

Hamsters in the 0·1 and 0·5% groups showed a transient reduction in food intake, this being about

Table 1. *Survival and incidence of various tumours and hepatic lesions in hamsters fed coumarin at 0–0·5% in the diet for 2 yr*

		Values for groups of hamsters fed coumarin at dietary levels (%) of:					
		0 (Control)		0·1		0·5	
		M	F	M	F	M	F
Initial group size		12	12	11	13	11	10
No. surviving at month	15	11	8	10	5	11	8
	18	10	7	7	2	11	7
	22	9	3	2	0	10	2
	24	8	2	1	0	4	1
No. of hamsters with:							
Forestomach papilloma			1		1	2	
Adrenal cortical adenoma		1	2	2		4	2
Adrenal phaeochromocytoma					1		
Adrenal haemangioma						1	
Pancreatic islet-cell carcinoma							2
Uterine leiomyoma					1		1
Thymic lymphoma (histiocytic)							1
Haemangioma						1	
Leukaemia						1	
Mesenteric lymph-node sarcoma			1				
Sarcoma in inguinal region			1				
Hepatic fatty degeneration		8	4	5	7	8	3
Cystic liver lesion		3	3	2		6	5
Total tumour-bearing animals (%)†		8	42	18	23	45*	40

†Expressed as a percentage of the initial group size. Some animals developed more than one type of tumour.

The value marked with an asterisk differs significantly (*$P < 0.05$ by the chi-square test) from that for the male control group.

20% lower than in controls at 1 month but returning to normality by month 5. Growth retardation was not apparent in either coumarin-treated group. Although survival in the 0·5% group was similar to that in controls, animals fed 0·1% coumarin showed poor survival (Table 1).

The incidence of hepatic lesions (fatty degeneration or cysts) and of tumours at various sites in the control and test groups is also shown in Table 1. Although the number of tumour-bearing animals in the males fed 0·5% coumarin was greater than in control males (45 *v.* 8%, expressed as a percentage of initial group size), it was of the same order as that in control females (42%). There was no consistent difference in the incidence of any given tumour between test and control hamsters.

Of particular note was the absence of cholangioadenoma or cholangiocarcinoma in test animals and the fact that the minimal bile-duct proliferation observed in some animals on 0·1 or 0·5% coumarin was also seen in the controls. These negative findings contrast strikingly with observations in coumarin-fed rats, notably the bile-duct carcinomas reported by Bär & Griepentrog (1967) and Griepentrog (1973) and the focal bile-duct proliferation of atypical appearance with fibrosis (cholangiofibrosis) observed by Hagan *et al.* (1967).

Liver cysts develop spontaneously in Syrian golden hamsters (Fortner, 1957; Della Porta *et al.* 1959). In the present study cysts, all with the same histological structure, occurred with similar frequency in test and control animals (25, 18 and 55% in males fed 0·0, 0·1 and 0·5% coumarin and 25, 0 and 50% in the corresponding female groups). Liver cysts appeared earlier in females on 0·5% coumarin than in other groups of either sex.

Fatty degeneration has been induced in rats fed coumarin at and above 2500 ppm for 90 days (Hazleton *et al.* 1956) or 2 yr (Hagan *et al.* 1967). In the hamster, the magnitude and frequency of this lesion were similar in the control and test groups, the incidence being 67, 45 and 73% in males on 0·0, 0·1 and 0·5% coumarin and 33, 54 and 30% in females on the corresponding levels.

Only limited conclusions can be drawn from this study because of the relatively small number of animals used. Nevertheless, it does suggest that the Syrian golden hamster is refractory to the hepatotoxic action of coumarin, and in particular fails to react like the rat in the development of cholangiofibrosis or supposed cholangiocarcinoma.

REFERENCES

Bär, F. & Griepentrog, F. (1967). Die Situation in der gesundheitlichen Beurteilung der Aromatisierungsmittel für Lebensmittel. *Medizin Ernähr.* **8**, 244.

Booth, A. N., Masri, M. S., Robbins, D. J., Emerson, O. H., Jones, F. T. & DeEds, F. (1959). Urinary metabolites of coumarin and *o*-coumaric acid. *J. biol. Chem.* **234**, 946.

Cohen, A. J. (1979). Critical review of the toxicology of coumarin with special reference to interspecies differences in metabolism and hepatotoxic response and their significance to man. *Fd Cosmet. Toxicol.* **17**, 277.

Della Porta, G., Shubik, P. & Scortessi, V. (1959). The action of N-2-fluorenylacetamide in the Syrian golden hamster. *J. natn. Cancer Inst.* **22**, 463.

Fortner, J. G. (1957). Spontaneous tumors, including gastrointestinal neoplasms and malignant melanomas in the Syrian hamster. *Cancer N.Y.* **10**, 1153.

Gangolli, S. D., Shilling, W. H., Grasso, P. & Gaunt, I. F. (1974). Studies on the metabolism and hepatotoxicity of coumarin in the baboon. *Biochem. Soc. Trans.* **2**, 310.

Griepentrog, F. (1973). Pathologisch-anatomische Befunde zur karzinogenen Wirkung von Cumarin im Tierversuch. *Toxicology* **1**, 93.

Hagan, E. C., Hansen, W. H., Fitzhugh, O. G., Jenner, P. M., Jones, W. I., Taylor, J. M., Long, E. L., Nelson, A. A. & Brouwer, J. B. (1967). Food flavourings and compounds of related structure. II. Subacute and chronic toxicity. *Fd Cosmet. Toxicol.* **5**, 141.

Hazleton, L. W., Tusing, T. W., Zeitlin, B. R., Thiessen, R., Jr. & Murer, H. K. (1956). Toxicity of coumarin. *J. Pharmac. exp. Ther.* **118**, 348.

Hirono, I., Mori, H., Yamada, K., Hirata, Y., Haga, N., Tatematsu, H. & Kanie, S. (1977). Carcinogenic activity of petasitenine, a new pyrrolizidine alkaloid isolated from *Petasites japonicus* Maxim. *J. natn. Cancer Inst.* **58**, 1155.

Homburger, F. (1979a). Chemical carcinogenesis in Syrian hamsters: A review (through 1976). In *Progress in Experimental Tumor Research. Vol. 23. Oncogenesis and Natural Immunity in Syrian Hamsters.* Edited by J. J. Trentin. p. 100. S. Karger, Basel.

Homburger, F. (Ed.) (1979b). *Progress in Experimental Tumor Research. Vol. 24. The Syrian Hamster in Toxicology and Carcinogenesis Research.* S. Karger, Basel.

Kaighen, M. & Williams, R. T. (1961). The metabolism of [3-^{14}C]coumarin. *J. mednl pharm. Chem.* **3**, 25.

Roe, F. J. C. & Salaman, M. H. (1955). Further studies on incomplete carcinogenesis: Triethylene melamine (T.E.M.), 1,2-benzanthracene and β-propiolactone, as initiators of skin tumour formation in the mouse. *Br. J. Cancer* **9**, 177.

Shilling, W. H., Crampton, R. F. & Longland, R. C. (1969). Metabolism of coumarin in man. *Nature, Lond.* **221**, 664.

Van Sumere, C. F. & Teuchy, H. (1971). The metabolism of [2-^{14}C]coumarin and [2-^{14}C]-7-hydroxycoumarin in the rat. *Archs int. Physiol. Biochim.* **79**, 665.

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DIMINUTION PAR UN DITHIOCARBAMATE FONGICIDE, LE ZINEBE, DE L'ACTIVITE DES OXYGENASES MICROSOMALES DU FOIE CHEZ LE RAT: EFFETS D'UN REGIME A 9% DE CASEINE

M. A. PÉLISSIER, F. FAUDEMAY, E. DOOH-PRISO, S. ATTÉBA et R. ALBRECHT

Laboratoire de Biologie du Conservatoire National des Arts et Métiers et Groupe de Recherches sur la Physiopathologie de la Nutrition, U 177 INSERM, 75141 Paris Cedex 03, France

(*Reçu le 27 novembre 1980*)

Résumé—De jeunes rats mâles reçoivent une alimentation équilibrée ou à 9% de caséine. Après 2 semaines, chaque groupe est divisé en deux lots: témoin et traité au zinèbe (1,2-éthylènebis(dithiocarbamate) de zinc). Le traitement consiste en l'addition aux régimes de 600 ppm de pesticide (quantité exprimée en produit pur), durant 4 semaines. Le régime hypoprotéique abaisse les activités de la *N*-déméthylase de l'aminopyrine (APDM) et de la NADPH-cytochrome *c* réductase quand on les exprime par rapport aux protéines, mais il augmente leurs activités moléculaires exprimées par unité de *P*-450. Le régime déficient n'a pas d'effet sur l'activité spécifique de l'hydroxylase de l'aniline (AH) mais il augmente l'activité par unité de *P*-450. Le régime hypoprotéique réduit les teneurs en cytochromes *P*-450 et b_5. La durée du sommeil provoqué par l'hexobarbital est prolongée chez le rat au régime déficient. L'ingestion de zinèbe abaisse les activités de l'ADPM et de l'AH rapportées aux protéines mais elle n'a pas d'effet sur leurs activités exprimées par unité de *P*-450. Le traitement ne modifie pas significativement les teneurs en cytochromes *P*-450 et b_5, non plus que l'activité de la NADPH-cytochrome *c* réductase. Le fongicide augmente le temps de sommeil provoqué. Le régime hypoprotéique ne modifie pas les effets du zinèbe sur les oxygénases microsomales et *vice versa*; de sorte que le rat traité au zinèbe et nourri avec le régime déficient métabolise très lentement les xénobiotiques.

Abstract—Young male rats were fed a balanced diet or one containing 9% casein. After 2 wk, each group was divided, one half being maintained as the control and the other half being fed diet containing zineb, zinc 1,2-ethylenebis(dithiocarbamate), at a level of 600 ppm (of the pure product) for 4 wk. The low-protein diet lowered the activities of aminopyrine *N*-demethylase (APDM) and NADPH-cytochrome *c* reductase expressed in relation to protein, but increased their molecular activities per unit of *P*-450. The protein-deficient diet had no effect on the specific activity of aniline hydroxylase (AH) but increased the activity per unit of *P*-450. It also reduced the levels of cytochromes *P*-450 and b_5. Rats on this diet showed a prolonged hexobarbital-induced sleeping time. Ingestion of zineb lowered the activities of APDM and AH, calculated on the basis of protein, but had no effect on their activities expressed in terms of *P*-450 units. The treatment did not modify the cytochrome *P*-450 and b_5 levels significantly, nor the activity of NADPH-cytochrome *c* reductase. The fungicide augmented the hexobarbital-induced sleeping time. The low-protein diet did not modify the effects of zineb on the microsomal oxygenases or *vice versa*, so the zineb-treated rat fed on a deficient diet metabolizes xenobiotics very slowly.

Introduction

Les sujets présentant des carences protéiques sont particulièrement sensibles aux diverses agressions, et par suite ils sont (ou ils devraient être) soumis à des traitements médicamenteux. Or, l'expérience acquise avec les animaux de laboratoire montre que souvent la toxicité des xénobiotiques s'élève quand l'alimentation devient pauvre en protéines (Boyd & Dubos, 1969; Drill, 1952; Kato, Oshima & Tomizawa, 1968; McLean & McLean, 1969). On sait que les modifications de toxicité de ces molécules sont en partie liées à des altérations de leur métabolisme dans les microsomes hépatiques (Campbell & Hayes, 1974 & 1976).

Au laboratoire, nous avons antérieurement établi que l'activité des oxygénases microsomales est abaissée, après 4 ou 8 semaines, chez le rat nourri avec des régimes comportant 3600 ou 7200 ppm de zinèbe, 1,2-éthylènebis(dithiocarbamate) de zinc (Albrecht, Pélissier, Manchon & Dupuis, 1975). Une méthode statistique, originale dans le cas des données de mesure, nous a permis de calculer que la plus petite dose ayant un effet significatif ($P = 0,05$) est 50 ppm dans l'alimentation soit une ingestion de 4 mg/kg rat/jour (Albrecht, 1979; Lowy, Albrecht, Pélissier & Manchon, 1977).

Par la présente expérience, nous étudions l'influence d'un régime à 9% de caséine sur la diminution d'activité des oxygénases par le zinèbe (600 ppm).

Methodes Expérimentales

Les animaux sont des rats mâles Wistar U. A l'âge d'un mois, ils sont répartis en deux groupes. Le premier reçoit une alimentation équilibrée, le second un régime hypoprotéique (Tableau 1). Après 2 semaines, chaque groupe est divisé en deux lots: témoin et traité

au zinèbe (92% pur et fourni par Société Pepro, Lyon). Le traitement par le pesticide consiste en l'addition de 600 ppm (exprimée en produit pur) dans la nourriture, durant 4 semaines.

Après un jeûne d'une nuit, nous tuons les animaux par ponction cardiaque et nous préparons les microsomes hépatiques suivant le procédé que nous avons décrit (Albrecht, Pélissier, Manchon & Rospars, 1973). Nous apprécions dans ces microsomes la teneur en protéines, la *N*-déméthylation de l'aminopyrine, l'hydroxylation de l'aniline, la NADPH-réduction du cytochrome c^{3+} ainsi que les quantités de *P*-450 et de cytochrome b_5 (Pélissier, Faudemay, Manchon & Albrecht, 1978).

Sur un autre lot, nous déterminons le temps de sommeil provoqué par l'administration d'hexobarbital. Le barbiturique sodé, en solution à 2,5%, est injecté par voie intrapéritonéale à la dose de 100 mg/kg rat.

Resultats et Discussion

Les effets du régime hypoprotéique

Dans nos conditions expérimentales, 6 semaines d'administration d'un régime à 9% de caséine, l'oxydation de l'hexobarbital et la *N*-déméthylation de l'aminopyrine sont en partie réprimées (Tableau 2). Cependant, nous constatons une différence entre les effets de la carence suivant le type de substrat. Ainsi, l'activité spécifique, rapportée aux protéines, de l'hydroxylation de l'aniline n'est pas modifiée chez les rats au régime hypoprotéique alors que la déméthylation de l'aminopyrine est réduite de 23%.

Nous constatons que la diminution, sous l'effet de la déficience protéique, du métabolisme des xénobiotiques est liée à une répression de la biosynthèse du *P*-450 et de la NADPH-cytochrome *c* réductase. Nos résultats sont en cela en accord avec les données de la littérature (Kato *et al.* 1968; Marshall & McLean, 1969). L'effet du régime déséquilibré sur l'hémoprotéine est plus important qu'il ne l'est pour le métabolisme des substrats, de sorte que les activités moléculaires spécifiques (activité par unité de *P*-450) sont accrues par la carence protéique. D'après Hayes, Mgbodile & Campbell (1973) et Mgbodile, Hayes & Campbell (1973) une partie des sites de liaison du *P*-450 avec les substrats de type I serait liée à la phosphatidylcholine; cette fraction serait proportionnellement plus grande lors de la déficience protéique. Nous pouvons admettre que l'activité moléculaire spécifique de la déméthylation de l'aminopyrine est accrue par la carence protéique parce que celle-ci élève la proportion des sites de liaisons du *P*-450 avec ce substrat, mais dans notre travail, l'augmentation d'activité moléculaire porte aussi sur le métabolisme de l'aniline; or on considère que les sites de liaison pour les substrats de type II sont indépendants de l'association *P*-450–phosphatidylcholine (Mgbodile *et al.* 1973).

Soulignons que, malgré de nombreux travaux, on ne connaît encore qu'imparfaitement les mécanismes des effets d'une diminution de l'apport alimentaire en protéines sur les oxygénases microsomales de foie. D'autant que les récentes recherches de Nerurkar, Hayes & Campbell (1978) suggèrent que non seulement la carence protéique affecte les activités spécifiques de chaque composant de la chaîne polyenzymatique mais qu'elle modifie l'interaction entre le *P*-450 et la cytochrome *c* réductase.

Les effets du zinèbe

Nous confirmons un travail antérieur du laboratoire (Lowy *et al.* 1977; Pélissier, Attéba, Manchon & Albrecht, 1976) l'administration de zinèbe ralentit *in vitro* le métabolisme des substrats de type I (aminopyrine) et de type II (aniline) dans les microsomes hépatiques du rat (Tableau 2). Par la présente expérience, nous remarquons que cet effet semble s'appliquer aussi à l'animal entier puisque le temps de sommeil provoqué par l'hexobarbital est prolongé chez le rat traité. C'est, à notre connaissance, la première fois que l'on signale qu'une molécule organique de synthèse utilisée comme pesticide diminue l'activité des oxygénases, après plusieurs semaines d'administration.

Le régime hypoprotéique ne modifie pas les effets du zinèbe sur l'activité des oxygénases microsomales et *vice versa*. Les effets des deux facteurs vont dans le même sens, la diminution d'activité enzymatique; ils s'ajoutent l'un l'autre, de sorte que le rat traité et nourri avec le régime déficient métabolise très lentement les xénobiotiques.

Le zinèbe ou ses métabolites, réprime-t-il la synthèse protéique dans le foie? Les récentes

Tableau 1. *Composition pondérale centésimale et énergétique des régimes*

Composants	Régime équilibré		Régime hypoprotéique	
	Teneur (% en poids)	Energie* (%)	Teneur (% en poids)	Energie* (%)
Protéines (caséine)	18,2	22,0	9,1	10,9
Lipides (saindoux)	3,5	9,6	3,1	8,4
Glucides assimilables (farine de blé, sucre et semoule)	56,4	68,6	67,0	80,7
Eau	10,8		10,2	
Ballast†	5,7		5,5	
Sels minéraux	4,9		4,6	
Vitamines	0,5		0,5	

*Energie totale des régimes: 330 Kcal/100 g du régime équilibré et 332 Kcal/100 g du régime hypoprotéique.

†Poudre de cellulose purifiée pour chromatographie (fournie par Durieux, Paris).

Tableau 2. *Activités des microsomes*

Paramètre	Moyenne, écart type sur la moyenne†				Analyse de variance‡		
	Régime équilibré		Régime hypoprotéique				
	Témoin	Zinèbe	Témoin	Zinèbe	Interaction	Effet zinèbe	Effet régime
Protéines microsomales: mg/100 mg foie	4,29 ± 0,226	3,99 ± 0,158	3,73 ± 0,225	3,47 ± 0,225	NS	*	**
N-Déméthylation de l'aminopyrine:							
nmol aminoantipyrine/min/100 mg protéines	13,82 ± 0,756	11,32 ± 0,747	10,1 ± 1,06	9,14 ± 0,669	NS	*	**
nmol aminoantipyrine/min/nmol *P*-450	0,263 ± 0,0129	0,229 ± 0,0136	0,333 ± 0,0315	0,346 ± 0,0228	NS	NS	**
Hydroxylation de l'aniline:							
nmol *p*-aminophénol/min/100 mg protéines	16,5 ± 1,22	12,94 ± 0,923	16,6 ± 2,59	10,9 ± 1,14	NS	**	NS
nmol *p*-aminophénol/min/nmol *P*-450	0,314 ± 0,0186	0,262 ± 0,0149	0,548 ± 0,0683	0,413 ± 0,0345	NS	NS	**
P-450: nmol/100 mg protéines	52,6 ± 4,05	49,4 ± 5,23	30,3 ± 1,55	26,4 ± 4,00	NS	NS	**
Cytochrome b_5: nmol/100 mg protéines	55,1 ± 3,32	52,8 ± 3,85	35,1 ± 4,49	38,5 ± 2,82	NS	NS	**
NADPH-cytochrome *c* réductase:							
µmol/min/100 mg protéines	5,05 ± 0,232	4,94 ± 0,266	4,10 ± 0,266	4,39 ± 0,512	NS	NS	**
nmol/min/nmol *P*-450	95,6 ± 4,41	100 ± 10,3	135,3 ± 8,78	166 ± 19,4	NS	NS	**
Temps de sommeil§: min	15,4 ± 2,91	19,64 ± 0,857	29,2 ± 3,04	36,9 ± 2,62	NS	*	**

†Huit rats/groupe.
‡Test F: $*P < 0,05$; $**P < 0,01$; NS = $P > 0,05$.
§Effecteur, hexobarbital.

recherches de Moulé, Rousseau & Darracq (1978) indiquent que l'ingestion répétée du fongicide pendant 10 semaines (régime à 5200 ppm) déclenche une répression des synthèses de l'ordre de 50%; toutefois, si l'ingestion est limitée à 4 semaines, les effets observés ne sont pas significatifs (Moulé *et al.* 1978). Le zinèbe (et/ou ses métabolites) est donc très vraisemblablement un répresseur enzymatique 'potentiel' mais nous ne pouvons pas affirmer que dans nos conditions expérimentales (régimes à 600 ppm pendant 4 semaines) la biosynthèse des oxygénases microsomales soit effectivement ralentie. L'essentiel de la diminution du métabolisme des xénobiotiques, sous l'effet de l'ingestion de zinèbe, pourrait résulter d'une inhibition directe des oxygénases dans les microsomes hèpatiques. L'idée d'une inhibition directe des enzymes liées au *P*-450 repose sur trois ensembles de faits expérimentaux: nos propres observations avec le zinèbe lui-même, quand on l'ajoute au milieu d'incubation de l'aniline (Albrecht, 1979); les travaux concernant des molécules de structure chimique proche du zinèbe tels que le disulfirame (Lang, Marselos & Törrönen, 1976; Zemaitis & Greene, 1976), et le diéthyldithiocarbamate (Honjo & Netter, 1969; Zemaitis, Blackborn & Greene, 1973); enfin les quelques recherches portant sur certains métabolites du pesticides, sulfure d'éthylènebisisothiocyanate (Yoshida, Jordan & Neal, 1978), éthylène-thiourée (Hunter & Neal, 1975), sulfure de carbone (Bond & De Matteis, 1969; Hunter & Neal, 1975; Järvisalo, Savolainen, Elovaara & Vainio, 1977). Il apparaît que le zinèbe peut inhiber les oxygénases (1) en libérant du soufre élémentaire, lequel peut réagir sur les sites nucléophiles des protéines microsomales et en particulier dégrader et détruire le *P*-450, (2) en réagissant directement sur les groupes cystéine de l'hémoprotéine (et d'autres protéines des microsomes). Nous n'excluons pas que le zinèbe puisse aussi réprimer la biosynthèse de RNA et de protéines; on peut considérer, avec Moulé *et al.* (1978), que l'inhibition des oxygénases précède leur répression.

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REFERENCES

Albrecht, R. (1979). Influence des Pesticides, Lindane et Zinèbe, sur les Monoxygénases Microsomiques du Foie chez le Rat. Thèse Doctorat d'Etat ès Sciences, Université Paris VII.

Albrecht, R., Pélissier, M. A., Manchon, Ph. & Dupuis, D. (1975). Influence de l'administration de parathion-méthyle ou de zinèbe sur l'activité de quelques enzymes hépatiques chez le rat. *Annls Nutr. Aliment.* **29,** 223.

Albrecht, R., Pélissier, M. A., Manchon, Ph. & Rospars, J. P. (1973). Obtention de microsomes par coprécipitation avec Ca^{2+} et mise en évidence d'induction enzymatique par le phénobarbital. *C.r. Séanc. Soc. Biol.* **167,** 1552.

Bond, E. J. & De Matteis, F. (1969). Biochemical changes in rat liver after administration of carbon disulphide, with particular reference to microsomal changes. *Biochem. Pharmac.* **18,** 2531.

Boyd, E. D. & Dubos, I. (1969). Protein deficiency and tolerated oral doses of endosulfan. *Archs int. Pharmacodyn. Thér.* **178,** 153.

Campbell, T. C. & Hayes, J. R. (1974). Role of nutrition in the drug-metabolizing enzyme system. *Pharmac. Rev.* **26,** 171.

Campbell, T. C. & Hayes, J. R. (1976). The effect of quantity and quality of dietary protein on drug metabolism. *Fedn Proc. Fedn Am. Socs exp. Biol.* **35,** 2470.

Drill, V. A. (1952). Hepatotoxic agents: mechanism of action and dietary interrelationship. *Pharmac. Rev.* **4,** 1.

Hayes, J. R., Mgbodile, M. U. K. & Campbell, T. C. (1973). Effect of protein deficiency on the inducibility of the hepatic microsomal drug-metabolizing enzyme system—I. Effect of substrate interaction with cytochrome P-450. *Biochem. Pharmac.* **22,** 1005.

Honjo, T. & Netter, K. J. (1969). Inhibition of drug demethylation by disulfiram *in vivo* and *in vitro*. *Biochem. Pharmac.* **18,** 2681.

Hunter, A. L. & Neal, R. A. (1975). Inhibition of hepatic mixed-function oxidase activity *in vitro* and *in vivo* by various thiono-sulfur-containing compounds. *Biochem. Pharmac.* **24,** 2199.

Järvisalo, J., Savolainen, H., Elovaara, E. & Vainio, H. (1977). The *in vivo* toxicity of CS_2 to liver microsomes: binding of labelled CS_2 and changes of the microsomal enzyme activities. *Acta pharmac. tox.* **40,** 329.

Kato, R., Oshima, T. & Tomizawa, S. (1968). Toxicity and metabolism of drugs in relation to dietary protein. *Jap. J. Pharmac.* **18,** 356.

Lang, M., Marselos, M. & Törrönen, R. (1976). Modifications of drug metabolism by disulfiram and diethyldithio-carbamate. I. Mixed-function oxygenase. *Chemico-Biol. Interactions* **15,** 267.

Lowy, R., Albrecht, R., Pélissier, M. A. & Manchon, Ph. (1977). Determination of the "no-effect levels" of two pesticides, lindane and zineb, on the microsomal enzyme activities of rat liver. *Toxic. appl. Pharmac.* **42,** 329.

Mgbodile, M. U. K., Hayes, J. R. & Campbell, T. C. (1973). Effect of protein deficiency on the inducibility of the hepatic microsomal drug-metabolizing enzyme system—II. Effect on enzyme kinetics and electron transport system. *Biochem. Pharmac.* **22,** 1125.

McLean, A. E. M. & McLean, E. K. (1969). Diet and toxicity. *Br. med. Bull.* **25,** 278.

Marshall, W. J. & McLean, A. E. M. (1969). The effect of oral phenobarbitone on hepatic microsomal cytochrome P-450 and demethylation activity in rats fed normal and low protein diets. *Biochem. Pharmac.* **18,** 153.

Moulé, Y., Rousseau, N. & Darracq, N. (1978). Action d'un dithiocarbamate, le zinèbe, sur les synthèses de RNA et de protéines dans le foie de rat. *Toxicol. Eur. Res.* **1,** 283.

Nerurkar, L. S., Hayes, J. R. & Campbell, T. C. (1978). The reconstitution of hepatic microsomal mixed function oxidase activity with fractions derived from weanling rats fed different levels of protein. *J. Nutr.* **108,** 678.

Pélissier, M. A., Attéba, S., Manchon, Ph. & Albrecht, R. (1976). Diminution d'activité des oxygénases microsomales du foie chez le rat nourri avec un régime additionné de zinèbe. *Annls Nutr. Aliment.* **30,** 45.

Pélissier, M. A., Faudemay, F., Manchon, Ph. & Albrecht, R. (1978). Induction par le lindane des monoxygénases microsomales du foie chez le rat: Effets d'un régime hypocalcique. *Fd Cosmet. Toxicol.* **16,** 27.

Yoshida, T., Jordan, L. & Neal, R. A. (1978). Effect of ethylenebisdithiocyanate sulfide (EBIS) on hepatic monoxygenase activity. *Toxic. appl. Pharmac.* **46,** 215.

Zemaitis, M. A., Blackborn, W. R. & Greene, F. E. (1973). Some effects of disulfiram on drug metabolism and hepatic cell ultrastructure in the rat. *Pharmacologist* **15,** 191.

Zemaitis, M. A. & Greene, F. E. (1976). Impairment of hepatic microsomal drug metabolism in the rat during daily disulfiram administration. *Biochem. Pharmac.* **25,** 1355.

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Review Section

MULTI-STAGE DOSE-RESPONSE MODELS IN CARCINOGENESIS

F. W. CARLBORG
400 South Ninth Street, Saint Charles, Illinois 60174, USA

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Summary—A frequently proposed multi-stage dose-response model for carcinogenesis is evaluated theoretically and on the basis of existing experimental results. Several defects in this model are revealed. A modified form of the Weibull model, without these defects, is proposed as an alternative.

The development of cancer in man and in laboratory animals in response to chemical carcinogens has long been described qualitatively as a multi-stage process (Farber & Cameron, 1980) occurring over an extended period of time. The number of stages appears to depend on many factors: species, strain, sex, site in the body, nature of the carcinogen, route of exposure, and others.

Those interested in quantitatively relating the probability of cancer to the dose of the carcinogen and/or to the time to a tumor have attempted to make use of this observed multi-stage phenomenon in the construction of mathematical models. In such a model, the number of stages (an integer) appears as a parameter in the model, usually as an exponent. With experimental results, the estimated value of the parameter (an integer, again) then has a biological interpretation as the estimated number of stages. The early work in this area was done by Armitage & Doll (1954). Originally, they were interested in the development of the stages as a function of time (age). More recently, the development of the stages has also been considered as a function of the dose.

At present, the most commonly mentioned form of the multi-stage model for carcinogenesis as a function of the dose is

$$P = 1 - e^{-(\alpha + \beta_1 d + \beta_2 d^2 + \ldots + \beta_m d^m)}, \quad (1)$$

where P is the probability of a tumour, d is the dose, m is a parameter (integer) for the number of stages, and α and β_i are other parameters, all non-negative. The parameter α is related to the background tumour rate P_0 by

$$P_0 = 1 - e^{-\alpha} \text{ or } \alpha = -\ln(1 - P_0).$$

This model is used, for example, to analyse the experimental results from a lifetime exposure study with laboratory animals. In a typical case of this kind two of the βs might be positive and the others equal to zero. The estimated value of the parameter m would be the larger exponent. An extended form of this model, which includes the time (age of the animal), is discussed below. This version of the multi-stage model is heavily endorsed by the Interagency Regulatory Liaison Group (IRLG, 1979) of the US Government for low-risk assessment of cancer (see also Guess, Crump & Peto, 1977; Hartley & Sielken, 1977).

Models not containing a parameter associated with the number of stages have also been proposed. For example, in the Weibull model, carcinogenesis as a function of the dose is expressed as

$$P = 1 - e^{-(\alpha + \beta d^m)} = P_0 + (1 - P_0)(1 - e^{-\beta d^m}), \quad (2)$$

where P, P_0, d, α and β are defined as above and m (not necessarily an integer) is a shape parameter. The principal negative feature of the Weibull model is the fact that no biological interpretation of the important parameter m has been offered. This will be further discussed below. There is also an extended form of this model which includes the time (age of the animal).

Five defects in the form of the multi-stage model set out above will now be discussed. The Weibull model will also be considered in relation to each of these defects. Then, a different form of the multi-stage model will be given, and its relation to the Weibull model will be explored. This will lead to a new interpretation of the shape parameter m in the Weibull model and to a new interpretation of a well-known relation between the dose and the time to a tumour.

The first defect in the multi-stage model (1) is that it does not fit the observed data from one category of animal experiments. These are data sets in which the dose-response function rises very steeply and then levels off. (Mathematically, these are concave functions.) Table 1 gives a summary of the results of fitting the multi-stage model to the data from four such experiments: with DDT, vinyl chloride, diethylstilboestrol and ethylene dibromide. The multi-stage model comes closest to fitting the data in each set with the parameter m equal to 1. Consider, for example, the first row of Table 1 for DDT. This was an ingestion experiment with male mice (CF1 strain). The pathological endpoint was a liver tumour. The *P*-value for measuring the goodness of the fit of the multi-stage model is 0·01, indicating a very poor fit in the statistical sense. Notice in the column labelled '*P*(M)' that the multi-stage model fails to fit in every case at the traditional 0·05 level of significance.

The Weibull model (2), however, does fit these data sets. The penultimate column in Table 1 gives the statistical best estimate of the shape parameter m (using weighted least squares), and the last column gives the *P*-value for the goodness of the fit. (With

Table 1. *Experimental results which the multi-stage model does not fit*

Chemical	Route	Species	Strain	Sex	Endpoint	P (M)*	Estimated m†	P(W)‡
DDT	Ingestion	Mouse	CF1	M	Liver tumour	0·01	0·33	0·19
VC	Inhalation	Rat	Sprague–Dawley	M + F	Liver angiosarcoma	0·05	0·48	0·50
DES	Ingestion	Mouse	C3H	F	Mammary carcinoma	0·04	0·51	0·65
EDB§	Inhalation	Rat	Osborne–Mendel	M + F	Nasal adeno-carcinoma	0·01	0·36	

*The P-value from the chi-square test for goodness of fit of the multi-stage model.
†The statistical best estimate of the shape parameter in the Weibull model.
‡The P-value from the chi-square test for goodness of fit of the Weibull model.
§This experiment had only three groups of animals with following carcinoma proportions and doses: 0/100 at 0 ppm, 40/100 at 10 ppm, and 57/100 at 40 ppm.

EDB, there were only three doses, and no statistical test of the fit is possible.)

In an earlier communication (Carlborg, 1980), the Weibull Model (2) was applied to 27 large data sets reported in the literature. For all 27 sets, the fit was very good in the statistical sense. The first three studies in Table 1 here (DDT, VC and DES) were included in the 27. The reported experimental results and the references for these three are given in that article. The ethylene dibromide study (National Cancer Institute, 1981) was not included in that survey because the experiment contained only three dose groups, including the control group.

The second defect in the multi-stage model (1) is that it is mathematically undefined and, therefore, that the choice of terms in the polynomial exponent is arbitrary. To illustrate this, consider a hypothetical experiment with three dose groups: d = 0, d = 0·5 and d = 1·0. Suppose further that this is an infinitely large experiment producing tumour proportions of 0, 0·10 and 0·70, respectively. (That is, these are the 'true' responses, free of experimental error.) Under the multi-stage model, there are many (probably, infinite) ways to fit the model to the data. One is $\alpha = 0$, $\beta_1 = 0{\cdot}069$ and $\beta_4 = \beta_m = 1{\cdot}135$, suggesting that this is a four-stage process. Table 2 gives this solution in the top left-hand corner and gives eleven other solutions ($\alpha = 0$ in all cases). There are more solutions with higher values of m.

The need for low-risk extrapolation from measured responses at high doses to unmeasurable responses at low doses is a principal reason for fitting mathematical models to results from cancer studies. With this in mind, let us extend the hypothetical situation of the preceding paragraph and suppose we are interested in the extrapolated risk at d = 0·001, that is, at two to three orders of magnitude below the doses with measured responses. Table 2 also gives these extrapolated risks for all twelve solutions ($6{\cdot}9 \times 10^{-5}$ for the data mentioned above). Notice that there is a huge variation, depending primarily on the smaller exponent in the fitted model.

Thus, the multi-stage model (1) with its polynomial exponent ($\beta_1 d + \beta_2 d^2 + \ldots + \beta_m d^m$) leads to almost total uncertainty about the number of stages (m) and to similar uncertainty about the extrapolated risk at a low dose. With actual data from finite experiment, the uncertainties are even greater. This defect is a consequence of the polynomial in the exponent, and it is a very familiar problem in statistics. In a slightly different context, Cochran & Cox (1957) summarize it clearly:

"First, a word of caution. Polynomial response surfaces have the great advantage that they are easy to fit. With a suitable choice of design, even a quadratic surface in 6 variables, which contains 28 coefficients to be estimated, is not too formidable a

Table 2. *Results of fitting the multi-stage model to a hypothetical data set including extrapolated risks*

Smaller exponent in fitted model	Larger exponent in fitted model...	4	5	6	7
1		0·069	0·144	0·179	0·195
		1·135	1·060	1·025	1·009
		$6{\cdot}9 \times 10^{-5}$	$1{\cdot}4 \times 10^{-4}$	$1{\cdot}8 \times 10^{-4}$	$1{\cdot}9 \times 10^{-4}$
2		0·161	0·310	0·369	0·396
		1·043	0·894	0·834	0·808
		$1{\cdot}6 \times 10^{-7}$	$3{\cdot}1 \times 10^{-7}$	$3{\cdot}7 \times 10^{-7}$	$4{\cdot}0 \times 10^{-7}$
3		0·482	0·722	0·791	0·819
		0·722	0·482	0·413	0·385
		5×10^{-10}	7×10^{-10}	8×10^{-10}	8×10^{-10}

Explanation: consider for example the group of three figures in the top left-hand corner (smaller exponent—1, larger exponent—4); 0·069 is β_1 that is the coefficient of d; 1·135 is β_4 that is the coefficient of d^4 and $6{\cdot}9 \times 10^{-5}$ is the extrapolated risk at d = 0·001. Please see text for further explanation.

task. On the other hand, polynomials are notoriously untrustworthy when extrapolated. A polynomial surface should be regarded only as an approximation to ϕ *within the region covered by the experiment.* Any prediction made from the polynomial about the response outside the region should be verified by experiments before putting reliance on it."

Algorithms for using the multi-stage model with actual data are heavily biased toward the top left-hand corner in Table 2, especially toward including the linear term $\beta_1 d$, which produces the highest extrapolated risks. In fact, when the actual data do not require a linear term, proponents of the multi-stage model argue that the linear term might have been excluded by chance, that an upper confidence limit for the missing linear term should be used to introduce it into the model, and that low-risk extrapolations should be made including this linear term (Crump, Hoel, Langley & Peto, 1976).

There is no corresponding lack of definition or arbitrariness with the Weibull model (2). For the hypothetical example mentioned above, the value of the shape parameter m is 3·51, and the extrapolated risk at the low dose (d = 0·001) is $3{\cdot}6 \times 10^{-11}$ ($\alpha = 0$ and $\beta = 1{\cdot}204$).

The third defect in the multi-stage model (1) with the bias in the top left-hand corner is that low-risk extrapolation is insensitive to the shape of the dose-response function in the observable range. Experiments which show very steep dose-response curves over the observable range produce extrapolated risks which are essentially equal to those produced by experiments with very flat dose-response curves over the observable range.

To illustrate this defect, let us return to the hypothetical example introduced above. Assuming an infinitely large experiment with tumour proportions of 0 at d = 0 and 0·10 at d = 0·5 let us vary the assumed tumour proportion at the high dose (d = 1·0) as shown in the second column of Table 3. For example, take case 11, which is the previous example with an assumed response of 0·70 at d = 1. Under the multi-stage model with the top left-hand-corner bias, the fitted polynomial has terms with exponents of 1 and 4 ($\beta_1 d$ and $\beta_4 d^4$) and an extrapolated response of $6{\cdot}9 \times 10^{-5}$ at the low dose of d = 0·001, all as before.

Now let us consider the other extrapolated risks in the fourth column of Table 3. The flattest dose-response curve is case 1 with assumed responses of 0 at d = 0, 0·10 at d = 0·5 and 0·20 at d = 1·0 and with an extrapolated risk of $2{\cdot}0 \times 10^{-4}$ at d = 0·001. The steepest dose-response curve is case 17 with the same assumed responses at d = 0 and d = 0·5, but with a response of 0·98 at d = 1·0 and an extrapolated risk of $9{\cdot}1 \times 10^{-5}$ at d = 0·001, which is essentially equal to the extrapolated risk for case 1. In fact, all the extrapolated responses in this column are essentially the same. This implies that the multi-stage model produces the same extrapolated risk at a low dose, regardless of the shape of the dose-response function in the observable range.

The Weibull model (2) is sensitive to the shape of the dose-response function in the observed range. The last two columns of Table 3 give the value of the shape parameter m and the extrapolated risk at the low dose of d = 0·001 for each case. Notice here that the extrapolated risk decreases steadily as the steepness of the dose-response curve increases.

The fourth defect in the multi-stage model (1) is that it is unstable. This was apparent in the fourth column of Table 3. To be specific, compare the extrapolated risks for cases 8 ($1{\cdot}5 \times 10^{-5}$) and 9 ($1{\cdot}1 \times 10^{-4}$). The risk for case 9 is one order of magnitude greater than for case 8, when in fact it should be lower because the dose-response curve is steeper in case 9 than in case 8. Of course, the explanation is that different terms (exponents) happen to enter the fitted models in these two cases.

Table 3. *Extrapolated risks (responses) for various hypothetical data sets under the multi-stage and Weibull models*

		Multi-stage model		Weibull model	
Case	Assumed response at d = 1*	Exponents in fitted model	Extrapolated response at d = 0·001	Value of M	Extrapolated response at d = 0·001
1	0·20	1, 2	$2{\cdot}0 \times 10^{-4}$	1·08	$1{\cdot}3 \times 10^{-4}$
2	0·25	1, 2	$1{\cdot}3 \times 10^{-4}$	1·45	$1{\cdot}3 \times 10^{-5}$
3	0·30	1, 2	$6{\cdot}5 \times 10^{-5}$	1·76	$1{\cdot}8 \times 10^{-6}$
4	0·35	1, 3	$1{\cdot}4 \times 10^{-4}$	2·03	$3{\cdot}5 \times 10^{-7}$
5	0·40	1, 3	$1{\cdot}1 \times 10^{-4}$	2·28	$7{\cdot}5 \times 10^{-8}$
6	0·45	1, 3	$8{\cdot}2 \times 10^{-5}$	2·50	$1{\cdot}8 \times 10^{-8}$
7	0·50	1, 3	$5{\cdot}0 \times 10^{-5}$	2·72	$4{\cdot}9 \times 10^{-9}$
8	0·55	1, 3	$1{\cdot}5 \times 10^{-5}$	2·92	$1{\cdot}4 \times 10^{-9}$
9	0·60	1, 4	$1{\cdot}1 \times 10^{-4}$	3·12	$4{\cdot}0 \times 10^{-10}$
10	0·65	1, 4	$9{\cdot}1 \times 10^{-5}$	3·32	$1{\cdot}2 \times 10^{-10}$
11	0·70	1, 4	$6{\cdot}9 \times 10^{-5}$	3·51	$3{\cdot}6 \times 10^{-11}$
12	0·75	1, 4	$4{\cdot}3 \times 10^{-5}$	3·72	$1{\cdot}1 \times 10^{-11}$
13	0·80	1, 4	$1{\cdot}1 \times 10^{-5}$	3·93	$< 1 \times 10^{-11}$
14	0·85	1, 5	$9{\cdot}8 \times 10^{-5}$	4·17	$< 1 \times 10^{-11}$
15	0·90	1, 5	$7{\cdot}1 \times 10^{-5}$	4·45	$< 1 \times 10^{-11}$
16	0·95	1, 5	$2{\cdot}5 \times 10^{-5}$	4·83	$< 1 \times 10^{-11}$
17	0·98	1, 6	$9{\cdot}1 \times 10^{-5}$	5·22	$< 1 \times 10^{-11}$

*All of these cases assume a response of 0 at d = 0 and a response of 0·10 at d = 0·5.

The Weibull model (2) is completely stable, as shown in the last column of Table 3.

The fifth defect in the multi-stage model is that it is inconsistent with the important observations of Druckrey and his many colleagues concerning the time to a tumour. To show this, one must consider the extended form of the multi-stage model (Hartley & Sielken, 1977):

$$P = 1 - e^{-(\alpha + \beta_1 d + \ldots + \beta_m d^m)H(t)}, \quad (3)$$

where t is the time (age) and H(t) is some function of t, typically a polynomial again. The corresponding extended form of the Weibull model is

$$P = 1 - e^{-(\alpha + \beta d^m)t^k}, \quad (4)$$

where k is a shape parameter for the time.

The discovery of Druckrey (1967) is that within a particular experiment, the daily dose (d) multiplied by the median time to a tumour (t) raised to a power (n) is a constant across all dose groups:

$$\text{constant} = dt^n.$$

The power (n) varies from one experiment to another depending on the chemical, the species, the route of exposure, and so on.

It is easier to explain the fifth defect of the multi-stage model (3) by discussing the Weibull model (4) first. In Druckey's experiments, the background tumour rate was zero or essentially zero. In the Weibull model, this means that the parameter α is zero. If t is the median time to a tumour, then the extended Weibull model (4) in this situation becomes

$$0{\cdot}50 = 1 - e^{-\beta d^m t^k}.$$

This reduces to

$$(-\ln 0{\cdot}50/\beta)^{1/m} = dt^{k/m} = dt^n,$$

where $n = k/m$ and the left side is a constant for a given experiment. But this is exactly Druckrey's empirically discovered relationship. That is, Druckrey's result is a corollary of the extended Weibull model.

Now turn to the extended multi-stage model (3). In the general case, it is not possible to derive Druckrey's relationship from this model. Druckrey's relation is implied by the extended multi-stage model only in the very special case when exactly one of the β's is not zero and when exactly one of the polynomial terms in H(t) is not zero.

In spite of these defects in the multi-stage model, the fundamental concept is appealing. That is, carcinogenesis is believed to be a multi-stage process in some way, and a mathematical model with a parameter (an integer) measuring the number stages is appealing. The remainder of this article presents an alternative model which has such a parameter and which retains all the benefits of the Weibull model.

The second, third, fourth and fifth defects in the multi-stage model (1) are all caused by the polynomial exponent involving the dose ($\beta_1 d + \beta_2 d^2 + \ldots + \beta_m d^m$). Temporarily, consider a simplified multi-stage model:

$$P = 1 - e^{-(\alpha + \beta d^I)}, \quad (5)$$

where I is a positive integer (the number of stages). Such a model would be free of defects 2–5, on the assumption that it does fit the data. It would, however, not fit the observed results from many large experiments. For example, it would not fit the results for those experiments mentioned in Table 1. For another example, take the 24-month results from the very large experiment with 2-AAF in female mice with a liver neoplasm as the pathological endpoint, performed at the National Center for Toxicological Research (Staffa & Mehlman, 1979). With this data set, the original multi-stage model (1) fits very well with two terms in the exponent ($\alpha + \beta_1 d + \beta_2 d^2$, or m = 2). The Weibull model (2) also fits very well with a fractional value of the shape parameter (exponent of $\alpha + \beta d^{1{\cdot}49}$, or m = 1·49). However, the simplified multi-stage model (5) does not fit these data.

Consider a modification of the preceding simplified multi-stage model (5). Suppose that it is the square root of the dose rather than the dose itself which is the critical measure of exposure. In symbols, let D be the square root of d. Then the simplified multi-stage model becomes

$$P = 1 - e^{-(\alpha + \beta D^I)} = 1 - e^{-(\alpha + \beta d^{I/2})}, \quad (6)$$

where I is an integer indicating the number of stages. This model (6) is also free of defects 2–5, again on the assumption that it fits the data.

It remains to check this model (6) with respect to existing experimental results, that is, with respect to defect 1 mentioned above. This model (6) says that the original Weibull model (2) should fit experimental results with the shape parameter m limited to values like m = 1/2, 2/2, 3/2, and so on. However, an earlier paper (Carlborg, 1980), which showed that the Weibull model (2) fits 27 data sets from the literature, also showed exactly this. In fact, it showed further that all the data sets could be fitted in the statistical sense with the shape parameter m = 1/3, 3/2, 5/2, ..., 13/2, that is, with a shape parameter m = I/2 where I is an odd positive integer. It can therefore be concluded that this model (6) has none of the defects of the original multi-stage model (1); it has all the benefits of the original Weibull model (2); the parameter I (a positive integer) is the number of stages; perhaps only odd integers are needed for the parameter I.

Finally, consider an extended form of model (6) which includes time (age of the animal). The Weibull model has long been used to relate the probability of a tumour to the time to a tumour

$$P = 1 - e^{-\beta t^k}.$$

Fractional values for the shape parameter k have long been accepted. A k of 6·5 occurs often; Druckrey (1967) mentions it. Results for a liver neoplasm in the NCTR experiment with 2-AAF (see p. 95 of Staffa & Mehlman, 1979) are also close to 6·5. To be a little speculative, suppose that the shape parameter k for the time is J/2, where J is an integer. This is mathematically equivalent to supposing that the basic measure of time is the square root of the actual time (say, $T = t^{1/2}$). Then the relation of the probability of a tumour to the time is

$$P = 1 - e^{-\beta T^J}.$$

Under this speculative hypothesis, the extended form of model (6) is

$$P = 1 - e^{-(\alpha + \beta D^I)T^J},$$

where $D = d^{1/2}$ and $T = t^{1/2}$. This may be written more suggestively as

$$P = P_0 + (1 - P_0)(1 - e^{-J(DT^n)^I}) \quad (7)$$

where $P_0 = 1 - e^{-\lambda T^J}$ and $n = J/I$. This n is still Druckrey's n. This leads to the conclusion that the fundamental carcinogenic variable is DT^n, where D is the square root of the actual dose, T is the square root of the actual time, n is that of Druckrey, and I is the number of stages (perhaps always odd).

In the author's final evaluation of model (7), the identification of the parameter I with the number of stages is highly tentative at best. What is important is that this model fits all the data sets so far encountered. Furthermore, it offers a reasonable way to separate results from animal experiments with dangerous low-dose implications (small value of I) from those results without dangerous low-dose implications (large value of I). In a subsequent article, this model (7) will be fitted successfully to all the data from the scheduled sacrifices in the NCTR experiment with 2-AAF. It will also be generalized to include another dimension: the effects of partial lifetime exposure.

REFERENCES

Armitage, P. & Doll, R. (1954). The age distribution of cancer and a multi-stage theory of carcinogenesis. *Br. J. Cancer* **8**, 1.

Carlborg, F. W. (1981). Dose-response functions in carcinogenesis and the Weibull model. *Fd Cosmet. Toxicol.* **19**, 255

Cochran, W. G. & Cox, G. M. (1957). *Experimental Designs*. 2nd Ed. p. 335. John Wiley & Sons, Inc., New York.

Crump, K. S., Hoel, D. G., Langley, C. H. & Peto, R. (1976). Fundamental carcinogenic processes and their implications for low dose risk assessment. *Cancer Res.* **36**, 2973.

Druckrey, H. (1967). Quantitative aspects in chemical carcinogenesis. In *Potential Carcinogenic Hazards from Drugs. Evaluation of Risks*. Vol. 7. Edited by R. Truhaut. UICC Monograph Series. p. 60. Springer-Verlag, Berlin.

Farber, E. & Cameron, R. (1980). The sequential analysis of cancer development. *Adv. Cancer Res.* **31**, 125.

Guess, H., Crump, K. & Peto, R. (1977). Uncertainty estimates for low-risk extrapolations of animal carcinogenicity data. *Cancer Res.* **37**, 3475.

Hartley, H. O. & Sielken, R. L., Jr (1977). Estimation of "safe doses" in carcinogenic experiments. *Biometrics* **33**, 1.

Interagency Regulatory Liaison Group (1979). Scientific bases for identification of potential carcinogens and estimation of risks. *Federal Register* **44**, pp. 39858–39879.

National Cancer Institute (1981). *Bioassay of 1,2-Dibromoethane for Possible Carcinogenicity*. Washington, DC.

Staffa, J. A. & Mehlman, M. A. (1979). *Innovations in Cancer Risk Assessment*. Pathotox Publishers, Inc., Park Forest South, IL.

Fd Cosmet. Toxicol. Vol. 19, pp. 367 to 371, 1981
Printed in Great Britain.

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2-ACETYLAMINOFLUORENE AND THE WEIBULL MODEL

F. W. CARLBORG
400 South Ninth Street, Saint Charles, IL 60174, USA

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Summary—The Weibull model for dose-response functions in carcinogenesis is explored with respect to the results from the large experiment with 2-acetylaminofluorene (2-AAF). Several generalized forms of the Weibull model are presented. Also, the hypothesis of 'low-dose linearity' is tested with the data for 2-AAF.

The largest experiment ever performed in carcinogenesis is now complete, and the results are available. These results are particularly useful for checking various mathematical dose-response models, in this case the Weibull model. In the experiment (Staffa & Mehlman, 1979) female mice (BALB/c strain) ingested 2-acetylaminofluorene (2-AAF). Liver and bladder neoplasms were the principal pathological outcomes. The results for liver neoplasms are especially valuable for checking a mathematical model because significant responses were found in many different experimental groups. Bladder neoplasms, on the other hand, tended to appear only at the extremes of the experiment. For this reason, primary consideration will be given here to the results for liver neoplasms. The statistical details are given in the Appendix.

For the purpose of checking a dose-response model, there were three dimensions to the 2-AAF experiment: there were eight levels of the dose ranging from 0 to 150 ppm; there were nine serial kills ranging from 9 to 33 months after the start of treatment; some of the mice were exposed to 2-AAF for only a fraction of their time in the experiment and were then removed from exposure until their later scheduled kills. A general mathematical dose-response model for such an experiment would contain a variable for each of these three dimensions. This general model could then be specialized to contain only one variable or to contain any pair of the three, making a total of seven versions of the model.

Start with a simple and familiar version of the Weibull model:

$$P = 1 - e^{-(\alpha + \beta d^m)}, \qquad (W1)$$

where P is the probability of a tumour, d is the dose, and m, α and β are parameters. The parameter α is determined by the background tumour probability; β is a scale parameter related to the units measuring the dose (ppm for 2-AAF); m is the important shape parameter. At very low doses, the excess probability (risk) over the background is $e^{-\alpha}\beta d^m$. Since α is usually near zero, this reduces to βd^m. Then, only with m = 1 (the one-hit model) is there 'low-dose linearity'. With actual data sets from other lifetime experiments with other chemicals, the value of m ranges from about 0·5 to about 6·5 (Carlborg, 1980).

When version W1 is extended to include the time (t), it becomes

$$P = 1 - e^{-(\alpha + \beta d^m)t^k}, \qquad (W2)$$

where k is a new shape parameter for the time. In the analysis of the 2-AAF results, time (t) will be taken as the time to a scheduled kill. (In other contexts, it may be the time to the appearance of a tumour.) To simplify the interpretation and the computing for the 2-AAF data, take t = time to killing (months)/24 (months). Thus, t = 1 for the results from the 24-month kill. Notice that version W2 reduces to version W1 when t = 1.

When version W2 is further extended to include the duration of the exposure (f), it becomes

$$P = 1 - e^{-(\alpha + \beta f^h d^m)t^k}, \qquad (W3)$$

where h is a new shape parameter for the duration. This is the most general form of the Weibull model. Again, to simplify the interpretation and the computing for the 2-AAF data, take f = duration of exposure (months)/time to killing (months). Then f = 1 means that the group was exposed continuously at the dose d up to the kill. Similarly, f = 0·5 means that the group was exposed at dose d only for the first half of its scheduled time to killing. Notice that version W3 reduces to version W2 when f = 1.

Each of the other versions of the Weibull model is a special case of version W3. As a function of the time (t) only, it is

$$P = 1 - e^{-\delta t^k}, \qquad (W4)$$

where $\delta = \alpha + \beta f^h d^m$. The version W4 has long been used to describe the time to a tumour. As a function of the duration (f) only, the Weibull model is

$$P = 1 - e^{-(\alpha + \gamma f^h)}, \qquad (W5)$$

where $\gamma = \beta d^m$. A few important animal experiments have been run in this context (rather than that of W1). That is, only the duration of the exposure has been varied with the dose held constant; examples will be mentioned later. As a function of the duration (f) and the time (t), the version is

$$P = 1 - e^{-(\alpha + \gamma f^h)t^k}, \qquad (W6)$$

where $\gamma = \beta d^m$ again. Finally, there is the version

with the dose (d) and the duration (f) varied:

$$P = 1 - e^{-(\alpha + \beta f^h d^m)}, \qquad (W7)$$

where t = 1 for the case of a nominal full lifetime is assumed.

All the reported data (Staffa & Mehlman, 1979) for all the scheduled kills are given in Table 1. To illustrate, consider the column for t = 1 and f = 1, which is in the middle of the table. Since t = 1, this is the 24-month kill; since, f = 1, the mice were exposed over the entire 24 months. The first triplet of numbers in this column is for the control group (dose = d = 0). There were 383 mice sacrificed, and nine had liver neoplasms. (The "9·3" is the corresponding calculated frequency under Weibull model W3, which will be discussed later.) All the results for mice killed at 24 months with exposure up to the time of killing appear in this column. The first nine columns (all headed "f = 1") give the results for all mice exposed up to the time of their killing. The last six columns (f < 1) give the results for mice who were killed some time after their exposure had been stopped.

Three versions of the Weibull model have been fitted to the reported data in Table 1: (i) W1 with only the dose (d) as a variable, (ii) W2 with the dose (d) and the time (t) as variables and (iii) W3 with the dose (d), the time (t) and the duration (f) as variables. The results are given in Table 2. As indicated by its *P*-value, the fit of each version of the Weibull model to the data is very good. Also, notice the stability in the estimated parameters (α, β and m) as more general versions are introduced.

Version W3, which is the most general form, is especially interesting. The three shape parameters (m, k and h) are most important and deserve further comment. At the highest dose (d = 150) with 24-months continuous exposure (t = 1 and f = 1), the calculated probability of a liver neoplasm is 0·395. Figures 1, 2 and 3 show three different curves leading to this probability. Figure 1 shows the curve with only the dose (d) varied (t = 1 and f = 1 held constant). Figure 2 shows the curve with only the killing time (t) varied (d = 150 and f = 1 held constant). Figure 3 shows the curve with only the duration (f) varied (d = 150 and t = 1 held constant).

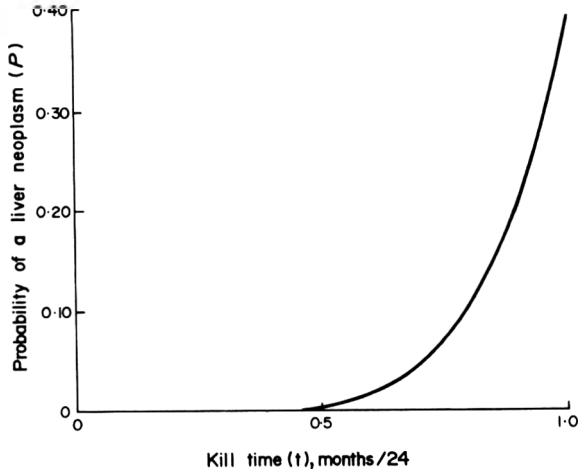

Fig. 2. Probability of a liver neoplasm (*P*) as a function of the kill time (t) for the maximum dose (d = 150 ppm) and with exposure for the whole period (f = 1·0).

Now consider the relation between the shape parameter for the dose (m = 1·44) and the shape parameter for the duration (h = 0·72). Assume two hypothetical experiments. In the first, the mice are exposed continuously at d = 75 for an entire nominal lifetime (t = 1 and f = 1); the calculated probability of a liver neoplasm is 0·18. In the second, the mice are exposed at d = 150 for only the first 12 months and later killed at 24 months (t = 1 and f = 0·5); the calculated probability of a liver neoplasm is 0·27. In each of these two experiments, the total lifetime exposure is the same (75 × 1 = 150 × 0·5), but the final probabilities are quite different (0·18 versus 0·27). The implication is consistent with a generally held belief: Early shorter exposure at a higher dose is more likely to produce a tumour than longer exposure at a lower dose.

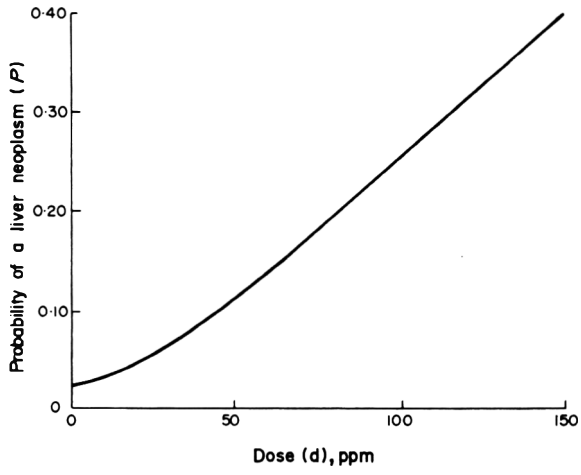

Fig. 1. Probability of a liver neoplasm (*P*) as a function of the dose (d) for a 24-month kill (t = 1·0) and with exposure for the whole period (f = 1·0).

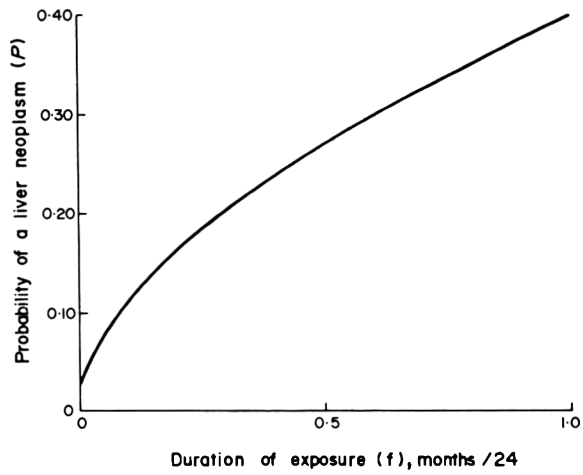

Fig. 3. Probability of a liver neoplasm (*P*) as a function of the duration of exposure (f) for the maximum dose (d = 150 ppm) and with a 24-month kill (t = 1·0).

Table 1. *Application of the Weibull model to the 2-AAF liver neoplasm data of Staffa & Mehlman (1979)*

Dose level* (ppm)		t ... 0·375 f ... 1·0	0·500 1·0	0·583 1·0	0·625 1·0	0·667 1·0	0·708 1·0	0·750 1·0	1·0 1·0	1·333 1·0	0·750 0·500	1·0 0·375	0·750 0·667	1·0 0·500	0·750 0·833	1·0 0·625
0	N	142†	140†	113†	88†	183†	128†	401	383	23						
	O	0	0	0	0	1	0	1	9	8						
	C	0·0	0·0	0·1	0·1	0·3	0·3	1·4	9·3	3·5						
30	N							1573	900	92						
	O							17	55	44						
	C							16·4	62·3	35·3						
35	N						389	792	639	45						
	O						2	7	55	20						
	C						3·2	9·6	51·2	19·4						
45	N					272	264	383	445	12						
	O					2	5	7	57	5						
	C					2·0	2·8	6·1	46·0	6·2						
60	N	279†	268†	224†	182	265	206	268	415	11	186	108	190	118	196	114
	O	0	2	0	1	4	6	7	71	7	1	13	1	11	4	15
	C	0·1	0·4	0·9	1·2	2·7	3·1	5·9	58·7	7·1	2·8	9·1	3·3	11·5	3·9	12·5
75	N	139†	137†	110†	94	174	134	267	311	12	128	66	132	74	130	86
	O	0	0	0	1	5	5	6	62	8	3	10	6	14	5	14
	C	0·0	0·3	0·6	0·8	2·3	2·6	7·7	56·6	8·9	2·4	7·0	3·0	9·1	3·4	12·0
100	N	142	138	117	90	90	67	131	160	10	64	35	65†	33	64	35
	O	1	3	1	4	1	1	6	47	8	1	5	0	5	1	6
	C	0·1	0·4	0·9	1·1	1·7	1·9	5·5	40·4	8·6	1·7	5·1	2·1	5·6	2·4	6·8
150	N	140†	141	114	90	86	65†	121	130		63	33	63	29	65	65
	O	0	1	1	1	3	0	7	56		4	9	2	9	4	6
	C	0·1	0·7	1·5	1·9	2·8	3·2	8·6	51·3		2·8	7·6	3·4	7·8	4·1	8·6

t = Time to killing (months/24 months) f = Duration of exposure (months)/time to killing (months)
N = No. of mice at risk O = Observed frequency of liver neoplasms C = Calculated frequency of neoplasms under the Weibull model
*Dose level while exposed.
†Data not used in fitting the Weibull model.

Table 2. *Results of fitting the Weibull model to the data for liver neoplasms in mice exposed to 2-AAF*

Model	*P*-value	Estimated value of parameter	Approximate SE of estimate
W1	0·56	α = 0·021	0·0076
		β = 0·000339	0·000202
		m = 1·49	0·13
W2	0·66	α = 0·024	0·0065
		β = 0·000343	0·000189
		m = 1·45	0·12
		k = 6·53	0·21
W3	0·59	α = 0·025	0·0064
		β = 0·000354	0·000190
		m = 1·44	0·12
		k = 6·69	0·20
		h = 0·72	0·19

If the shape parameter for the dose and the shape parameter for the duration were equal (m = h), then these two hypothetical experiments would produce the same probability of a tumour. If the shape parameter for the dose were less than the shape parameter for the duration (m < h), then the first hypothetical experiment would produce a greater probability of a tumour than the second.

With bladder carcinoma as the pathological endpoint, the 2-AAF experiment provides another comparison of the shape parameter for the dose (m = 6·46) to the shape parameter for the duration (h = 2·23). The former was computed using model W1 with the results for continuous exposure to the 24-month sacrifice. The latter was computed using model W5 with the results for mice exposed to 150 ppm (Carlborg, 1980).

Two other large experiments have been reported for which only model W5 is appropriate for the data. With inhalation of asbestos by Wistar rats (lung carcinoma), the estimated value of the shape parameter for the duration is h = 1·20. With inhalation of bis(chloromethyl)ether (BCME) by Sprague–Dawley rats (respiratory cancer), the estimated value of the shape parameter for the duration is h = 1·83 (Carlborg, 1980).

Table 3. *Statistical tests of 'low-dose linearity' for mice fed 2-AAF (24-month kill with continuous exposure)*

Dose groups included	*P*-value for liver neoplasm	*P*-value for liver carcinoma*
All 8 (0–150)	0·002	<0·001
Lowest 7 (0–100)	0·007	<0·001
Lowest 6 (0–75)	0·014	<0·001
Lowest 5 (0–60)	0·014	<0·001
Lowest 4 (0–45)	0·050	<0·007
Lowest 3 (0–35)	0·19	<0·16

*The observed responses with a liver carcinoma as the endpoint were: 7/383 for 0 ppm, 24/900 for 30 ppm, 27/639 for 35 ppm, 36/445 for 45 ppm, 47/415 for 60 ppm, 42/311 for 75 ppm, 27/160 for 100 ppm, and 42/130 for 150 ppm. These data were supplied by the Freedom of Information Officer of the US NCTR.

Humans are exposed by inhalation to low levels of asbestos, which was widely used in the construction of buildings in recent decades. Asbestos is a known human carcinogen at high doses. To extrapolate the risk to low levels of exposure, one needs the shape parameter for the dose (m), not the shape parameter for the duration (h). Some speculations can be made. With liver neoplasms for 2-AAF, the ratio of m to h is 2·0 (1·44/0·72). With a bladder carcinoma for 2-AAF, the ratio of m to h is 2·9 (6·46/2·23). Suppose that the ratio for asbestos is m/h = 2·0, as with the liver neoplasms. Then the corresponding estimated value of the shape parameter for the dose for asbestos is m = 2·4 (2 × 1·20). With an m of 2·4, the virtual safe dose (the VSD) for a one-in-a-million risk is about three orders of magnitude below the dose which produces 50% tumours (the TD_{50}). That is, persons exposed continuously at 0·001 TD_{50} would have about a one-in-a-million risk of developing cancer from asbestos (all on the assumption that the rats are predictors for humans). With a higher ratio (m/h > 2·0), the risk at 0·001 TD_{50} would be less; with a lower ratio (m/h < 2·0), the risk at 0·001 TD_{50} would be greater.

The final topic is a discussion of the hypothesis of 'low-dose linearity' in relation to the observed experimental results for 2-AAF. Loosely, this hypothesis states that at sufficiently low doses the dose-response function is linear. Experimentally, of course, the hypothesis is irrefutable because every experiment has its lowest dose, which may not be 'sufficiently low'.

The most important manifestation of the hypothesis is through its relation to the multi-stage model:

$$P = 1 - e^{-(\alpha + \beta_1 d + \beta_2 d^2 + \cdots + \beta_m d^m)}, \qquad (M1)$$

where the parameter m (an integer) is the number of stages. In practice, the α, the β's and the m are estimated from the data (Hartley & Sielken, 1977). Some of the β's may be zero, but none can be negative. At low doses, only the linear term has any importance:

$$P = 1 - e^{-(\alpha + \beta_1 d)}, \qquad (M2)$$

and this is the one-hit model. At these low doses, the excess risk over the background risk is $e^{-\alpha}\beta_1 d$, which is then a linear function of the dose (d).

With actual data, the linear term in M1 may not be present in the statistical best estimate of the model. When the background tumour rate (α) is not zero, a mathematical 'proof' has been offered showing that the linear term must be present. The assumption is then that the experimental error has masked the presence of the linear term and that it should be introduced through an upper confidence limit (Crump, Hoel, Langley & Peto, 1976). Low-risk extrapolation, therefore, should always include this linear term. The Interagency Regulatory Liaison Group of the U.S. Government (1979) has heavily endorsed this method of low-risk assessment.

The 2-AAF experiment is the largest ever run in carcinogenesis. The primary goal of the experiment was to determine the shape of the dose-response function to as low a dose as possible. With a liver tumour as the endpoint, there is a non-zero background tumour rate. Therefore, the best available check of the hypothesis of 'low-dose linearity' is to test model M2 successively against lower portions of the observed results. These reported data are given in the middle column of Table 1. The second column of Table 3 gives the results of testing model M2 against these data. With all eight doses included, model M2 fails to fit the data at the 0·002 level of significance. With only the lowest seven doses, model M2 fails to fit the data at the 0·007 level. At the traditional 0·05 significance level, model M2 also fails to fit with the lowest six doses, with the lowest five doses and with the lowest four doses. It is important to note that the increasing *P*-values going down the second column reflect the increasing meagerness of the data, rather than increasing linearity. In fact, the Weibull model fitted to only the lowest three doses is

$$P = 1 - e^{-(\alpha + \beta d^{3.4})}$$

with $\alpha = 0{\cdot}024$ and $\beta = 0{\cdot}000000386$.

The third column of Table 3 gives the similar results with a liver carcinoma as the pathologic endpoint. Model M2 fits even more poorly here. The conclusion from Table 3 is that the results for the liver from the 2-AAF experiment are inconsistent with the hypothesis of 'low-dose linearity'. (On page 27 of Staffa & Mehlman (1979) there is a graph of the observed data for the liver neoplasm. That graph is rather inaccurately drawn, suggesting more low-dose linearity than it should.)

APPENDIX

The Weibull model was fitted to the data by non-linear weighted least squares with a preliminary transformation. For example, take model W1:

$$Y = -\log_e(1 - P) = \alpha + \beta d^m$$

the weight for an observed Y is $n(1 - P)/P$, where P is the observed tumour rate and n is the number of animals at risk. The BMDP3R computer program was used. The *P*-values in Table 2 are based on the chi-square test applied to the error sum of squares.

In fitting versions W2 and W3, several low responses at the experimental extremes were omitted because they cause technical problems and contain no useful information. Two other data points, not at the experimental extremes, were omitted because of a zero response (yielding an infinite weight). These are: d = 150, t = 0·708, f = 1 and d = 100, t = 0·750, f = 0·667. These two omissions appear to have only a small effect on the overall result. If the error sum of squares is increased by adding the contributions of these two points (using the calculated P's to get the weights), then the *P*-value for the fit of model W3 is 0·48, instead of the 0·59 reported in Table 2.

The *P*-values reported in Table 3 are from the chi-square test applied to the error sum of squares from fitting the one-hit model (M2).

REFERENCES

Carlborg, F. W. (1981). Dose-response functions in carcinogenesis and the Weibull model. *Fd Cosmet. Toxicol.* **19,** 255.

Crump, K. S., Hoel, D. G., Langley, C. H. & Peto, R. (1976). Fundamental carcinogenic processes and their implications for low dose risk assessment. *Cancer Res.* **36,** 2973.

Hartley, H. O. & Sielken, R. L. (1977). Estimation of "safe doses" in carcinogenic experiments. *Biometrics* **33,** 1.

Interagency Regulatory Liaison Group (1979). Scientific bases for identification of potential carcinogens and estimation of risks. *Federal Register* **44,** pp. 39858–39879.

Staffa, J. A. & Mehlman, M. A. (1979). *Innovations in Cancer Risk Assessment.* Pathotox Publishers, Inc., Park Forest South, IL.

Fd Cosmet. Toxicol. Vol. 19, pp. 373 to 379, 1981
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IN UTERO EXPOSURE IN CHRONIC TOXICITY/ CARCINOGENICITY STUDIES

H. C. GRICE*, I. C. MUNRO and D. R. KREWSKI

FDC Consultants, 71 Norice Drive, Ottawa, Ontario K2H 2X7

I. C. MUNRO and D. R. KREWSKI

Food Directorate, Health Protection Branch, Health and Welfare Canada, Ottawa, Ontario K1A OL2, Canada

and

H. BLUMENTHAL

Division of Toxicology, Bureau of Foods, Food and Drug Administration, 200 C Street S.W., Washington, DC 20204, USA

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Summary—The need for *in utero* exposure in chronic toxicity/carcinogenicity is reviewed but more comparative data are required to evaluate fully the need for such protocols in safety evaluation programmes. Practical considerations including cost, selection of the second generation animals and dose levels to be used are discussed. In evaluating the results of studies involving perinatal exposure it is also necessary to consider such factors as the additional tissues through which the test chemical passes and differences in susceptibility between developing and mature tissues.

Introduction

Considerable attention is currently being paid by toxicologists to chronic toxicty/carcinogenicity studies involving perinatal exposure of experimental animals to the test compound. In such studies the parent or F_0 generation is dosed with test chemical for some time before impregnation as well as during pregnancy and lactation. The offspring in the F_1 generation are then dosed with the chemical for the remainder of their lifetimes or for a period thereof. The second generation is thus exposed to the chemical *in utero* and through the mother's milk (if the chemical or its metabolites are able to enter the foetus or newborn animal by these routes) as well as through the diet after weaning. The object of this paper is to review the rationale for requiring these studies, to assess practical problems associated with their conduct and interpretation, and to suggest areas where additional information and further research are required.

In the following discussion it is assumed that considerable information concerning the subchronic and reproductive toxicity of the compound and its maternal and foetal pharmacokinetic characteristics would be available before the initiation of an *in utero* exposure chronic study. This information must be available before a viable protocol for a chronic study involving perinatal exposure can be developed.

The need for *in utero* exposure studies

Rationale

In the practical sense, the *in utero* exposure model mimics the human situation, particularly in the case of food additives and certain environmental contaminants. These chemicals are often consumed unknowingly and may be ingested by humans throughout their lifetimes, including pregnancy and lactation periods. Thus, the perinatal human may receive the chemical or its metabolites *via* the placenta or from the mother's milk.

The use of the model is also based on the expectation that *in utero* exposure may increase the sensitivity of cancer bioassays. This increased sensitivity may be expressed as an increased incidence or reduced latent period of tumours seen in the offspring or in the induction of unique tumours not frequently seen in the parents. However, this assertion is based on somewhat limited published data. The incidence of tumours in animals exposed *in utero* to aflatoxin, saccharin or the ethyl derivatives of the nitrosamides is somewhat greater than that in animals treated only from weaning (Arnold, Moodie, Grice, Charbonneau, Stavric, Collins, McGuire, Zawidzka & Munro, 1980; Druckrey, Schagen & Ivankovic, 1970; Grice, Moodie & Smith, 1973). However, substances such as methylnitrosourea, 1,2-dimethylhydrazine, 1-phenyl-3,3-dimethyltriazene and azoxymethane appear to be more carcinogenic in the adult than in those exposed only *in utero* (Ivankovic, 1973).

The hypothesis of increased sensitivity is supported by the observation that diethylstilboestrol induces tumours of the vagina in young women whose mothers were given this drug for pregnancy maintenance (Herbst, Robboy, Scully & Poskanzer, 1974; Herbst, Ulfelder & Poskanzer, 1971). Although this appears to be a unique carcinogenic event not yet observed in the parent there is some suggestion

*Present address: FDC Consultants, 71 Norice Drive, Ottawa, Ontario K2H 2X7.

(Bibbo, Haenszel, Wied, Hubby & Herbst, 1978; Ryan, 1978; U.S. DHEW, 1978) that DES may increase the incidence and decrease the latent period of breast cancer in the mother. Thus it cannot be concluded from these data that DES is carcinogenic only in the offspring. A somewhat analogous situation has been reported by Druckrey (1973) who observed that rats exposed *in utero* to certain nitroso compounds developed mainly neurogenic tumours whereas tumours at other sites predominated in animals treated after weaning.

These apparent differences in sensitivity may be due to a variety of factors including the pharmacokinetics and metabolism of the test compound and the immunologic competence of the host. In addition, the rapidly dividing cells of the foetus may be more or less susceptible to carcinogenic substances depending upon their metabolic capability and stage of development.

Regulatory considerations

With the possible exception of saccharin, there are no examples presently available where the *in utero* exposure model has formed the basis for regulatory decisions. Despite this limited history, it may be helpful to examine briefly the saccharin data in an attempt to assess the usefulness of the *in utero* exposure model. In the Canadian study (Arnold *et al.* 1980), the excess incidence of bladder tumours in treated parent males was of only marginal statistical significance while in the offspring the tumour incidence in treated males was markedly increased relative to controls. This latter finding confirmed similar results found in two previous studies involving *in utero* exposure (National Academy of Sciences, 1978; U.S. Congress, 1977).

If the data on tumour incidence for each generation is used to determine a virtually safe level of exposure using mathematical modelling procedures, however, then it could be argued that the apparent difference in sensitivity between the F_0 and F_1 generations is of little consequence. For example, the estimated virtually safe dose shown in Table 1 obtained *via* linear extrapolation (Gaylor & Shapiro, 1979) of the results in the F_1 generation is half that based on the results in the F_0 generation. (Direct linear extrapolation of the observed results is used here. The use of confidence limits would lead to slightly lower results.) This difference is somewhat insignificant in view of the fact that both safe doses are less than 1 ppm in the diet. In this example, the safety factors imposed by the extrapolation procedure are so great that the difference between the estimated safe doses for the F_0 and F_1 generations is irrelevant. Further data of this type involving a variety of test compounds would help in evaluating the need for *in utero* exposure studies in safety evaluation. Such data should include results at several dose levels in order that extrapolation procedures which take into account the shape of the dose-response curve may be used (Food Safety Council, 1980).

From the regulatory viewpoint, the *in utero* exposure model could ultimately prove to be most useful as an instrument for detecting those compounds which may be carcinogenic only in the offspring. If the test compound is found to be carcinogenic in the parent as well as the progeny, regulatory action will be based primarily on the fact that it is a carcinogen, not because it induces tumours at different doses or with different latent periods or even at different sites in the F_0 and F_1 generations.

Selection of chemicals for an in utero *exposure study*

In order to determine whether an *in utero* exposure study or a conventional study involving only post-weaning exposure is most appropriate, a number of factors should be taken into account. If the population at risk does not include pregnant or lactating females, then *in utero* exposure may not be relevant. Except in instances such as this, where the need for *in utero* exposure is contraindicated, this protocol should be considered in the case of substances that are absorbed from the gastro-intestinal tract and traverse the placental or mammary barriers. In this connection, particular attention should be paid to substances that concentrate in these two compartments. In addition, special consideration should be given to nutrients for both the parent and the foetus as well as substances affecting reproductive processes.

Considerations in the conduct of *in utero* exposure studies

Cost

A conventional chronic toxicity study conducted at the Health Protection Branch costs about $250,000. (This figure includes the initial cost of the animals and dose-selection studies, but excludes major overhead items.) For the *in utero* exposure protocol, the additional cost of maintaining the parent animals from weaning until their offspring are weaned (about 100 days) may range from $20,000 to $35,000. In addition, the costs of feeding the test compound to the parent generation and the determination of the level of the chemical in the food must be taken into account. The final cost depending on these and other variables would be in the range of $30,000–$45,000 or 12%–18% more than that of a conventional chronic

Table 1. *Estimated virtually safe doses of saccharin based on linear extrapolation*

Generation	Incidence of bladder tumours among male rats		Safe dose at an added risk of 10^{-6} over background (ppm)
	Controls	Animals given test diet (5% saccharin)	
F_0	1/50	7/50	0·4
F_1	0/50	12/50	0·2

toxicity study. (Industrial sources have indicated that their costs for a two-generation chronic study are about 21% higher than those for a conventional study.)

Selection of the F_1 generation animals

One of the major considerations in the design of an *in utero* exposure study involves the selection of the second generation animals. As a result of inter-litter differences in both transplacental or milk exposure and genealogy, the litter rather than the individual pup may be the appropriate experimental unit for purposes of statistical analysis (Haseman & Kupper, 1979).

In order to assess the degree of inter-litter variation in tissue levels of chemical substances following exposure *via* the placenta, tissue distribution studies were undertaken with saccharin, amaranth and styrene. In the first experiment, five pregnant Wistar rats were administered a single dose of radiolabelled (^{14}C) saccharin (5·6 μCi/200 g body weight) through a jugular cannula on day 20 of gestation. The amount of saccharin in the foetal tissues was measured by scintillation counting 30 min after dosing. In the second experiment, six female Sprague–Dawley rats were fed daily doses of 2000 mg of amaranth/kg body weight in the diet for 9 days. The animals were then mated and maintained on their respective diets until day 21 of gestation. At this time, the concentration of naphthionic acid (a metabolite of amaranth) in the foetal blood was measured. In the third experiment, five pregnant Sprague–Dawley rats were placed in a vapour chamber containing 2000 ppm styrene for 5 hr on day 17 of gestation. The concentration of styrene in the foetal tissues was measured immediately after the exposure.

The results of these three experiments are shown in Fig. 1, where the differences among the litter means reflect the inter-litter variation in transplacental exposure. (Here, each individual bar represents a single pup with the results within each litter arranged in decreasing order of magnitude showing the intra-litter variation.) Statistical analysis of these data indicates that this inter-litter variation is highly significant

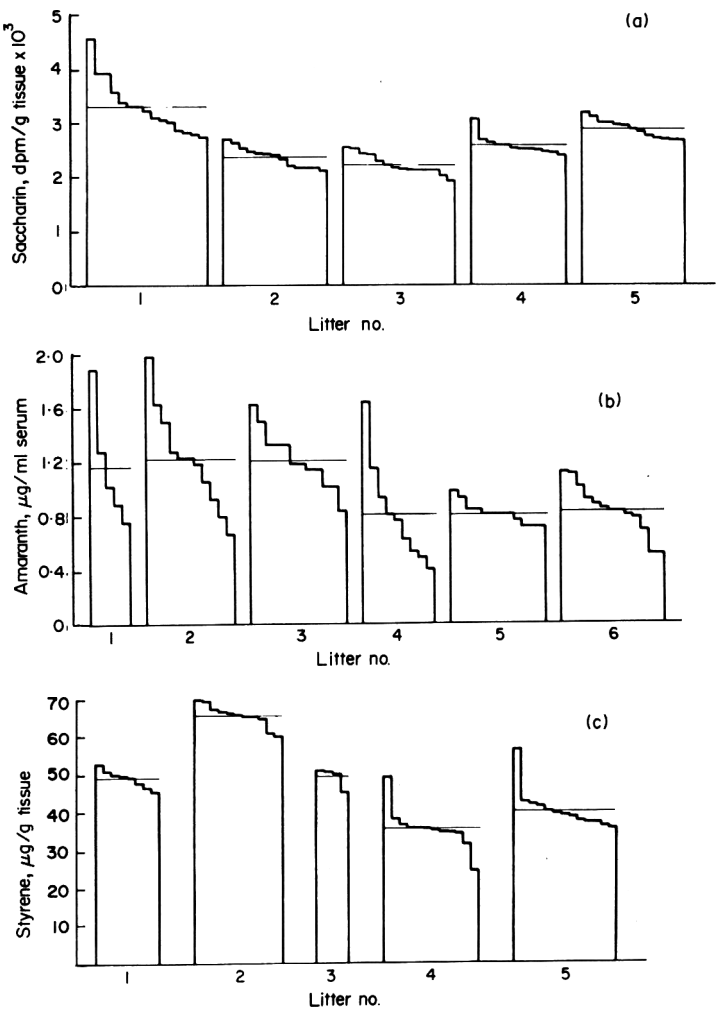

Fig. 1. Intra- and inter-litter variation in foetal tissue concentrations of (a) saccharin, (b) amaranth and (c) styrene following intra-uterine exposure to the compounds. The fine horizontal lines indicate litter means.

($P < 0{\cdot}002$) for all three compounds, with the ratio of the variance between litters to that within litters (Searle, 1971) estimated to about 2·3, 0·5 and 8·1 for saccharin, amaranth and styrene respectively. The corresponding intra-litter correlation coefficients (Scheffé, 1959), which measure the degree of homogeneity in exposure among litter mates, were estimated to be about 0·7, 0·3 and 0·9 respectively. (Although the standard analysis of variance model used here is based on the assumption that the intra-litter variation is constant across litters, this is not the case for either saccharin or amaranth. An analysis of these data excluding litter one in the case of saccharin and litter five in the case of amaranth, however, will yield essentially the same results.)

Further data of this type are required to determine whether other compounds are handled in a similar fashion. In addition, actual bioassay data are needed in order to establish whether or not the litter effects noted above are also characteristic of the toxic endpoints of interest in a chronic study. Such effects may not be unexpected in cases where the toxic response of interest is induced or enhanced as a result of exposure *in utero*.

In the presence of appreciable litter effects, the statistical sensitivity of a chronic *in utero* exposure study will depend on the number of pups selected from each litter. In order to illustrate the effects of intra-litter correlation on statistical sensitivity, consider a simple hypothetical experiment in which 60 animals of the same sex are to be selected in the second generation from each of a control and a test group. The 60 animals required in each group could be obtained on the basis of one per litter from 60 litters or two per litter from 30 litters. Fewer than 30 litters would be required if more than two pups per litter were chosen.

The approximate probability of detecting a carcinogenic compound inducing a tumour incidence rate of 20% at a site where the spontaneous tumour incidence rate is 5% is shown in Table 2 for various possible values of the intra-litter correlation coefficient in the test group. (Details of the statistical test procedure on which these results are based are given in the Appendix.) With increasing intra-litter correlation, statistical sensitivity is reduced by selecting more than one pup from each litter, with the losses increasing substantially as the intra-litter correlation increases. Note that when only one pup is selected from each litter, however, the sensitivity is unaffected by the degree of intra-litter correlation. (While the goal of maximum sensitivity is achieved through the use of an increased number of dams in the F_0 generation, the total cost of a two-generation bioassay depends more on the number of F_1 animals than the number of F_0 animals.)

The method of selection of the F_1 generation animals should ensure that each pup in a given litter has an equal chance of inclusion. One procedure which may be used involves culling pups to a maximum of eight per litter at 4–5 days of age in order to balance the burden on the dams and prevent the natural selection of the more vigorous offspring. Culling should be on a random basis within each sex, balancing the number of males and females within each litter as nearly as possible. Finally, one male and one female should be selected randomly from each litter at weaning to continue on test in the F_1 generation.

Dose selection

The selection of the dose given to the dam, to the neonate, to the juvenile and to the F_1 adult should be considered. There are a number of factors to be taken into account in each instance. For example, how long before breeding should dosing start? In cases where the dose to the parent must be limited because of foetotoxic effects, what dose should be given to the offspring? Should the dose administered to the dam be adjusted because of the additional caloric requirements during gestation which result in increased food consumption? Since very few studies in this area have been undertaken, the establishment of guidelines on dose selection should prove to be challenging.

In dealing with new compounds much of the information needed for dose selection will be derived from multi-generation reproduction and teratology studies. These studies will assist in determining dose levels that should be avoided in order to prevent reproductive or teratogenic effects in the F_1 generation that would adversely affect survival.

Pharmacodynamic studies will provide information on whether or not metabolic processes in the dam are saturated. Knowledge of the rate of uptake, the pattern of distribution and the rate of elimination of the test compound is important in determining the effective dose to the litter. Pharmacodynamic studies may be used to define the dose level, dosing interval and

Table 2. *Probability of significance (%), for a hypothetical two-generation cancer bioassay, and its relationship to intra-litter correlation**

No. of pups selected per litter	No. of litters	Intra-litter correlation coefficient in test group...	Probability of significance (%)		
			0·1	0·5	0·9
1	60		82	82	82
2	30		79	70	62
3	20		77	61	50
4	15		74	54	42

*Assuming the intra-litter correlation in the control group is 0·01 and testing at the 5% significance level. (The response probabilities in the control and test groups were taken to be 5% and 20% respectively.)

period of exposure that will produce a dam in pharmacokinetic equilibrium at the time of mating.

In general, the high dose administered to the F_0 generation should not affect the viability of the offspring nor should it markedly affect their fertility or reproductive capability. In some instances, animals treated after weaning only may tolerate a considerably higher dose than that which could be administered to the pregnant dams. In such instances, a higher dose might be given to the F_1 generation to comply with generally accepted guidelines on dose selection (Food Safety Council, 1980; Munro, 1977; Sontag, Page & Saffiotti, 1976). However, such practice may lead to difficulty in assessing dose-response because of the different doses administered to the parents and the offspring.

Potential problems in the interpretation of *in utero* studies

Transplacental effects

The main reason why the results from conventional chronic studies and *in utero* exposure studies are likely to differ arises from the fact that in the latter case the test chemical is processed by additional organs including the placenta, mammary gland and gastro-intestinal tract. There is also the potential for the chemical to affect the developing tissue or organ before the animal is weaned because of a particular sensitivity or a preferential concentration of the substance or its metabolites at a particular locus in the foetus.

The species used for *in utero* testing and the relationship of its placenta to that of man is particularly important. According to Hamilton, Boyd & Mossman (1962), the yolk sac is the most variable of all mammalian foetal membranes. The selective uptake of chemicals by foetal tissues may be affected by the capacity of the yolk sac, with its endodermal lining resembling the microvilli and endoplasmic reticulum of the adult liver, to metabolize and secrete chemicals. In the rat, the lining of the yolk sac secretes chemicals into the uterine lumen in much the same way as the liver secretes substances in the bile. It is not known whether the human yolk sac, which appears to be a vestigial organ, plays a similar role in excreting drugs from the developing foetus (Waddell, 1972). The yolk sac of rodents and rabbits has been shown to absorb and accumulate a number of chemical agents from the uterine lumen and maternal blood (Brambell, 1958; Butt & Wilson, 1968; Deren, Padykula & Wilson, 1966; Everett, 1935). These may be transferred to the foetus by the vitelline circulation. The comparative histology of the placenta indicates many species differences which may be important in relation to the transplacental pharmacokinetic properties of the chemical (Wynn, 1968).

Indirect effects whereby chemically induced physiological changes in the placenta adversely affect the foetus should also be considered. For example, ergot causes spasmodic contractions in placental circulation and induces abortion at high doses (Grauwiler & Schon, 1973). However, most such situations may be avoided if the selection of the highest dose given to the F_0 generation meets the dose selection criteria outlined previously.

Metabolism by the foetus

Foetal metabolism of chemicals differs markedly between man and many of the species that are used in long-term toxicity tests. In animals such as the rat, mouse and rabbit, enzyme systems such as aryl hydrocarbon hydroxylase and the mixed-function oxidases do not develop appreciably until after birth (Short, Kinden & Stith, 1976). In the human foetal liver, however, some of these enzyme systems develop as early as the first trimester. In addition, the development of some mixed-function oxidases has been shown to depend on genetic factors. Such genetic differences would appear to influence the susceptibility to certain carcinogens. It is known for example that pulmonary tumours, either spontaneously occurring or chemically induced, are rare in C57 BL and NZW but common in Swiss and C3H strains of mice (Rice, 1973).

Mammary gland

There is little information concerning the capability of the mammary gland to convert chemicals passing through it to more or less toxic metabolites. However, it is recognized that chemicals tend to become concentrated in the milk. Chemicals in the mammary gland secretions would be mainly responsible for toxic effects in infants in those cases where the chemical crossed the placenta in small amounts but was secreted at high levels in the milk and where the target tissues were not fully developed at birth. The thymus of the rat, for example, begins to be populated with lymphocytes during days 14–16 of gestation and this continues until after birth. Tetrachlorodibenzo-*p*-dioxin (TCDD) has been shown to have profound effects on the thymus in the offspring of rats and mice treated postnatally with TCDD (Vos & Moore, 1974). Similarly, the concentrations of hexachlorobenzene (HCB) in the tissues of pups born to dams that are given HCB before and during parturition and lactation are derived almost entirely from the milk. In addition, transmission of HCB through milk has a greater effect on esterase activities in preweanling pups than does placental transmission (Mendoza, Collins, Shields & Laver, 1977; Mendoza, Collins, Shields & Laver, 1978).

Summary and conclusions

In utero exposure chronic toxicity/carcinogenicity studies provide an additional screen in the network of toxicity testing procedures. On the basis of our presently limited experience however, the extent to which decisions based on the results of such studies would conflict with those dictated by traditional toxicological tests is not clear. Further comparative data are required in order to evaluate fully the need for such protocols in the overall safety evaluation programme.

Special considerations in the conduct of these two generation studies include the selection of the second generation animals and the highest dose level to be administered to both the parent and the offspring. For specified group sizes in the second generation, the selection of one male and one female pup from each litter will generally ensure maximal statistical sensitivity for comparisons between groups of the same sex

even when correlations within litters are associated with perinatal exposure. In a screening study, the highest dose administered to the offspring need not correspond to that given to the parents. In a dose-response study, however, differences in dose between the two generations may obscure quantification of dose-related effects.

When an *in utero* exposure study is used instead of a conventional study, the test chemical passes through several additional tissues and organs which have the potential to render the chemical more or less toxic. The chemical or its metabolites may act on developing tissues that may be more or less susceptible to toxic effects than the mature tissue. These factors should be taken into account when the data are evaluated and attempts are made to extrapolate from one species to another.

Appendix

Intra-litter correlation and statistical sensitivity

This Appendix describes in detail how the results in Table 2 were obtained. Suppose that m pups are selected, from each of n litters born in a particular treatment group, to continue on test in the second generation. Suppose further that among those pups selected in the ith litter, tumours develop independently with probability $p_i (i = 1, \ldots, n)$, where the random variable p_i follows a given probability distribution with mean p. Significant litter effects will then be reflected by appreciable inter-litter variation in the litter specific response probabilities p_i.

An unbiased estimator of the overall response probability p is given by

$$\hat{p} = \frac{1}{n} \sum_{i=1}^{n} \hat{p}_i, \qquad (1)$$

where $\hat{p}_i$ denotes the observed incidence of tumours in the ith litter. The variance of $\hat{p}$ is given by

$$V(\hat{p}) = \{\rho(1 - \rho)/nm\}\{1 + (m - 1)\rho\} \qquad (2)$$

where ρ denotes the intra-litter correlation coefficient (Scheffé, 1959), and may be estimated by

$$v(\hat{p}) = \frac{1}{n} \sum_{i=1}^{n} \frac{(\hat{p}_i - \hat{p})^2}{(n - 1)}. \qquad (3)$$

Provided $\rho > 0$, it follows from (2) that if the total number of animals required (nm) is fixed, then the precision of $\hat{p}$ may be maximized by choosing m = 1 pup from each litter. With this choice of m, moreover, the variance of $\hat{p}$ does not depend on the magnitude of the intra-litter correlation coefficient ρ. For $m > 1$ and $\rho > 0$, the variance of $\hat{p}$ increases as ρ increases.

To assess the effects of intra-litter correlation and the choice of m on the statistical sensitivity of a two-generation cancer bioassay, consider a test for increased tumour incidence based on the statistic

$$z = (\hat{p}_t - \hat{p}_c)/\{v(\hat{p}_t) + v(\hat{p}_c)\}^{\frac{1}{2}}, \qquad (4)$$

where the subscripts t and c are used to identify the results for the treated and control groups respectively. The power of this test (Table 2) may be easily evaluated using the fact that the distribution of z will be approximately normal, provided that the number of litters in both the treated and control group is sufficiently large.

In order to examine the adequacy of the normal approximation, the power of this test procedure was also evaluated empirically by simulating one thousand experimental outcomes under the β-binomial model (Williams, 1975) and calculating the proportion of those outcomes in which a statistically significant increase in tumour incidence was observed. (Assuming that the litter-specific response probability p_i follows a β distribution, the marginal distribution of the number of tumours within a litter is β-binomial. The two parameters in the β-binomial model are uniquely determined by the specified overall response probability p and the intra-litter correlation coefficient ρ.) Since the results obtained were in all but one case within $\pm 5\%$ of those given in Table 2, the use of the normal approximation would appear to be reasonable.

While the results in Table 2 are based on the particular test procedure used here, the use of other appropriate procedures may be expected to lead to the same general pattern. Analytical results on the power of an exact permutation test (Soms, 1977) have been obtained and will be reported elsewhere.

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REFERENCES

Arnold, D. L., Moodie, C. A., Grice, H. C., Charbonneau, S. M., Stavric, B., Collins, B. T., McGuire, P. F., Zawidzka, Z. Z. & Munro, I. C. (1980). Long-term toxicity of *ortho*-toluenesulfonamide and sodium saccharin in the rat. *Toxic. appl. Pharmac.* **52,** 113.

Bibbo, M., Haenszel, W. M., Wied, G. L., Hubby, M. & Herbst, A. L. (1978). A twenty-five year follow-up study of women exposed to diethylstilbestrol during pregnancy. *New Engl. J. Med.* **298,** 763.

Brambell, F. W. R. (1958). The passive immunity of the young mammal. *Camb. Phil. Soc. biol. Rev.* **33,** 488.

Butt, J. H. II & Wilson, T. H. (1968). Development of sugar and amino acid transport by intestine and yolk sac of the guinea pig. *Am. J. Physiol.* **215,** 1468.

Deren, J. J., Padykula, H. A. & Wilson, T. H. (1966). The development of structure and function of amino yolk sac. III. The development of amino acid transport by rabbit yolk sac. *Devl. Biol.* **13,** 370.

Druckrey, H. (1973). Chemical structure and action in transplacental carcinogenesis and teratogenesis. In *Transplacental Carcinogenesis.* Edited by L. Tomatis & U. Mohr. IARC Scient. Publ. no. 4, p. 45. International Agency for Research on Cancer, Lyon.

Druckrey, H., Schagen, B. & Ivankovic, S. (1970). Erzeugung neurogener Malignome durch einmalige Gabe von Äthyl-nitrosoharnstaff (ÄNH) an neugeborene und junge BD IX-Ratten. *Z. Krebsforsch.* **74,** 141.

Everett, J. W. (1935). Morphological and physiological studies of the placenta in the albino rat. *J. exp. Zool.* **70,** 243.

Food Safety Council (1980). Proposed System for Food Safety Assessment. Food Safety Council, 1725 K St. N.W., Washington, DC see also *Fd Cosmet. Toxicol.* 1978, **16,** Supplement 2.

Gaylor, D. W. & Shapiro, R. E. (1979). Extrapolation and

risk estimation for carcinogenesis. In *Advances in Modern Toxicology*. Vol. 1, *New Concepts in Safety Evaluation*. Part 2. Edited by M. A. Mehlman, R. E. Shapiro & H. Blumenthal. p. 65. Hemisphere Publishing Corporation, New York.

Grauwiler, J. & Schon, H. (1973). Teratological experiments with ergotamine in mice, rats and rabbits. *Teratology* **7**, 227.

Grice, H. C., Moodie, C. A. & Smith, D. C. (1973). The carcinogenic potential of aflatoxin or its metabolites in rats from dams fed aflatoxin pre- and postpartum. *Cancer Res.* **33**, 262.

Hamilton, W. J., Boyd, J. D. & Mossman, H. W. (1962). *Human Embryology*. 3rd Ed. Williams and Wilkins, Baltimore, Maryland, USA.

Haseman, J. K. & Kupper, L. L. (1979). Analysis of dichotomous data from certain toxicological experiments. *Biometrics* **35**, 281.

Herbst, A. L., Robboy, S. J., Scully, R. E. & Poskanzer, D. C. (1974). Clear-cell adenocarcinoma of the vagina and cervix in girls: Analysis of 170 registry cases. *Am. J. Obstet. Gynecol.* **119**, 713.

Herbst, A. L., Ulfelder, H. & Poskanzer, D. C. (1971). Adenocarcinoma of the vagina. Association of maternal stilbestrol therapy with tumor appearance in young women. *New Engl. J. Med.* **284**, 878.

Ivankovic, S. (1973). Experimental prenatal carcinogenesis. In *Transplacental Carcinogenesis*. Edited by L. Tomatis & U. Mohr. IARC Scient. Publ. no. 4. p. 92. International Agency for Research on Cancer, Lyon.

Mendoza, C. E., Collins, B. T., Shields, J. B. & Laver, G. W. (1978). Effects of hexachlorobenzene or hexabromobenzene on body and organ weights of preweanling rats after a reciprocal transfer between the treated and control dams. *J. agric. Fd Chem.* **26**, 941.

Mendoza, C. E., Collins, B., Shields, J. B. & Laver, G. W. (1977). Hexachlorobenzene residues and effects on esterase activities in pre-weanling rats after a reciprocal transfer between HCB-treated and control dams. *Archs Toxicol.* **38**, 191.

Munro, I. C. (1977). Considerations in chronic toxicity testing: the chemical, the dose, the design. *J. envir. Path. Toxicol.* **1**, 183.

National Academy of Sciences (1978). Saccharin: Technical Assessment of Risks and Benefits (Report No. 1 of the Committee for a Study on Saccharin and Food Safety Policy). NAS, Washington, DC.

Rice, J. M. (1973). The biological behaviour of transplacentally induced tumours in mice. In *Transplacental Carcinogenesis*. Edited by L. Tomatis & H. Mohr. IARC Scient. Publ. no. 4. p. 71. International Agency for Research on Cancer, Lyon.

Ryan, K. J. (1978). Diethylstilbestrol: twenty-five years later. *New Engl. J. Med.* **298**, 794.

Scheffé, H. (1959). *The Analysis of Variance*. p. 223. J. Wiley and Sons, New York.

Searle, S. R. (1971). *Linear Models*. p. 337. J. Wiley and Sons, New York.

Short, C. R., Kinden, D. A. & Stith, R. (1976). Fetal and neonatal development of the microsomal mono-oxygenase system. *Drug Metab. Rev.* **5**, 1.

Soms, A. P. (1977). An algorithm for the discrete Fisher's permutation test. *J. Am. Statist. Assoc.* **72**, 662.

Sontag, J. M., Page, N. P. & Saffiotti, U. (1976). Guidelines for Carcinogen Bioassay in Small Rodents. DHEW Publ. no. (NIH) 76–801. Department of Health, Education and Welfare, Washington, DC.

U.S. Congress, Office of Technology Assessment (1977). Cancer Testing Technology and Saccharin. U.S. Government Printing Office, Washington, DC.

U.S. Department of Health, Education and Welfare (1978). DES Task Force Summary Report. DHEW Publ. no. (NIH) 79–1688.

Vos, J. G. & Moore, J. A. (1974). Suppression of cellular immunity in rats and mice by maternal treatment with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Int. Archs Allergy appl. Immun.* **47**, 777.

Waddell, W. J. (1972). Localization and metabolism of drugs in the fetus. *Fedn Proc. Fedn Am. Socs exp. Biol.* **31**, 52.

Williams, D. A. (1975). The analysis of binary responses from toxicological experiments involving reproduction and teratogenicity. *Biometrics* **31**, 949.

Wynn, R. W. (1968). Morphology of the placenta. In *Biology of Gestation*. p. 93. Edited by N.S. Assali. Vol. 1. Academic Press, New York.

Fd Cosmet. Toxicol. Vol. 19, pp. 381 to 386, 1981
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REVIEWS OF RECENT PUBLICATIONS

Evaluation of Certain Food Additives. Twenty-third Report of the Joint FAO/WHO Expert Committee on Food Additives. Tech. Rep. Ser. Wld Hlth Org. 1980, no. 648, pp. 45. Sw.fr. 3.00 (available in the UK through HMSO).

Toxicological Evaluation of Certain Food Additives. Joint FAO/WHO Expert Committee on Food Additives. WHO Fd Add. Ser. 1979, no. 14, pp. 96 (available in the UK through HMSO).

These documents resulted from a meeting in Geneva in April 1979, when the substances evaluated included a number of food colourings, carrier and extraction solvents, flavourings and miscellaneous additives. The first publication contains a general summary of the recommendations. The second contains the monographs drawn up on some of the additives, including Red 2G, 1,3-butanediol, polyethylene glycols, methylene chloride, chloroform, *trans*-anethole, *d*- and *l*-carvones, cinnamaldehyde, estragole, α and β-ionones, methyl anthranilate, nonanal and octanal.

With regard to extraction solvents, the Committee noted that impurities and stabilizers may affect toxicity and in the case of 1,1,1-trichloroethane, trichloroethylene and tetrachloroethylene may have affected the results of carcinogenicity tests. Furthermore, such impurities and stabilizers may be less volatile than the solvent and remain in the food after the solvent has been removed. It was recommended that in future toxicity tests, food-grade rather than industrial-grade solvents should be used, and that any impurities should be fully identified.

The need for food additives and contaminants to be tested *in utero* and during lactation was confirmed by the Committee, but it was recommended that a meeting of experts should be convened to assess the value of such testing and to propose guidelines covering dosages, relative exposures of mother and foetus, the possibility of combining this test with reproduction studies, study length and the most appropriate species to use. The importance of possible interactions between food additives or contaminants and drugs was also emphasized, and active liaison between experts in food and drug toxicology was recommended. Another topic considered was the extrapolation of toxicological data from one compound to other structurally-related materials, a procedure that was condoned provided certain conditions were satisfied.

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

This handbook contains a comprehensive review of those biological effects of metals that are pertinent to the understanding and assessment of their toxicity. Part I of this useful compilation outlines general aspects of metal toxicology. The topics dealt with range from the environmental and ecological distribution of metals, and their analysis, metabolism and mode of action to discussions of health and safety standards and of the diagnosis and treatment of metal poisoning. The carcinogenic and mutagenic effects of metals are also covered.

Throughout Part I there is liberal illustrative reference to individual metals or groups of metals, but in Part II the topics dealt with broadly in the first part are applied specifically, each of the 28 chapters being devoted to a single metal. Thorough reviews are presented and a large number of literature references, generally dated up to and including 1978, are given. These chapters have been contributed by different authors and are of various lengths, with arsenic, cadmium, lead and mercury being covered in the greatest detail. It is admirable, therefore, that each of the chapters follows a similar format.

The book is generally well presented, and has a very good "Table of Contents" and index. It provides an excellent entry into the literature of the toxicological aspects of metals.

Toxicity of Heavy Metals in the Environment. Parts 1 and 2. Edited by F. W. Oehme. Marcel Dekker Inc., New York, 1978 & 1979. Part 1: pp. x + 515; Sw.fr. 106.00. Part 2: pp. x + 454. $45.00.

This multi-author treatise on heavy-metal toxicity is in two separate parts, with the index for both at the end of part 2. In part 1, the occurrence of heavy metals in the environment and in animal and human food chains is discussed. A chapter on the basic mechanism of heavy-metal toxicity precedes more detailed coverage of the metabolism, mode of action and clinical syndromes of toxicity of individual elements and their compounds (Pb, Cd, Hg, As, Se, Cu and Mo).

Part 2 surveys the toxicity of some (non-heavy) metals such as the group 1 and rare earth elements, and diverges even further from the title of the publication by discussing *inter alia* the effects of fluorides in domestic and wild animals and by presenting a detailed account of the analytical techniques used for determining heavy metals, unfortunately with little emphasis on the type of sample encountered in toxicological work (or its preparation).

Individually the 31 chapters that comprise the two parts of this book are authoritative, but ten of them were first published between 1972 and 1976 in *Clinical Toxicology*. Moreover, there is some lack of editorial co-ordination between the chapters, and the use of a uniform type face does little to distinguish sections within a chapter. For these reasons, the two books may not be the first choice of either the general or the specialist reader, although they do present a considerable amount of information.

IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Vol. 20. **Some Halogenated Hydrocarbons.** International Agency for Research on Cancer, Lyon, 1979. pp. 609. Sw.fr. 60.00 (available in the UK through HMSO).

IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Chemicals and Industrial Processes Associated with Cancer in Humans. Vols 1 to 20, Supplement 1. International Agency for Research on Cancer, Lyon, 1979. pp. xi + 71. Sw.fr. 10.00 (available in the UK through HMSO).

The twentieth volume in the series of *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans* deals with 28 halogenated hydrocarbons (mainly organochlorines) which are used as pesticides, industrial solvents and intermediates or flame retardants. Two brominated compounds are covered—the flame retardant tris-(2,3-dibromopropyl) phosphate (Tris) and a fumigant and nematocide, 1,2-dibromo-3-chloropropane, which is also an impurity in Tris. Several of the monographs update and re-evaluate data on compounds assessed previously: carbon tetrachloride was first considered in vol. 1, chloroform also in vol. 1, hexachlorocyclohexane (technical HCH and lindane), heptachlor and heptachlor epoxide, methoxychlor, and mirex all in vol. 5, trichloroethylene in vol. 11 and 1,2-dibromo-3-chloropropane in vol. 15.

The monograph on chloroform is particularly timely in view of the current debate about the carcinogenicity of this solvent. The IARC concludes that "there is *sufficient evidence* that chloroform is carcinogenic in mice and rats" and recommends that, in the absence of adequate data in humans, chloroform should be regarded as if it presented a carcinogenic risk to man. The other compounds that it is concluded should be regarded for all practical purposes in the same way as chloroform are chlordecone, hexachlorobenzene, mirex, toxaphene, carbon tetrachloride, 1,2-dichloroethane, 1,2-dibromo-3-chloropropane and Tris. Available data were insufficient for any evaluation of dichlorvos, pentachlorophenol, dichloromethane (methylene chloride), 1,1,1-trichloroethane, hexachlorophene and 2,4,5- and 2,4,6-trichlorophenol, although the assessment of the latter was made before the results of an NCI study (*Federal Register* 1979, **44**, 23582) became available and these have been added by the IARC Secretariat in a footnote.

The first supplement to the IARC monographs arose out of deliberations of an *ad hoc* Working Group who met in January 1979 to evaluate the data on human and experimental animal carcinogenicity for 54 chemicals, groups of chemicals and industrial processes. Some epidemiological studies or case reports concerning these compounds had appeared in the literature, and each had previously been the subject of a monograph, although the Working Group's review included any new data that had become available since the previous publication. Separate assessments of the human and animal evidence were made and an overall evaluation of carcinogenicity for man was presented on the basis of the combined evidence. Eighteen of the chemicals and industrial processes were concluded to be human carcinogens. On the basis of human studies, a further 18 were assessed as probable human carcinogens. Within the latter group there was a high degree of evidence for six, and less evidence for the others. There were insufficient data on the remaining compounds for any evaluation to be made. The evidence supporting each assessment is given briefly in an Appendix to the Supplement.

The cumulative index to all 20 volumes appears in both publications and now covers well over 400 chemicals or groups of chemicals. In the Supplement there is also an index of chemicals by possible target organs in humans. This should also prove a useful reference source.

Environmental Carcinogenesis. Occurrence, Risk Evaluation and Mechanisms. Edited by P. Emmelot & E. Kriek. Elsevier/North-Holland Biomedical Press, Amsterdam, 1979. pp. viii + 401. Dfl. 120.00.

It is axiomatic to most people that the primary objective of cancer research, however esoteric it may appear, is to reduce the incidence of tumours in man. The means by which it has been thought possible to achieve this objective are legion, although since the discovery that cancer could be induced in animals by certain chemicals, much effort has been made to identify carcinogenic factors in the environment, and to reduce or prevent man's exposure to them. Environmental chemical carcinogenesis is the subject of this volume which contains the proceedings of a conference held in May 1979 in Amsterdam under the auspices of The Netherlands Cancer Institute and The Netherlands Cancer Society.

The opening chapter by J. Higginson, entitled "Environmental carcinogenesis: a global perspective", illustrates the difficulties encountered by epidemiologists in identifying those factors responsible for variations in the incidence of particular types of cancer in various regions of the world. From this overall view of the problem, the succeeding chapters become narrower in scope. A general account of carcinogen metabolism, by J. A. Miller and E. C. Miller, is followed by chapters on the occurrence of *N*-nitroso compounds in the environment and *in vivo* and on the biochemical mechanisms of action of these and several other classes of chemical carcinogens, including fungal toxins, polycyclic hydrocarbons and aromatic amines. The emphasis then changes from the biochemical to the pathological, with chapters on the carcinogenicity and anticarcinogenicity of certain metals, the interpretation of pesticide-related liver lesions, transplacental carcinogenesis, and cancer of the gastro-intestinal tract (with particular emphasis on dietary factors).

The remainder of the contributions range more widely and cover such topics as inhibitors of chemical carcinogenesis, interpretation of animal data, two-stage carcinogenesis, chromosomal aberrations, DNA repair, short-term tests for carcinogenicity, the multi-hit concept of tumorigenesis and the power and limitations of epidemiology. The emphasis of the conference on the identification and mechanisms of action of potential human carcinogens, with the object of either eliminating or reducing human exposure to

them, is to be applauded. However, it is evident from many of the contributions that current knowledge is insufficient to allow the introduction of effective preventive measures in most instances, particularly if Dr Higginson's estimate that at least 40% of male and 60% of female cancers are of unknown aetiology is accurate.

The topics covered in this book are sufficiently wide-ranging to be of interest both to the scientist active in this area of research and to others who feel the need for an authoritative review in order to keep informed of current trends.

Recent Results in Cancer Research. 66. Carcinogenic Hormones. Edited by C. H. Lingeman. Springer-Verlag, Berlin, 1979. pp. 196. $42.90.

This book, the sixty-sixth volume of a series sponsored by the Swiss League against Cancer, presents the papers delivered at an American symposium conducted under the auspices of the Interagency Collaborative Group on Environmental Carcinogenesis, a group sponsored by the National Cancer Institute. Most of the authors are from the Armed Forces Institute of Pathology, Washington, or the National Cancer Institute.

The book starts with an exhaustive review of the mechanisms whereby oestrogens and other hormones act as carcinogens or co-carcinogens in man and animals, with emphasis on their chemistry, biology and pharmacology, their hepatic metabolism and their relation to cancers at specific sites, as well as on external sources of hormones and similarly acting compounds. Endogenous hormones, as well as environmental factors, play a role in breast cancer; cancer of the endometrium and possibly of the breast is more frequent in women taking exogenous hormones. Attention is drawn to the overlapping of oestrogenicity and carcinogenicity of oestrogens and polycyclic hydrocarbons.

Then follows a chapter on the pathological effects of oral contraceptives on the cervix, endometrium, breast, ovary and vascular system and on blood coagulation and carbohydrate and lipid metabolism. Oral contraceptives may decrease the risk of benign breast disease; they may also interact with other risk factors in the development of breast cancer and they may increase the risk of cervical dysplasia. Sequential oral contraceptives (no longer used in the USA) may increase the risk of endometrial cancer. However, the evidence on all these counts is far from conclusive. The authors recommend the creation of a national registry which would record all women who die while taking oral contraceptives. A separate chapter on hepatic neoplasms associated with contraceptive and anabolic steroids emphasizes the differences between hepatocellular adenoma and focal nodular hyperplasia. While oral contraceptives apparently increase the relative risk of the former, they have not been associated with the development of the latter conditions. Hepatocellular carcinomas have been reported in patients treated for anaemia with anabolic androgenic steroids.

A section on mammary neoplasms in animals mentions the interesting fact that non-human primates rarely develop mammary tumours although they have a menstrual cycle comparable to that of women. The following chapter considers abnormalities of the genital tract following exposure to diethylstilboestrol *in utero*. Vaginal and cervical clear-cell adenocarcinomas have been found in a number of young women so exposed, and such exposure is almost invariably followed by the development of one or more of the benign conditions, vaginal adenosis, cervical erosion and transverse vaginal and clinical ridges. The book ends with a short chapter on cancer and other lesions in mice receiving oestrogens.

Unfortunately the date of the symposium is not given. Moreover there are a number of printing errors, and it would perhaps have been better for the chapter on mammary neoplasia in animals to have followed that on abnormalities of the genital tract in women exposed *in utero* to diethylstilboestrol. These are minor criticisms, however, of a book that provides a comprehensive review of the various aspects of the carcinogenic potential of hormones in humans and animals. The text is supported by a large number of references and is illustrated with good photomicrographs.

Readers concerned with this topic will also be interested in a recent publication from the International Agency for Research on Cancer, Lyon. Volume 21 in the series of IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans was published in 1979 (pp. 583; price Sw.fr. 60.00.), is the second in the series to deal with sex hormones (the first having appeared in 1974), and is a useful complement to the text discussed above.

Chemical Porphyria in Man. Edited by J.J.T.W.A. Strik & J. H. Koeman. Elsevier/North-Holland Biomedical Press, Amsterdam, 1979. pp. xiii + 236. Dfl. 85.00.

Porphyria is a metabolic disorder that leads to the production and excretion of excessive amounts of some porphyrins. The different forms of chronic hepatic porphyria can be recognized on the basis of the varying proportions of the different types of porphyrin characteristically excreted in the urine. The introductory section of the book named above is concerned with the occurrence of chronic hepatic porphyria in man. The primary enzymatic defect in porphyria involves the inhibition of uroporphyrinogen decarboxylase; the disturbance in enzyme function may be hereditary (with possible potentiation by exogenous agents such as alcohol or oestrogens) or it may be caused by a porphyrogenic agent. These agents are halogenated hydrocarbons and include compounds of industrial and agricultural importance as well as drugs.

The case histories described in the first of the three main sections of the book report the finding of chronic hepatic porphyria Type A or secondary coproporphyrinuria in Michigan farm families who ingested meat and dairy products contaminated with polybrominated biphenyl, in workers with vinyl chloride-induced liver damage, and in people exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin after the Seveso accident. One contribution discusses the inadequacy of the criteria used by the Seveso authorities to define

the groups deemed to be at risk after the latter incident (and therefore to be included to varying degrees in the health-control programme and epidemiological research) and describes the evaluation of a further criterion involving the recording of subjective symptoms. Analytical data derived from some of the Seveso exposure groups indicated that the urinary porphyrin pattern was a more sensitive indicator of chronic TCDD exposure than was total porphyrin excretion. Studies of urinary porphyrin patterns as well as total excretion gave negative results, however, in workers exposed to allyl chloride, epichlorohydrin, hexachlorocyclopentadiene and endrin and in 'Yusho' patients, who had consumed rice oil contaminated with polychlorinated biphenyls.

In spite of the book's title, the second main section deals with studies in experimental animals, reporting the induction of erythropoietic protoporphyria in mice and quail fed 3,5-diethoxycarbonyl-1,4-dihydrocollidine, griseofulvin or lead, and the toxicity of hexachlorobenzene in rats, with particular reference to the induction of hepatic porphyria. The final section describes different methods for the analysis of porphyrins and porphyrin precursors in the urine.

This book gives the impression of having resulted from a meeting, although this is not mentioned. A high incidence of typographical errors certainly suggests hasty production. The introduction on hepatic porphyria is a good one, but the section on human aspects suffers from some overlap and lack of organization and relatively little attention is paid to the skin lesions associated in man with exposure to porphyrogenic chemicals. Perhaps, in view of the animal studies included, a more appropriate title would be 'Porphyria Induced in Man and Animals by Halogenated Hydrocarbons'.

Advances in the Study of Birth Defects. Vol. 1. **Teratogenic Mechanisms.** Edited by T. V. N. Persaud. MTP Press Ltd, Lancaster, 1979. pp. ix + 240. £16.95.

Defects existing at birth, irrespective of their cause, create considerable social problems. It is estimated that as many as 50% of all pregnancies end in miscarriages and that, in the majority of cases, this is the result of faulty development. Major congenital malformations are found in at least 2% of all liveborn infants. The importance of these statistics is reflected in the considerable amount of work carried out in the field of experimental teratology over the past two decades. As a result of the wide range of information that has accumulated, it has become possible to obtain an insight into the causes, mechanisms and prevention of birth defects. This book brings together some of the more recent and important research findings relating to the mechanism and pathogenesis of abnormal development.

A distinguished panel of fourteen authors contributed to the book, which covers a wide range of areas from embryology and pathology to genetic studies. However, the authors have not presumed that the reader is familiar with these diverse disciplines and, where necessary, the basic concepts of a research area are explained. The experimental techniques used are briefly described and there is an adequate bibliography at the end of each chapter. The book serves, therefore, not only to update those already involved in the study of birth defects but also as a useful introduction to current research in experimental teratology.

Inevitably, cleft palate and limb malformations form a salient part of the book and it is evident that progress has been made not only in understanding the mechanism in the development of these abnormalities but also in the process of normal development. W. G. McBride's chapter on the pathogenesis of thalidomide embryopathy illustrates this point. The author's investigations into the effect of thalidomide on sensory nerves led to further understanding of normal limb bud development and it is suggested that peripheral nervous tissue has an inductive influence on this process. Elizabeth Deuchar's chapter on new approaches to the study of malformations resulting from maternal diabetes gives an excellent example of the application of the relatively new technique of embryo culture in maternal serum to the investigation of maternal influences during early organogenesis.

The above illustrate just a few of the advances in research that are included in this book, which to the credit of the authors and the editor is unified, positive and encouraging in its approach.

Progress in Experimental Tumor Research. Vol. 24. **The Syrian Hamster in Toxicology and Carcinogenesis Research.** Edited by F. Homburger. S. Karger AG, Basel, 1979. pp. x + 439. Sw.fr. 163.00.

There seems little doubt in the minds of the contributors to this volume that the Syrian hamster is a very worthy alternative to rats and mice, traditionally the animals widely used in toxicology and cancer research. Certainly the book provides an opportunity to appraise the merits of the hamster as a laboratory animal by bringing together both fundamental information on its biology and studies reviewing its utility as an experimental model.

The first section is devoted to studies using cultures of hamster cells and organs. The two major areas considered are *in vitro* studies of neoplastic transformation, for which hamster embryo cells are particularly suitable, and the use of hamster tracheal organ cultures for studies of respiratory irritants and carcinogens. Perhaps one of the most useful sections of the book is the second. This comprises seven papers and provides extensive data on the reproductive performance, longevity, nutrition and susceptibility to disease of the major strains of Syrian hamster.

The remaining seven sections contain 23 papers on various aspects of carcinogenesis research and two papers under the heading of toxicology. There is considerable repetition in the sections on carcinogenesis but a number of important points emerge. For example, several authors point out that hamsters have a low incidence of spontaneous tumours compared with rats and mice but that they are nevertheless highly sensitive to chemical carcinogens. These are, of course, very desirable characteristics for long-term carcinogenicity testing. The particular suitability of hamsters for studies of organ-specific carcinogenesis is also emphasized. Of the common laboratory species, the hamster is the only one in which pancreatic tumours can readily be induced, whilst the hamster

cheek pouch provides a unique model for the study of oral carcinogenesis. The hamster's respiratory tract resembles that of man more closely than is the case with many other species and it is particularly sensitive to tumour induction by cigarette smoke. Because of this, hamsters have been used widely in studies on respiratory carcinogenesis and eight chapters are devoted to this topic.

This book is of value on two counts—it highlights those research areas in which hamsters are particularly suitable as experimental animals and it collates much basic biological data essential to any laboratory wishing to use this species. It is marred somewhat by an irritating amount of repetition (like so many other symposium proceedings) and by several omissions in cross-referencing. With more editing, it would have been shorter, more digestible and no less useful.

Banbury Report. 3. A Safe Cigarette? Edited by G. B. Gori & F. G. Bock. Cold Spring Harbor Laboratory, New York, 1980. pp. xi + 364. $54.00. ($45 within USA).

It is not uncommon nowadays for scientific campaigns to be established with an evangelical fervour akin to fanaticism, and too often, when this occurs, the scientific content is quickly devalued. It is regrettably true that such campaigns arise more commonly where human health is an issue, and particularly if the crusaders believe that the object of their attack is generating large profits for someone else. The chemical industry is one group that, in its many guises, has been subjected to such onslaughts.

Another is the tobacco industry, long admonished for making stupendous profits by offering for sale an addictive weed that is thought to cause substantial morbidity among its users. The anti-smoking lobby has been very active and vociferous in pursuit of its goal, and has skilfully managed to nullify any concept but an outright prohibition of smoking as a way of tackling the problem.

Science, however, is more resilient, and whatever problems arise there will be people who try to understand and resolve them (especially, let it be said, if substantial funds are available to finance the attempt). So the concept of a safer cigarette has been in existence for several years and has generated a great deal of excellent research related to the motivation of smokers, the toxicology of cigarette smoke, the methodology of investigation and the opportunities for improving the product, either by modifying the raw material or by modifying the results of pyrolysis. This book is an excellent account of the essential problems associated with smoking, of the biological components of that problem and of the possible procedures in its resolution. Its presentation is such that anyone new to the scientific basis of the smoking problem can readily acquire very up-to-date information, and it is particularly admirable in its presentation of a highly emotive topic in a way that neither belittles the human dimension nor betrays the investigative science. In a few words, it is a responsible account of a treacherous topic and is highly recommended.

Side Effects of Drugs Annual 4. A Worldwide Yearly Survey of New Data and Trends. Edited by M. N. G. Dukes. Excerpta Medica, Amsterdam, 1980. pp. xviii + 376. Dfl. 130.00.

Meyler's Side Effects of Drugs. An Encyclopaedia of Adverse Reactions and Interactions. 9th Ed. Edited by M. N. G. Dukes. Excerpta Medica, Amsterdam, 1980. pp. xx + 859. Dfl. 250.00.

Since our last review relating to the publications that have grown out of the original 'Meyler' (*Cited in F.C.T.* 1980, **18,** 89), two further volumes have appeared. The first, the fourth of the annual publications issued to complement the eighth edition of *Meyler's Side Effects of Drugs*, follows the general pattern of the earlier 'Annuals' (*ibid* 1978, **16,** 488) and reviews significant new evidence relating to adverse reactions to drugs. The review covers material published between 1 August 1978 and 21 July 1979, with the inclusion where possible of more recent papers. As before, the references are coded to indicate the type of paper or extent of the information provided. The drugs on which significant information has appeared are grouped in the text according to function, and many of the individual sections include, in addition to specific reports, critical reviews of conflicting evidence or changing views on specific topics. The cumulative indexes provided in Annual 3 (1979) have not been continued into this volume, but on the credit side, a fourth index, listing interactions between drugs, has been added to the previously available three, based on drug names, synonyms and side effects.

The ninth edition of this encyclopaedia of adverse reactions and interactions appeared more or less when anticipated. This is not merely an updated eighth edition (*ibid* 1977, **15,** 241), but an entirely replanned and rewritten text, reflecting the radical changes that have taken place recently in adverse-reaction monitoring.

The book is arranged systematically; homogeneous groups of drugs are dealt with together and the extent to which an individual member of a particular group differs from the general pattern is indicated. Each of the 51 monographs dealing with a group of drugs is presented in a standard format. The "adverse reaction pattern", shown in heavy type on a grey background, summarizes the principal data on general and toxic reactions, hypersensitivity reactions and tumour-inducing effects and is accompanied by an account of effects on organs and systems, risk situations, withdrawal effects, second-generation effects, overdosage, interactions and interference with diagnostic routines, where such information is available. As in the 1980 Annual, a fourth index, based on interacting drugs and the nature of the interaction, has been included, and references are coded according to type of publication and content. American spelling has been used in the indexes because they will eventually be stored in the Excerpta Medica database, but British spelling has been used in the text.

Because of its clarity and orderliness, its wide scope and the detailed subdivision of the information reported, this book must be regarded as one of the foremost reference books in the field.

Carcinogens and Related Substances. Analytical Chemistry for Toxicological Research. By M. C. Bowman. Marcel Dekker, Inc., New York, 1979. pp. vii + 316. Sw.fr. 78.00.

Three aspects of the role of analytical chemistry in toxicological testing are discussed in this account of current techniques written by the Director of Chemistry at the FDA's National Center for Toxicological Research (NCTR). These are: the quality control of animal feed (prior to mixing with the test substance) and of water, bedding materials and feeder boxes for essential and/or deleterious substances; the determination of the purity, stability, and concentration of test substances before and after mixing with the feed; the extension of these analytical methods to environmental and health considerations, e.g. the analysis of human urine and waste water for possible carcinogens.

The author has restricted his choice of test substances to some of those encountered at the NCTR over the last 5 years: 2-acetylaminofluorene, benzidine and analogues, 2-naphthylamine and analogues, diethylstilboestrol, oestradiol, zearalenone and zearalanol, 2,4,5-T, rotenone, 4-ethylsulphonylnaphthalene-1-sulphonamide and sodium phenobarbital. Each of these substances is dealt with separately in the lengthy chapter on the analysis of test compounds which makes up the bulk of the book. Literature on methods of analysis is briefly reviewed, and the analytical techniques used by the author and his coworkers are described in considerable detail. Each section of the chapter ends with a discussion of the results that have been obtained using these methods. The Ames test is also dealt with in this chapter.

Two minor criticisms of the text could be made. First, some of the tables included contribute little to the description and discussion of the analytical methods. Secondly, it is a pity that benzene is used as the extraction solvent in so many of the procedures.

This book, particularly if used in conjunction with the IARC series of methods of analysis for environmental carcinogens (*Cited in F.C.T.* 1979, **17**, 535; *ibid* 1980, **18**, 307) should provide valuable background reading for analytical chemists engaged in toxicological research.

BOOKS RECEIVED FOR REVIEW

Banbury Report. 5. Ethylene Dichloride: A Potential Health Risk? Edited by B. Ames, P. Infante & R. Reitz. Cold Spring Harbor Laboratory, New York, 1980. pp. xi + 350. $45.00.

Metal Contamination of Food. By C. Reilly. Applied Science Publishers Ltd, London, 1980. pp. xvi + 235. £17.00.

Antimicrobial Food Additives. Characteristics · Uses · Effects. By E. Lueck. Springer-Verlag, Berlin, 1980. pp. xviii + 280. DM 66.00.

Pesticide Residues in Food: 1979 Evaluations. The Monographs. Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Expert Group on Pesticide Residues, Geneva 3–12 December 1979. FAO Plant Prodn and Protection Paper 20 Sup. FAO, Rome, 1980. pp. ix + 560 (available in the UK through HMSO).

Environmental Health Criteria. 15. Tin and Organotin Compounds: A Preliminary Review. Published under the joint sponsorship of the United Nations Environment Programme and the World Health Organization. WHO, Geneva, 1980. pp. 109. Sw.fr. 7.00 (available in the UK through HMSO).

Handbook of Industrial Safety and Health. Trade and Technical Press Ltd, Morden, 1980. pp. vi + 576. £45.00.

Progress in Drug Metabolism. Vol. 4. Edited by J. W. Bridges & L. F. Chasseaud. John Wiley & Sons Ltd., Chichester, 1980. pp. ix + 335. £25.00.

Encyclopedia of Common Natural Ingredients Used in Food, Drugs, and Cosmetics. By A. Y. Leung. John Wiley & Sons, Ltd. Chichester, 1980. pp. xvi + 409. £25.60.

Residue Reviews. Vol. 73. **Residues of Pesticides and Other Contaminants in the Total Environment.** Edited by F. A. Gunther. Springer-Verlag, New York, 1980. pp. viii + 130. $26.00.

IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Suppl. 2. Long-term and Short-term Screening Assays for Carcinogens: A Critical Appraisal. International Agency for Research on Cancer, Lyon, 1980. pp. 426. Sw.fr. 40.00 (available in the UK from HMSO).

Toxic Constituents of Plant Foodstuffs. 2nd Ed. Edited by I. E. Liener. Academic Press, Inc., (London) Ltd, London, 1980. pp. xiv + 502. £27.80.

Persistent Poisons. Chemical Pollutants in the Environment. By M.-J. Schneider. The New York Academy of Sciences, New York, 1979. pp. 67. $4.00.

The Asbestos Hazard. By P. Brodeur. The New York Academy of Sciences, New York, 1980. pp. 93. $4.00.

Hazards to Your Health. The Problem of Environmental Disease. By J. Gorman. The New York Academy of Sciences, New York, 1979. pp. 70. $4.00.

Fd Cosmet. Toxicol. Vol. 19. pp. 387 to 392. 1981
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Pergamon Press Ltd

Information Section

ARTICLES OF GENERAL INTEREST

DIOXIN: INDUCTION OF XENOBIOTIC METABOLISM

The compound 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a nearly planar tricyclic aromatic molecule with twofold symmetry, is perhaps the most potent toxin of low molecular weight known to man. We have recently reviewed some of the various incidences of human exposure to TCDD together with animal studies on its carcinogenicity, teratogenicity and reproductive effects (*Cited in F.C.T.* 1980, **18,** 541 & 739; *ibid* 1981, **19,** 123). In this article, the effects of TCDD on xenobiotic-metabolizing enzymes are discussed.

The administration of single doses of TCDD to rats or mice produces a marked induction of cytochrome *P*-450-dependent mixed-function oxidase (m.f.o.) enzymes in the liver and in other tissues (Poland & Glover, *Molec. Pharmacol.* 1974, **10,** 349; *idem, ibid* 1975, **11,** 389; Hook *et al. Biochem. Pharmac* 1975, **24,** 335; *idem, Chemico-Biol. Interactions* 1975, **10,** 199). In addition to inducing Phase I xenobiotic-metabolizing enzymes, i.e. m.f.o. enzyme activities and levels of cytochrome *P*-450, TCDD also stimulates certain other enzymes involved in xenobiotic metabolism, including uridine diphosphate-glucuronosyltransferase (Lucier *et al. Biochem. Pharmac.* 1975, **24,** 325), glutathione *S*-transferase B (ligandin; Kirsch *et al. J. clin. Invest.* 1975, **55,** 1009) and aldehyde dehydrogenase (Roper *et al. Fedn Proc. Fedn Am. Socs exp. Biol.* 1976, **35,** 282). The potency of TCDD as an inducer of m.f.o. enzyme activities in experimental animals is indicated both by the very low doses required and by the persistence of the enzyme induction. For example, as an inducer of aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase (AHH) in the rat, TCDD is some 30,000 times more potent than 20-methylcholanthrene (Poland & Glover, *Molec. Pharmacol.* 1974, **10,** 349). The latter compound is the archetype of the polycyclic hydrocarbon (PCH) class of m.f.o. enzyme inducers (Conney, *Pharmac. Rev.* 1967, **19,** 317). Indeed, the dose of TCDD required for half-maximal induction of AHH in the rat is only 0·85 nmol/kg (0·27 μg/kg) as a single intraperitoneal (ip) dose, and in parallel studies on the induction of this enzyme in chicken eggs a doubling of activity could be obtained by a dose of TCDD of only 1·55 pmol/egg (0·5 ng; Poland & Glover, *Molec. Pharmacol.* 1973, **9,** 736; *idem, ibid* 1974, **10,** 349). In other studies in the rat, induction of AHH has been observed after single oral doses of TCDD as low as 2 ng/kg, and from radiotracer experiments it was estimated that only 65 molecules of TCDD per hepatocyte are necessary to initiate a significant increase in enzyme activity (Kitchin & Woods, *Toxic. appl. Pharmac.* 1979, **47,** 537).

Poland & Glover (*Molec. Pharmacol.* 1974, **10,** 349) found that induction of AHH by a single ip dose of 0·31 nmol TCDD/kg persisted for more than 35 days, whereas activity induced by a single ip dose of 0·75 μmol 20-methylcholanthrene/kg returned to control levels after only 8 days. Furthermore Hook *et al.* (*Biochem. Pharmac.* 1975, **24,** 335) observed that the inductive effect on rat hepatic m.f.o. enzyme activities persisted for up to 73 days following a single oral dose of 78 nmol TCDD/kg.

Inducers of hepatic m.f.o. enzyme activities belong to two main classes, namely those of the drug type as exemplified by sodium phenobarbital and those of the PCH type such as 20-methylcholanthrene (Conney, *Pharmac. Rev.* **19,** 317). TCDD is considered to be a very potent member of the latter class of enzyme inducers because it markedly induces AHH but does not affect certain other m.f.o. enzymes such as aminopyrine *N*-demethylase or the activity of the microsomal electron transport chain component NADPH-cytochrome *c* reductase. Like other PCH-type enzyme inducers, TCDD results in marked changes in the nature of the various microsomal cytochrome *P*-450 haemoprotein types, as indicated by changes in the ligand binding of either carbon monoxide or ethyl isocyanide with reduced hepatic microsomal preparations (Poland & Glover, *Molec. Pharmacol.* 1974, **10,** 394). In addition to spectral changes, electrophoretic studies of microsomal proteins also indicate that TCDD, like other PCH-type inducers, increases the synthesis of microsomal haemoproteins known as cytochrome *P*-448 (also termed cytochrome P_1-450) rather than the forms designated cytochrome *P*-450 induced by phenobarbital and related compounds (Haugan *et al. J. biol. Chem.* 1976, **251,** 1817; Johnson, *ibid* 1980, **255,** 304). Furthermore, TCDD appears to induce a different predominant haemoprotein type in foetal or neonatal as against adult rats, mice or rabbits (Guenthner & Nebert, *Eur. J. Biochem.* 1978, **91,** 449; Norman *et al. J. biol. Chem.* 1978, **253,** 8640). This differential stimulation of the cytochrome *P*-450 forms is particularly significant, since cytochrome *P*-448 inducers may modify not only the rate of xenobiotic metabolism but also the nature of the metabolites formed compared to the situation in either control or phenobarbital-induced animals (Nebert & Felton, *Fedn Proc. Fedn Am. Socs exp. Biol.* 1976, **35,** 1133). For example, TCDD pretreatment of the animals enhances the formation of toxic metabolites of benzo[*a*]pyrene by rat-tissue preparations (Uotila *et al. Toxic. appl. Pharmac.* 1978, **46, 671**) and also stimulates the mutagenicity of this and other PCHs in the Ames test using activation by various mouse-tissue preparations (DiGiovanni *et al. ibid* 1979, **50,** 229).

The potency and persistence of TCDD as an enzyme inducer in experimental animals is due to a number of factors, including its chemical structure, its

high affinity for binding to a specific protein in the cytosol fraction of the liver and its metabolism and pharmacokinetics. With regard to structure, the presence of a chlorine atom in at least three of the four lateral ring positions (carbons 2, 3, 7 and 8) with at least one free ring position elsewhere (carbons 1, 4, 6 and 9) is required for induction of aryl hydrocarbon hydroxylase (AHH). Thus dibenzo-*p*-dioxin and 1,2,3,4,6,7,8,9-octachlorodibenzo-*p*-dioxin (i.e. a fully chlorinated structure) are both inactive as enzyme inducers (Poland & Kende, *Fedn Proc. Fedn Am. Socs exp. Biol.* 1976, **35,** 2404). Bradlaw *et al.* (*Fd Cosmet. Toxicol.* 1980, **18,** 627) have tested 23 halogenated dibenzo-*p*-dioxin analogues for their ability to induce AHH in rat hepatoma cell cultures. Their results were in complete agreement with those of Poland & Kende (*loc. cit.*) except that they did find minimal induction with 1,2,3,4,6,7,9-hepta- and 1,2,3,4,6,7,8,9-octachlorodibenzo-*p*-dioxin (99·88% pure). However, these compounds were the least active of those tested (except for those that were completely inactive).

A number of studies have demonstrated the existence of a component of the cytosol fraction of rodent liver which has a high affinity for TCDD and appears to be the receptor for initiation of enzyme induction by TCDD and other polycyclic hydrocarbon(PCH)-type enzyme inducers. The affinity of TCDD and other dibenzo-*p*-dioxins for this cytosolic receptor correlates with their potency for induction of AHH and whilst PCBs compete with TCDD for binding to the cytosol receptor (of mice), drug-type enzyme inducers (e.g. phenobarbitone and pregnenolone-16α-carbonitrile) and steroid hormones have no specific binding (Poland *et al. J. biol. Chem.* 1976, **251,** 4936). Furthermore, age-related changes in the concentration of this cytosolic receptor have been reported to correlate to some extent with variations in the induction of AHH in the rat (Carlstedt-Duke *et al. Cancer Res.* 1979, **39,** 4653). The studies of Nebert and coworkers (Nebert & Gielen, *Fedn Proc. Fedn Am. Socs exp. Biol.* 1972, **31,** 1315) have classified various strains of mice as either genetically 'responsive' or 'nonresponsive' to induction of AHH by PCH-type inducers such as 20-methylcholanthrene. However, TCDD induces this enzyme in either genetically 'responsive' or 'nonresponsive' strains (Poland *et al. J. biol. Chem.* 1974, **249,** 5599), although higher doses are required for half-maximal induction of the enzyme in 'nonresponsive' strains (Poland & Glover, *Molec. Pharmacol.* 1975, **11,** 389). The difference in AHH inducibility between the two types of mice may well be due to much lower levels of the cytosolic receptor in the livers of 'nonresponsive' mice and this would account for the failure of PCH enzyme inducers, which are much less potent than TCDD, to induce in these strains (Poland *et al. J. biol. Chem.* 1976, **251,** 4936).

In addition to its high affinity for the hepatic cytosol receptor protein, TCDD accumulates in the rodent liver and is eliminated very slowly from the animal, with a half-life in the rat in the region of 16–31 days. Whilst the metabolism of TCDD awaits elucidation, it appears to be an extremely slow process, only small amounts of radioactivity being excreted in the urine of animals treated with the radiolabelled compound (Allen *et al. Fd Cosmet. Toxicol.* 1975, **13,** 501; Rose *et al. Toxic. appl. Pharmac.* 1976, **36,** 209). The detection of urinary radioactive TCDD residues presumably indicates that metabolism does occur, as TCDD itself is very insoluble in water (Poland & Kende, *Fedn Proc. Fedn Am. Socs exp. Biol.* 1976, **35,** 2404). The low rate of TCDD metabolism also indicates that it is the parent compound and not a metabolite that initiates the induction process. In the case of the rat, TCDD would appear to be the active toxin since its acute toxicity is inversely related to the activites of cytochrome *P*-450 dependent mixed-function oxidase (m.f.o.) enzymes, suggesting that TCDD is metabolized by the m.f.o. enzymes to a less toxic form (Beatty *et al. Toxic. appl. Pharmac.* 1978, **45,** 513).

Whilst TCDD is a known enzyme inducer in many animal species, there does not appear to be any clear evidence that TCDD is or is not an inducer of xenobiotic metabolism in man apart from an observed elevation of serum γ-glutamyltranspeptidase in about 10% of the Seveso subjects exposed to TCDD (Homberger *et al. Ann. occup. Hyg.* 1979, **22,** 327). Although increased serum γ-glutamyltranspeptidase may be associated with induction of hepatic xenobiotic metabolism in man (Whitfield *et al. Br. med. J.* 1973, **1,** 316) it is also elevated in various liver disorders, including alcohol-induced liver damage, and is not therefore totally reliable as an indicator of m.f.o. enzyme induction by TCDD. Since other PCH-type inducers such as those present in certain foods or cigarette smoke induce both hepatic and extra hepatic (e.g. intestinal and placental) xenobiotic metabolism in man (Conney *et al. Fedn Proc. Fedn Am. Socs exp. Biol.* 1977, **36,** 1647; Fraser *et al. Br. J. clin. Pharmac.* 1979, **7,** 237), TCDD may be expected to have a similar effect. Enzyme induction should certainly be considered as a possible consequence of human exposure to TCDD since enzyme induction in animals is produced at dosages either similar to or lower than those needed for teratogenicity and reproductive effects and possibly carcinogenicity (*Cited in F.C.T.* 1980, **18,** 739; *ibid* 1981, **19,** 123). The average human exposure to TCDD in Zone A (the highest exposure zone) of Seveso has been calculated to be 7·49 mg per person (Reggiani, *Arch. Tox.* 1978 **40,** 161), and in a female subject who died 7 months after TCDD exposure, tissue analysis revealed 40 μg of TCDD (equivalent to about 0·57 μg/kg body weight) present in the body at the time of death (Reggiani, *Arch. Tox.* 1979, Suppl. 2, 291).

In recent years attention has been drawn to the adverse effects of induction of xenobiotic-metabolizing enzymes in man with respect to the metabolism of endogenous substrates of these enzymes. For example, induction by anticonvulsant drugs such as phenobarbitone and diphenylhydantoin has been implicated in derangements in the metabolism of corticosteroids, vitamin D_3 and other compounds (Brooks *et al. New Engl. J. Med.* 1972, **286,** 1125; Stamp *et al. Br. med. J.* 1972, **4,** 9). Whilst the effects of TCDD on the metabolism of endogenous molecules in the rat have not been studied extensively, TCDD appeared not to stimulate the m.f.o. metabolism of testosterone (Hook *et al. Biochem. Pharmac.* 1975, **24,** 335) or the conjugation of testosterone or oestrone (Lucier *et al. Biochem. Pharmac.* 1975, **24,** 325). Animal studies have, how-

ever, demonstrated the transplacental passage of TCDD leading to subsequent enzyme induction in the liver of the foetus (Berry *et al. Toxic. appl. Pharmac.* 1976, **36,** 569; Lucier *et al. Chemico-Biol. Interactions* 1975, **11,** 15), which could lead to induction of the metabolism of endogenous substrates.

It is difficult, however, to extrapolate to man the rodent enzyme induction/toxicity data as TCDD appears to exhibit a number of species differences. For example, TCDD produces marked liver damage in the rat and large amounts of TCDD are retained in the liver, whereas in the monkey little evidence of morphological changes and lower concentrations of TCDD residues are found (Allen *et al. loc. cit.*; Jones & Butler, *J. Path.* 1974, **112,** 93; McConnell *et al. Toxic. appl. Pharmac.* 1978, **43,** 175; Seefeld *et al. Toxicology* 1979, **14,** 263; Van Miller *et al. Fd Cosmet. Toxicol.* 1976, **14,** 31). Whilst laboratory and industrial workers exposed to TCDD suffer from chloracne-type skin lesions, also found in monkeys (McConnell *et al. Toxic. appl. Pharmac.* 1978, **43,** 175) but not in rats (Van Miller *et al. Fd Cosmet. Toxicol.* 1976, **14,** 31), no evidence of marked liver damage has been obtained (May, *Br. J. ind. Med.* 1973, **30,** 276; Oliver, *ibid* 1975, **32,** 49). In hamsters TCDD does not produce liver damage, and the fact that it seems to be more rapidly metabolized in this species may contribute to its relatively low acute toxicity in the hamster (Olson *et al. Toxic. appl. Pharmac.* 1980, **55,** 67, & **56,** 78). Thymic atrophy, however, is a consistent finding in all TCDD-exposed species examined (Neal *et al. Ann. N.Y. Acad. Sci.* 1979, **320,** 204).

The elegant studies of Poland and coworkers have demonstrated good correlations between chlorinated dibenzo-*p*-dioxin structure, induction of AHH, binding to the cytosolic receptor and toxic responses, including thymic atrophy and possibly teratogenicity (Poland & Kende, *Fedn Proc. Fedn Am. Socs exp. Biol.* 1976, **35,** 2404; Poland *et al. J. biol. Chem.* 1976, **251,** 4936; Poland *et al. Ann. N.Y. Acad. Sci.* 1979, **320,** 214; Poland & Glover, *Molec. Pharmacol.* 1980, **17,** 86). Indeed, this group suggests that the correlation between enzyme induction and the toxic effects of TCDD and related compounds is more than fortuitous and that the initial event in the toxic response produced is the stereospecific recognition and binding of the compound to the cytosolic receptor species (Poland *et al. Ann. N.Y. Acad. Sci.* 1979, **320,** 214). Thus genetically 'responsive' mice, which have high levels of the hepatic cytosolic receptor, are more susceptible both to thymic atrophy and to teratogenicity produced by TCDD than genetically 'nonresponsive' strains (Poland & Glover, *Molec. Pharmacol.* 1980, **17,** 86).

The possibility of induction of xenobiotic-metabolizing enzymes in humans exposed to TCDD needs further investigation. The existence (or otherwise) of a relationship between the inductive and the toxic effects of TCDD in man also merits study.

[B. G. Lake—BIBRA]

DEALING WITH THEOPHYLLINE

Theophylline (1,3-dimethylxanthine) is isomeric with theobromine (3,7-dimethylxanthine) and closely related to caffeine (1,3,7-trimethylxanthine). All three compounds occur in small amounts in tea, but theophylline is not found in coffee, or in the cacao bean, in which theobromine constitutes the principal alkaloid at levels of up to about 3%. Theophylline is a potent central nervous system and cardiovascular stimulant, its diuretic action is more powerful than that of either theobromine or caffeine, and its ability to relax the bronchial smooth muscles has led to its use in the treatment of asthma and, increasingly, in the management of neonatal apnoea. Like other methylxanthines, it has been shown to induce hepatic microsomal enzymes in rats (*Cited in F.C.T.* 1976, **14,** 513) and it has demonstrated some rather puzzling pharmacokinetic characteristics when administered therapeutically.

Bell *et al.* (*Ann. Allergy* 1980, **44,** 67), concerned with the difficulties of establishing effective theophylline therapy in asthma, demonstrated significant differences in theophylline pharmacokinetics between two groups of asthmatic children matched by age and body weight. Twelve children in a Denver hospital for moderately severe, chronic asthmatics and 12 from an out-patients department in Philadelphia were given oxtriphylline (choline theophyllinate) syrup every 6 hr for 3 days, in oral doses of 8 mg/kg for the former group and 6 mg/kg for the latter. Serum theophylline concentrations were measured after doses 1, 5 and 9. Absorption was more rapid in the Philadelphia group, but the Denver group showed greater total body clearance and apparent volume of distribution (a measure of the amount in intra- and extracellular water, and therefore inversely of plasma-protein and tissue binding).

Since the groups differed in many respects, the factors influencing these parameters could not be identified readily. Earlier work (Ellis *et al. Pediatrics, Springfield* 1976, **58,** 542) did not suggest a difference in the sex ratio as a likely influence. While the authors have some evidence that theophylline is not readily distributed into adipose tissue, no correlation could be demonstrated in this study between the pharmacokinetic differences observed and the inter-group differences in lean body mass, and therefore degree of obesity. A low-fat diet given to the Philadelphia children during the treatment period may have been responsible for their more rapid absorption of theophylline, and possible differences in dietary protein-to-carbohydrate ratios may also have been reflected in the divergent findings. The study provided no evidence of a dose-dependent kinetic pattern, since the higher clearance rate and shorter elimination half-life in the higher dose group did not indicate any saturation of metabolic enzymes. Other unmatched factors that

may have been involved in the variation in drug disposition between the two groups included concurrent corticosteroid therapy, physical stress, cardiac output, and liver and renal disease, as well as a difference in altitude of some 5000 ft between the locations of the two groups.

Whatever the factors most responsible for the differences between these two groups, it is evident that very variable responses to a given dose of theophylline must be anticipated in children. Nielsen-Kudsk *et al.* (*Acta pharmac. tox.* 1980, **46**, 205) found variations in response also in six adult hospital patients who showed normal renal, hepatic and pulmonary function but required theophylline therapy for central nervous system dysfunction of ischaemic origin. Following a single oral (1·8–4·2 mg/kg) or intravenous (iv; 3·2–4·2 mg/kg) dose of theophylline, the half-life in these patients ranged from 3 to 16 hr, with plasma clearance values of 16·5–115 ml/kg/hr and apparent volumes of distribution during the elimination phase of 0·394–0·616 litre/kg. Maximum serum levels of theophylline were within or very close to the 5–10 μg/ml range and the only adverse effect reported was a slight and transient nausea after the iv dose in two subjects whose maximum serum levels were 3·1 and 4·9 μg/ml. Serum levels in the 24 children in the study by Bell *et al.* (*loc. cit.*) ranged from 5 to 33 μg/ml, but again no serious toxicity was encountered, the only adverse effects being transient nausea and headache in two patients with serum levels of 18 and 23 μg/ml.

The elimination rates demonstrated by Nielsen-Kudsk *et al.* (*loc. cit.*) were little affected by the route of administration but there was wide variation between individuals, not only in the disposition rate and biological half-life but, in the oral experiments, in the rate of absorption. The drug underwent two-compartment distribution in five patients after iv infusion but in only one after oral dosage. Nielsen-Kudsk *et al.* attribute the wide range of half-lives and clearance rates in these patients to variations in the hepatic degradation of the drug, since only a small proportion of administered theophylline is excreted unchanged. They also comment briefly on conflicting reports regarding the possible dose-dependency of theophylline clearance.

These aspects have been considered in more detail by Lesko (*Clin. Pharmacokinet.* 1979, **4**, 449), who comes down in favour of parallel Michaelis-Menten and first-order kinetics for theophylline elimination. He reports growing evidence that increasing doses may overload the elimination pathway, decreasing the elimination rate and leading to a disproportionate increase in the steady-state serum concentration. Since renal clearance of unchanged theophylline accounts for only about 8% of the plasma clearance and apparently proceeds by glomerular filtration, which is a passive diffusion process and therefore not saturable, this kinetic pattern is probably due to a change in hepatic clearance associated with complete or partial saturation of a metabolic pathway. Metabolism of the drug involves the microsomal mixed-function oxidase system and the main urinary metabolites have been identified as 3-methylxanthine (36% of total urinary elimination), 1,3-dimethyluric acid (39%) and 1-methyluric acid (17%) in man (Jenne *et al. Clin. Pharmac. Ther.* 1976, **19**, 375). Evidence so far available suggests 1-demethylation as a potentially saturable pathway.

In view of the structural similarity of theophylline, theobromine and caffeine, some competition between them for metabolic pathways must be a strong possibility. Both caffeine and theobromine have been found to inhibit the metabolism of theophylline by rat-liver slices (Lohmann & Miech, *J. Pharmac. exp. Ther.* 1976, **196**, 213) and Caldwell *et al.* (*Br. J. clin. Pharmac.* 1977, **4**, 637P) showed that the elimination half-life of theophylline in volunteers given a single iv dose was reduced from about 10 to 7 hr by consumption of a methylxanthine-restricted diet. There are at least five hepatic metabolites of caffeine, and two of these are also products of theophylline metabolism. It appears, moreover, that theophylline itself may be produced metabolically in adults by demethylation of caffeine (Sved *et al. Res. Commun. chem. Path. Pharmac.* 1976, **13**, 185). In premature newborn babies, however, the reverse reaction seems to occur, theophylline administration having been found to lead to increased plasma concentrations of caffeine (Bory *et al. Lancet* 1978, **I**, 1204). Aranda *et al.* (*Science, N.Y.* 1979, **206**, 1319) have shown that the human foetal liver can methylate theophylline as early as wk 12 of gestation. In liver explants from foetuses aged 12–20 wk, incubation with theophylline yielded caffeine; methylation was the predominant metabolic pathway, although some 1,3-dimethyluric acid and 3-methylxanthine were produced. After a lag phase of 4–8 hr, the rate of caffeine production remained linear until about 52 hr, with a mean production of 1·25 nmol caffeine/mg protein each 24 hr. Theophylline methyltransferase activity in the foetal liver was 0·23–2·36 nmol/mg protein/24 hr and showed some increase with foetal age. It is possible, therefore, that theophylline acquired transplacentally may contribute to the caffeine load of a foetus and neonate, and that pharmacological effects in neonates treated with theophylline may, in part, be attributable to caffeine of metabolic origin.

[P. Cooper—BIBRA]

MUTAGEN DETECTION IN THEORY AND PRACTICE

Chemical attack on DNA is now regarded as one of the most dangerous and insidious properties of toxic substances. Because DNA carries the basic genetic information controlling not only the activities of every cell but also the characteristics of the whole organism, chemical alteration of DNA can have drastic biological consequences. Many agents that react with DNA have already been identified, and their toxic effects

include carcinogenicity, teratogenicity and the induction of heritable mutations. The classical methods for detecting a chemical's ability to induce these effects involve the use of experimental animals and are fraught with difficulty. This is due, at least in the case of the induction of cancer and of heritable mutations, to the long period that must elapse before the effect is seen and also to the need, on statistical grounds, to use large numbers of animals.

For some years, a search has been going on for quicker, more sensitive and cheaper methods of detecting genetic toxicity. Judging from the resulting plethora of testing methods, one might conclude that this endeavour had met with unqualified success, and from a scientific point of view this may well be true. In many respects, however, the advent of 'short-term mutagenicity tests' has caused more headaches than it has cured. For those who have not made a continuous effort to keep abreast of developments, the field of genetic toxicology can appear to be a chaos of incomprehensible methodology, contributing little of value to toxicological decision making. Indeed, even those who are *au fait* with the basic technology of mutagenicity testing are faced with formidable problems in translating the results obtained by the new methods into practical decisions about the future of specific chemicals.

The successful development of rapid sensitive tests for genetic toxicity has depended on two strategies. In some cases, instead of using the relevant toxicological endpoint (such as cancer), the test method will use an effect that is thought to be a closely linked side-effect or an early stage in the induction of the important lesion. Thus, detection of chromosome damage in the bone-marrow cells of a mouse is used as an indicator of genetic damage that might be associated with the much later expression of cancer or heritable genetic disease. Damaged chromosomes can be detected within days of treating the animal with a mutagen, and often very many cells in each sample are affected. Other test systems, probably the majority, do away with mammals altogether and use organisms such as yeasts, fungi or bacteria (as well as tissue cells in culture), all of which multiply very rapidly and are available in vast numbers. Mutations can be detected in such populations within a short time, even if they occur at very low frequencies.

The advantages of these test systems are gains only at the cost of a loss of direct relevance to toxicological assessment. The results of any short-term test are only suggestive. It is reasonable for example to question the relevance of an observation of induced chromosome damage in mice or of mutation in bacteria to the possible effects of a chemical on human health. In an attempt to understand this controversial but undoubtedly important field, many people will turn to the recently published books on genetic toxicology. Readers who fall into that category may find the following comments helpful, but it must be stressed that this is not an exhaustive survey, or even a studied selection of the books available.

Mutagenesis in Sub-mammalian Systems, Status and Significance (edited by G. E. Paget; MTP Press Ltd, Lancaster, 1979; pp. xiv + 231, £8.95) is based on the third meeting in a series entitled *Topics in Toxicology*, organized and sponsored by Inveresk Research International. This comparatively small book is admirably presented, and is provided with many clear and informative diagrams and illustrations. The individual papers are generally more readable than most scientific presentations and constitute a more useful introduction to the subject than the exhaustively detailed discussions to be found in the other publications reviewed here. While the text is concerned with sub-mammalian test systems, a section entitled "General background to mutagenesis studies" provides an opportunity for a lucid explanation of the significance of DNA damage for man. This contribution, by B. A. Bridges, forms an excellent introduction to descriptions of the main methods currently used to detect genetic toxicity. Although a paper by C. Auerbach on the role of Drosophila in mutagenicity testing appears to occupy a somewhat anomalous position in the 'General background' section, no one would deny Prof. Auerbach precedence in any book on mutagenesis.

The section devoted to bacterial mutagenesis focuses, commendably, on the practical usefulness of these tests in identifying real genetic hazards to man. The article by B. N. Ames is particularly interesting and comprehensive. Under the heading "Other mutagenesis test systems", D. Anderson presents a clear and useful account of a wide range of methods, and this is followed by papers on cytogenetics and cell transformation. It might have been better if Dr Anderson had been allowed to review the whole range of test methods somewhat earlier in the book, and the selection of only two techniques for further discussion may be rather misleading, particularly in the case of cell transformation (the Styles test). The final section of this book discusses what the results of the various tests may mean in terms of assessing and minimizing the effects of mutagens on human health. Despite some deficiencies in the organization of the material, leading to omissions and some repetition, this book can be thoroughly commended for its clarity (and perhaps its brevity).

For those less enamoured of biological models and more interested in the effects of chemicals on man, *Genetic Damage in Man Caused by Environmental Agents* (edited by K. Berg; Academic Press, New York, 1979; pp. xiii + 511, £15.60) will be more acceptable. Aimed primarily at workers in preventive medicine, public health and industrial medicine, this book contains a wealth of information of the detection of heritable and somatic mutations, chromosome damage and sister chromatid exchange in exposed humans. Also included are discussions of epidemiology, inherited susceptibility to toxic agents and antenatal diagnosis of congenital malformations. Non-human test systems are described only briefly and almost apologetically.

This is certainly not a laboratory handbook of genetic test methods. Instead, it concentrates on the uses to which such techniques can be put in the effort to determine the impact of environmental chemicals on human genetics. There are numerous descriptions of attempts to monitor the genetic health of workers in the chemical industry and of sections of the general public exposed to environmental pollutants. The substantial contribution from Scandinavian scientists is impressive, but the USA is also well represented. The

individual contributions are generally written, organized and presented well, and the inclusion of reports and recommendations of study groups for each of the main topics considered rounds off and summarizes the book very satisfactorily. This publication will undoubtedly be a valuable acquisition for any industrial toxicologist or public health official.

Some time ago (*Cited in F.C.T.* 1978, **16,** 382) we published a review of *Handbook of Mutagenicity Test Procedures* (edited by B. J. Kilbey, M. Legator, W. Nichols & C. Ramel; Elsevier/North-Holland, Amsterdam, 1977; pp. xiv + 485, $59.95). As its name implies, this is a book for those who intend actually to perform mutagenicity tests in the laboratory. Most of the short-term tests for genetic damage are described in great detail, often with comprehensive instructions, and although the methods given are already showing some signs of age, the Handbook continues to be a very valuable source of instruction in methodology. However, it includes nothing in the way of introduction or summary and those in search of enlightenment about genetic toxicology may well be discouraged by the diversity of methods discussed. At first sight. *Progress in Genetic Toxicology* (edited by D. Scott, B. A. Bridges & F. H. Sobels; Elsevier/North-Holland, Amsterdam, 1977; pp. xi + 335, Dfl. 96.00) is rather similar to the Handbook. In contrast, however, it is not meant as a laboratory manual, but is an attempt to summarize the status of genetic toxicology at the time of the 1977 conference that provided its test. Although, again, some aspects are a little out of date, most of the material presented is still valid today. In an introductory section, the questions of the importance of genetic damage in man and the nature of the damage leading to cancer, mutation and teratogenicity are discussed in papers by C. O. Carter, P. N. Magee, J. E. Cleaver, J. W. Drake and H. J. Evans. Together, these five contributions provide a valuable introduction to the basic concepts underlying genetic toxicology.

The organization of the bulk of the book is somewhat arbitrary, a situation that is not helped by some over-optimistic section headings. Nevertheless, there are many very useful papers to be found. Descriptions of most of the important test systems appear, intermingled with discussions on the problems of extrapolation and threshold levels and with reports of practical experience with the tests. Studies in human subjects and the problems of metabolic activation are also touched upon. It is unfortunate that so much information should be both difficult to read in sequence and also difficult to locate.

Inherent in testing chemicals *in vitro* is the problem that most of the organisms used lack the enzyme systems responsible for activating carcinogens *in vivo*. While this question is touched upon in most publications on mutagenicity testing, it forms the main subject of an earlier Elsevier publication entitled In vitro *Metabolic Activation in Mutagenesis Testing* (edited by F. J. de Serres, J. R. Fouts, J. R. Bend & R. M. Philpot; North Holland Publishing Company, Amsterdam, 1976; pp. vii + 363, Dfl. 75.00). This book reports the proceedings of a meeting held in 1976 and discusses in detail the metabolism of a range of important classes of mutagens. The mutagenicity test systems themselves are not dealt with in any great detail and, once again, some of the sections are rather poorly constructed—a frequent hazard of symposium proceedings. Most of the useful information presented relates to work on the metabolic transformation of specific chemicals, such as polycyclic hydrocarbons and nitrosamines; there is no detailed discussion about methods available for mutagen activation *in vitro*, nor guidance on the activating systems most appropriate for mutagenicity screening. Consequently, while much useful background information on the metabolism of carcinogens can be obtained from this book, its practical usefulness for genetic toxicologists is extremely limited.

Finally we should perhaps draw attention again to two books reviewed in these pages several months ago (*Cited in F.C.T.* 1981, **19,** 121). These two "Banbury Reports" presented the proceedings of symposia concerned, on the one hand, with assessing the risks of chemical mutagens to man and, on the other, with the use of mammalian-cell systems for detecting mutagens. Together, these and the five publications discussed above should provide a fair amount of worthwhile reading for those interested in genetic toxicology, whatever their particular approach to the subject.

[B. J. Phillips—BIBRA]

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ABSTRACTS AND COMMENTS

FOOD AND FOOD ADDITIVES

More light on migraine

Monro, J., Brostoff, J., Carini, C. & Zilkha, K. (1980). Food allergy in migraine. Study of dietary exclusion and RAST. *Lancet* **II**, 1.

Food allergy is considered by many to be a cause of migraine, and the study cited above adds to the evidence that has accumulated in support of this theory.

In the first phase of the study, 47 patients with severe migraine were given rotation and elimination diets for 2 yr. Of the 33 patients who completed the study, migraine-provoking foods were identified for 23, no dietary factors were found in six cases, and in four, food factors were suspected but eliminating them from the diet did not help. In the 23 subjects for whom migraine-provoking foods were identified, the elimination of those foods from the diet resulted in relief from the migraine within 2 wk. The relief was usually complete. At the end of the phase, tests were carried out for specific IgE antibodies to foods using radioallergosorbent tests (RAST). A good correlation was observed between high RAST values and the foods identified by the elimination diets in the 23 patients, although RAST values to other foods were higher than normal. All six patients for whom dietary factors were not detected showed low RAST values. The foods tested were milk, cheese, eggs, chocolate, tea, coffee, fish, shellfish, wheat, rice, tomato, apple and orange, and it was found that patients were generally allergic to more than one food group (usually three).

In the second phase of the study the predictive value of RAST was investigated in 26 patients. Foods with the highest RAST titre were eliminated from the patients' diets. Only three of the subjects failed to benefit from the elimination diets. A group of ten patients was challenged with identified migraine-inducing foods after pretreatment with oral sodium cromoglycate. One was completely protected by 400 mg sodium cromoglycate daily for 7 days before challenge, eight were partially protected by 800 or 1600 mg daily, and one remained unprotected (but also did not keep to his diet). It is suggested that the initial specific allergic reaction in the gut may increase mucosal permeability, which in turn allows food antigens, complexes or mediators to be absorbed, provoking the migraine syndrome.

Premature antioxidant ingestion

Nitzan, M., Volovitz, B. & Topper, E. (1979). Infantile methemoglobinemia caused by food additives. *Clin. Toxicol.* **15**, 273.

Methaemoglobin, formed when the iron of haemoglobin is oxidized to the ferric state, cannot interact with oxygen, and its presence in the blood is therefore potentially life-threatening. The paper cited above describes an outbreak of toxic methaemoglobinaemia amongst hospitalized infants.

Within a period of 1 month, methaemoglobinaemia was diagnosed in a group of nine infants in an Israeli paediatric ward. Whereas typical neonates have methaemoglobin levels in the blood some two to five times the normal adult figure of 1%, the average level in these nine, who were all 6–15 weeks old was 18%. All the infants had recently recovered from a bout of acute gastro-enteritis and had as a precaution been fed Hyprovit, a brand of soya-bean product formulated for babies with milk allergy or lactose and sucrose intolerance. Replacing the Hyprovit with cows' milk or other infant formulas led to a rapid return to normal methaemoglobin levels. Although Hyprovit had been sold in Israel for about 10 yr without any reported toxicological problems, the fact that signs of methaemoglobin returned in those particular infants with its reintroduction into the diet strongly suggested that it was the cause of the observed effects.

In an attempt to confirm these suspicions, a number of the components of the Hyprovit, including soya-bean concentrate, vitamins and minerals were given separately to some of these infants and to several other babies of the same age group, but this produced no toxic effects. Enquiries to the suppliers of the fatty component, soya-bean oil, revealed a recent change in the oil's antioxidant to a BHA–BHT–propyl gallate mixture. On ethical grounds, the investigators could not justify feeding the soya-bean oil containing this mixture to infants; however as no further cases of methaemoglobinaemia were reported once these antioxidants were removed from the Hyprovit, they felt the circumstantial evidence of guilt was sufficient. Of the three antioxidants, propyl gallate was favoured as the specific causative agent, because the structurally related pyrogallol had been implicated as a methaemoglobinaemic agent by an earlier report (Konrady, *Arch. Tox.* 1936, **7**, 179).

Although the affected infants were shown to have a fully functional methaemoglobin-reductase system—reduced diphosphopyridine nucleotide diaphorase in the red blood cells was in the normal range—the fact that haem iron of foetal haemoglobin is known to be susceptible to oxidation could well predispose the newborn to methaemoglobinaemia. Furthermore, it has long been known that neonates have a lower metabolic capability than do older infants and therefore xenobiotics requiring metabolism prior to excretion could be present for a relatively long period in the blood. Both these factors may explain why two older babies (each about 6 months old) at the hospital in question did not develop overt signs of toxicity when fed the suspect soya-bean preparation.

[British babies should be protected from a fate similar to that which befell these Israeli infants. The UK food additive regulations do not permit the use of BHA, BHT or the gallates in food specifically designated for babies.]

NATURAL PRODUCTS

Carcinogenicity of betel quid ingredients

Bhide, S. V., Shivapurkar, N. M., Gothoskar, S. V. & Radadive, K. J. (1979). Carcinogenicity of betel quid ingredients: feeding mice with aqueous extract and the polyphenol fraction of betel nut. *Br. J. Cancer* **40,** 922.

The chewing of betel quid, (a mixture of betel nut fragments and lime with or without added tobacco and spices, wrapped in a betel vine leaf) is a popular and long-established custom in India and the Far East. However, the practice has been implicated as an aetiological factor in the development of cancer of the upper alimentary tract and results of experiments in which betel extracts have been applied to the buccal epithelia of hamsters and baboons tend to support this (*Cited in F.C.T.* 1971, **9,** 919; *ibid* 1973, **11,** 926). *In vitro*, betel leaf extracts have been shown to increase the frequency of chromatid aberrations in cultured human lymphocytes (Sadasivan *et al. Mutation Res.* 1978, **57,** 183). Although the causative element has yet to be identified, arecoline, the main alkaloid present, and its metabolite arecaidine, have both been shown to be alkylating agents (*Cited in F.C.T.* 1970, **8,** 114).

Groups of approximately 10-wk-old male mice were dosed with 0·1 ml of various betel fractions once daily, 5 days/wk, throughout life. The fractions were aqueous extracts of betel nuts or leaves, or a polyphenol fraction of the nuts extracted in ethyl acetate. Controls were treated with distilled water. No tumours were observed in the controls (20 Swiss mice and 20 C17 mice). Tumours were not apparent in the 15 Swiss mice treated with the aqueous leaf extract; indeed previous unpublished studies were said to have suggested that the leaf might have a protective effect against simultaneous administration of nut extract.

Of the 21 Swiss mice dosed with the aqueous nut extract, seven developed liver tumours (five hepatocellular carcinomas and two haemangiomas). Two lung adenocarcinomas, one squamous-cell carcinoma, one adenocarcinoma of the stomach, and one case of leukaemia also occurred in this group. In the C17 mice (30) treated similarly, three squamous-cell carcinomas of the forestomach, two adenocarcinomas of the glandular stomach, one lung adenocarcinoma and two cases of leukaemia were observed. The authors explain that these species differences may reflect a higher liver-tissue susceptibility to the effects of a weak carcinogen in the Swiss mice or reflect a lack of the enzymes required for carcinogen activation or formation in the C17 mice. The 18 Swiss mice treated with the polyphenol fraction did not develop tumours of the gastro-intestinal tract. However, two tumours of the salivary gland and one haemangioma of the liver were observed.

OCCUPATIONAL HEALTH

Biomethylation of inorganic arsenic

Odanaka, Y., Matano, O. & Goto, S. (1980). Biomethylation of inorganic arsenic by the rat and some laboratory animals. *Bull. envir. Contam. Toxicol.* **24,** 452.

Rodent experiments have often failed to reproduce effects such as the increased incidence of lung, lymphatic and skin cancer (*Federal Register* 1975, **40,** 3392; *Cited in F.C.T.* 1981, **19,** 132) or angiosarcoma of the liver (*ibid* 1976, **14,** 507) observed in arsenic-exposed humans. Methylation has been suggested as a mechanism of detoxification of inorganic arsenic in mammals (*ibid* 1976, **14,** 507; *ibid* 1980, **18,** 102) and the present authors have investigated interspecies differences in the excretion of methylated and non-methylated arsenic metabolites.

Within 48 hr of the oral administration, in water, of a single dose of 5 mg arsenic acid/kg, urinary excretion accounted for 17·2, 48·5 and 43·8% and faecal excretion for 33·0, 48·8 and 44·1% of the original dose in rats, mice and hamsters, respectively. In these animals, and others treated with a single intravenous (iv) injection of 1 mg arsenic acid/kg in saline solution, totals of 88 and 97% of the dose were found within 48 hr in the urine and faeces of the mice and hamsters but only 50% was recovered from the rat in the same time, most of the remainder being found in the blood.

Three metabolites, containing respectively inorganic arsenic, dimethylated arsenic (DMAs) and monomethylated arsenic (MMAs), were found in the urine and faeces of all three species. After oral dosing the urine of all three contained 14·1–17·7% inorganic arsenic and 0·9–4·6% MMAs, but the rat excreted only 2·2% of the dose as DMAs in the urine whereas the equivalent values for the mouse and hamster were 30 and 21·5% respectively. The faeces of all three species contained 24·2–28·8% inorganic arsenic and 7·7–13·9% MMAs, but here again the DMAs values differed widely, being 1·1% for the rat, 14·4% for the mouse and 1·4% for the hamster.

After iv dosing, 42·4–47·6% of the dose was present in the urine of all three species as inorganic arsenic and 0·7–2·1% as MMAs. The urinary values for DMAs were 2·7% for the rat, 37·4% for the mouse and 39·7% for the hamster. Total metabolites found in the faeces after iv dosing amounted to less than 4% of the dose for all three species. Similar iv studies in rabbits and cats showed that the chief urinary metabolites were DMAs and inorganic arsenic, as in the mice and hamsters.

Two days after oral or iv treatment the tissue distribution of arsenic metabolites in the rats, mice and hamsters was examined. The total amount of arsenic in rat blood (c. 40% of the dose) greatly exceeded that in the other species and was virtually all present as DMAs. About 2% of the dose was found in the liver and 0·6–0·7% in the kidneys; here again DMAs was the major metabolite. The residual arsenic levels in the tissues of the mice and hamsters were too low to

be identified. The biliary excretion of arsenic in rats following oral dosing was found to represent 4–5% of the original dose in 24 hr and occurred mainly as MMAs.

Thus the rat is distinguished from the mouse and the hamster in that a large amount of DMAs is bound in the blood and consequently far less is excreted in the urine.

Cancer and asbestos workers

Weill, H., Hughes, J. & Waggenspack, C. (1979). Influence of dose and fiber type on respiratory malignancy risk in asbestos cement manufacturing. *Am. Rev. resp. Dis.* **120,** 345.

Because of the extremely serious health hazard that asbestos presents, it is particularly important to ensure that current exposure standards have the best possible scientific foundations. The study described here attempts to relate the risk both to levels and to types of fibre exposure. The authors investigated the effects of asbestos on mortality in 5645 men who had worked for at least 1 month in one of two plants manufacturing asbestos-cement building materials in New Orleans. The cohort study was limited to men with a follow-up of at least 20 yr. An attempt to trace identified personnel *via* the Social Security Administration identified 11% as having died by the end of 1974 and death certificates were traced for 91% of these. It was assumed that all other members of the study were alive at that time although they were not all positively traced. Estimates of dust exposure were based on total airborne particulate measurements using the midget impinger at various locations throughout the two plants. It was assumed that pre-1950 levels did not differ from those recorded when sampling was initiated in the early 1950s.

The sampling data resulted in a table of dust concentrations and estimated fibre content for each job by month and year. These were combined with work histories to give an exposure profile for each subject. In addition, a worker's total dust exposure was recorded in millions of particles per cubic foot-year (mppcf-yr) for the first 20 yr of his employment. Subjects were classified into five total-dust categories, ≤ 10, 11–50, 51–100, 101–200 and >200 mppcf-yr. Mean length of follow-up and mean age at initial exposure were comparable in these groups. No data on smoking habits were available. The data from the death certificates were compared with standard race–age–cause-specific rates for US and Louisiana male populations for 1950–1970.

Standard mortality ratios (SMRs) ($100\times$ observed number of deaths/no. expected) were obtained for various causes for each of the five exposure categories. Deaths for which certificates were not located were allocated among causes in the same proportions as those with certificates. No excess mortality occurred in any exposure group for any cause other than respiratory neoplasms. There was no excess risk of gastro-intestinal neoplasms, as had been found in some previous studies (*Cited in F.C.T.* 1972, **10,** 575). In the two highest exposure groups (total dust exposures of 101–200 and >200 mppcf-yr) the SMRs for malignant neoplasms of the respiratory system were significantly increased to values of 29 and 226. The arbitrary setting of the 20-yr minimum for follow-up may have underestimated the extent of respiratory tumours in this population, since some deaths occurring 18 and 19 yr after initial exposure were due to this cause. The results followed a similar pattern when different approaches to the analysis of the data were used.

However, the study showed that it was not just total dust exposure that was important. The group was divided into three according to duration of exposure and subdivided according to average concentration of exposure to give nine groups. There was a general indication of increasing risk with duration that was concentration-dependent and of increasing risk with concentration that was duration-dependent. The results showed the importance of both factors in estimating risk.

To determine the possible influence of fibre type on respiratory malignancy, workers with exposure to chrysotile only were compared with two groups of workers who were also exposed to crocidolite, the first during steady employment in the pipe plant and the second during occasional maintenance work there. Workers exposed to amosite were excluded from this study. It was found that crocidolite enhanced the risk of respiratory malignancy particularly for workers intermittently exposed, generally to high concentrations of dust for short periods of time. This confirmed results previously reported in rats (*ibid* 1974, **12,** 592).

Acrylamide neurotoxicity in rats

Tilson, H. A., Cabe, P. A. & Spencer, P. S. (1979). Acrylamide neurotoxicity in rats: a correlated neurobehavioral and pathological study. *Neurotoxicology* **1,** 89.

The neurotoxicity of acrylamide in laboratory animals, and as a result of industrial exposure, in man is well documented (*Cited in F.C.T.* 1978, **16,** 188). In chronic poisoning the effects appear to be cumulative, but often reversible, producing a distal to proximal 'dying-back' peripheral neuropathy. Most previous investigations of acrylamide neurotoxicity have involved either pathological bioassay or behavioural studies. The study cited above compares simultaneous morphological and behavioural effects at different stages of intoxication of acrylamide-treated rats.

Groups of ten male Fischer albino rats were dosed with 0, 5, 10 or 20 mg acrylamide/kg by gavage in distilled water three times a week for 13 wk, whilst rats in another control group were handled but were otherwise untreated. Body weights were monitored and behavioural tests were carried out during the week prior to dosing and after 1, 4, 7, 10 and 13 wk of dosing, the latter to assess any weakness in hindlimbs, forelimbs or overall motor activity. At the end of the dosing period all ten rats in the 10-mg/kg group and half of the rats in the 20-mg/kg and in the distilled-water groups were killed and examined for histopathological evidence of damage in the medulla oblongata, mid-thigh sections of the sciatic nerve and individual tibial nerve branch fibres. The remaining rats from the high-dose group and the water-treated controls were given further behavioural tests 1 and 5 wk after the end of dosing and were then killed and morphologically examined. No effects were observed

at the 5-mg/kg level. Average body weight was significantly decreased in the groups given the 10- and 20-mg/kg doses; the effect was early and prolonged in the high-dose group, the rats which only regained normal weight 5 wk after cessation of dosing. Measurement of the hindlimb thrust against a strain gauge as the animals jumped onto an adjacent platform showed the hindlimb extensor response (HLER) was significantly decreased through wk 7–14 of the study in the high-dose group but not in the groups on the lower doses. Locomotor activity was monitored automatically and in the high-dose group it was decreased during wk 10–14. Forelimb grip strength was not affected at any level except in the first week after cessation of dosing at the 20-mg/kg level. Despite the absence of behavioural effects in rats receiving 10 mg/kg, histopathology revealed signs of early nerve-fibre degeneration in all ten animals examined at wk 13. More severe damage, characterized by fibre regeneration and remyelination together with fibre degeneration and formation of Schwann cell columns, was apparent when the high-dose animals were examined at wk 13, while 5 wk later there was evidence of distal degeneration in large-diameter myelinated fibres and of clusters of small regenerating myelinated fibres in peripheral nerves.

In this study morphological changes correlated well with the acrylamide-induced motor dysfunction indicated by the HLER. Loss of body weight appeared to be an even more sensitive measure of subchronic exposure to acrylamide, with significant changes observed as early as wk 4 of dosing with 20 mg acrylamide/kg. This, with other findings, has led the authors to suggest that the most sensitive behavioural indicator of acrylamide toxicity may be changes in feeding pattern. [However, the effect of acrylamide on factors determining food intake and growth need not necessarily involve the nervous system.] Finally, the hind-limb weakness was not apparent until the rats had received a cumulative dose of 420 mg/kg on this spaced dosing regime, whereas in a previous study these authors observed an effect after a total dose of 50–100 mg/kg in animals treated on 5 days/wk. Thus the spacing of doses appears to affect the result.

A clean bill for dichlorvos

Schwetz, B. A., Ioset, H. D., Leong, B. K. J. & Staples, R. E. (1979). Teratogenic potential of dichlorvos given by inhalation and gavage to mice and rabbits. *Teratology* **20**, 383.

Dichlorvos (*O,O*-dimethyl-2,2-dichlorovinyl 1-phosphate) is an organophosphate insecticide and anthelmintic which has found extensive uses in the domestic sphere. It has been demonstrated that, under anticipated conditions of handling, and in some studies even at levels that produce some evidence of toxicity, dichlorvos offers no substantial evidence of being teratogenic (*Cited in F.C.T.* 1974, **12**, 769). Further evidence of its lack of teratogenic potential is now offered.

Dichlorvos was given by gavage at the maximum tolerated doses of 60 mg/kg/day to mice and 5 mg/kg/day to rabbits, and by inhalation in concentrations of 4 μg/litre for 7 hr daily to both species. Rabbits were dosed on days 6–18 and mice on days 6–15 of gestation. In mice, exposure to dichlorvos by either route produced no significant effect on the mean number of foetuses per litter, the incidence or distribution of resorptions, or foetal body measurements. The incidence of foetal malformations and minor alterations in the experimental groups did not differ from that in controls, and indeed lumbar spurs were less frequent in the treated animals. In rabbits, the only observed effect of dichlorvos was a three-fold increase in resorption rate in litters that showed this effect, though not in the number of litters in which resorption occurred. There were no major foetal malformations, apart from one six-lobed liver among the group that inhaled dichlorvos; minor malformations were similar in experimental and control groups.

Tracking the fate of petroleum hydrocarbons

Chin, B. H., McKelvey, J. A., Tyler, T. R., Calisti, L. J., Kozbelt, S. J. & Sullivan, L. J. (1980). Absorption, distribution, and excretion of ethylbenzene, ethylcyclohexane, and methylethylbenzene isomers in rats. *Bull. envir. Contam. Toxicol.* **24**, 477.

Toxicity studies on the petroleum hydrocarbon mixture "60 Solvent" were carried out by Carpenter *et al.* (*Toxic. appl. Pharmac.* 1975, **34**, 374). The solvent's principal components are ethylbenzene (EB), ethylcyclohexane (ECH) and methylethylbenzene (MEB). The principal urinary metabolite of inhaled EB in humans is mandelic acid (*Cited in F.C.T.* 1975, **13**, 480), but some 2-ethylphenol is also produced, and it has been suggested that aromatic epoxides may be formed as intermediates in the metabolism of EB to the phenolic compound (*ibid* 1980, **18**, 322). However, in general there has been little detailed study of the metabolism and disposition of the components of 60 Solvent and the latter aspect is dealt with in the paper cited above.

Groups of three male rats were exposed to ^{14}C-labelled EB, ECH or MEB at concentrations of 1 mg/litre for 6 hr, and their urine, faeces and cage-washings were collected at 6, 24 and 48 hr for measurements of radioactivity. The mean amounts of [^{14}C]EB, [^{14}C]ECH and [^{14}C]MEB absorbed per rat were 16, 17 and 19 mg, respectively. Excretion of all three compounds was essentially complete within 24 hr of the start of exposure. The major route of excretion was the urine. This route accounted for 83, 65 and 72% of the absorbed doses of EB, ECH and MEB, respectively. Lesser proportions of the absorbed doses were present in the expired air (EB, 8%; ECH, 11%; MEB, 1·2%), and there were only minor quantities in faeces (EB, 0·7%; ECH, 3·9%; MEB, 2·7%). Total mean recoveries of activity of 91, 80 and 76% were obtained for EB, ECH and MEB, respectively, and tissue residues of radioactivity 48 hr after termination of exposure (72 hr for the study using EB) were 0·2, 0·2 and 0·3%, respectively. The tissue residues were highest in the carcasses, gastro-intestinal tract and liver. Residual activity from EB and ECH was relatively high in the fat, and that from MEB was relatively high in blood plasma. Large standard deviations appeared in the data for the urinary recovery of MEB, and the cause of this poor reproducibility is unknown.

Occupational exposure to dimethylformamide

Lauwerys, R. R., Kivits, A., Lhoir, M., Rigolet, P., Houbeau, D., Buchet, J. P. & Roels, H. A. (1980). Biological surveillance of workers exposed to dimethylformamide and the influence of skin protection on its percutaneous absorption. *Int. Archs occup. envir. Hlth* **45,** 189.

Dimethylformamide (DMF) is an extensively used solvent, particularly in the acrylic fibre industry. Occupational exposure (through inhalation of vapour and skin contact) appears principally to affect the upper gastro-intestinal tract, pancreas and liver (Di Lorenzo & Grazioli, *Lavore Umano* 1972, **24,** 97; *Cited in F.C.T.* 1975, **13,** 289), and symptoms such as stomach pain, loss of appetite, nausea and headache have been experienced by workers exposed to 10–30 ppm DMF in the atmosphere (Massmann, *Zentbl. ArbMed. ArbSchutz* 1956, **6,** 207; *idem, ibid* 1967, **17,** 206). Intolerance to alcohol is another frequently reported symptom (*Cited in F.C.T.* 1978, **16,** 629). In man, *N*-methylformamide (NMF) appears to be the major urinary metabolite of DMF (Krivanek *et al. J. occup. Med.* 1978, **20,** 179), although the exact relationship between exposure to DMF and urinary NMF concentration is apparently not yet established. In the present investigation, two studies were carried out on workers exposed to DMF in an acrylic-fibre factory. The first attempted to evaluate the usefulness of urinary NMF determination as a biological indicator of occupational exposure to DMF and to determine whether under normal working conditions DMF was hepatotoxic, whilst the second attempted to define the main route of DMF exposure.

The first study involved 22 exposed workers, who wore gloves (to prevent skin contact with DMF solution) but not respiratory protective devices, and 28 control workers. Atmospheric DMF concentrations had been measured at different sites in the factory throughout the 5 yr preceding the study and during it the recordings were made more frequently. Levels of NMF in pre- and post-shift urine samples collected daily for 5 days showed that urinary NMF levels provided a sensitive biological indicator of DMF exposure. NMF was readily detected in the urine even when the average atmospheric DMF concentration was less than 30 mg/m^3, the current ACGIH TLV. Furthermore, on a group basis, there was a good correlation between the intensity of DMF exposure and the concentration of NMF in urine collected at the end of the shift, although on an individual basis this correlation was much less pronounced. Blood samples taken at the beginning and end of the working week for determination of biological signs of liver dysfunction indicated that exposure to DMF vapour for 5 yr at levels usually below 30 mg/m^3 did not cause liver cytolysis. Nevertheless, some workers reported experiencing alcohol intolerance at the end of a shift following exposure to peak concentrations of DMF vapour (for example during spinneret cleaning).

The second study was performed on seven workers who all had the same duties. Urine samples were collected before and after each shift and the NMF concentration was determined. During the first week of the study, when skin absorption of DMF was prevented by the use of protective gloves, an average daily integrated (i.e. cumulative) exposure to DMF vapour of 80 mg/m^3 × hr (equivalent to exposure to approximately 13 mg/m^3 for 6 hr) was found to correspond to a urinary concentration at the end of the working day of 40–50 mg NMF/g creatinine. During the second week, the workers did not wear gloves, but instead applied a glycerol-based protective cream to their hands and forearms. This time, the average NMF urinary concentration at the end of the working day was much higher (approximately 75 mg NMF/g creatinine), suggesting that the cream was less effective than gloves in preventing the percutaneous absorption of DMF. A silicone-based barrier cream was found to be rapidly dissolved in DMF solution, and thus could not be used to limit DMF skin absorption. During the third week, it was intended that each worker should wear a self-contained breathing apparatus, but that the hands and forearms should not be protected. The average urinary NMF concentration at the end of the first day was about three times higher than that found during the first week, and two workers complained of upper abdominal or gastric pain. Workers therefore resumed wearing gloves from the second day of the third week onwards, whereupon the urinary NMF concentrations returned progressively to values similar to those found during the first week. The authors concluded that skin absorption is the main route of occupational exposure to DMF.

Epoxy resins: a provisional discharge

Mitelman, F., Fregert, S., Hedner, K. & Hillbertz-Nilsson, K. (1980). Occupational exposure to epoxy resins has no cytogenetic effect. *Mutation Res.* **77,** 345.

Epoxy resins have been incriminated as a cause of contact allergy (*Cited in F.C.T.* 1980, **18,** 206). Weak carcinogenicity has been demonstrated when some epoxy resin monomers have been painted on the skin of mice (*ibid* 1980, **18,** 444), and diglycidyl ether of bisphenol A, a common component of such resins, has been found to be mutagenic in the Ames test (*ibid* 1979, **17,** 420). However, interpretation of such results is difficult, and a cytogenetic study of individuals occupationally exposed to epoxy resins should offer a more reliable picture of the possible in-use effects of such compounds.

Peripheral lymphocytes from four groups of nine workers were examined for frequency of chromosomal aberrations. Two of these groups had been occupationally exposed to epoxy resins of the diglycidyl ether bisphenol-A type, one group for 5–16 yr (median 6·5 yr) to a resin of low molecular weight (average below 900) with an aliphatic amine hardener, the other for 3–10 yr (median 7 yr) to a resin of average molecular weight of about 2000 containing dicyandiamide as hardener. No reactive diluent was used in either formulation. Each of these groups was matched for sex and age with a control group of metal workers not occupationally exposed to epoxy compounds. No differences in terms of frequency of chromosomal aberrations (breaks, gaps and exchanges) were apparent between the exposed groups and their controls.

It is possible that the 72-hr culture method used may have resulted in a loss of cells showing aberrations, and the fact that assays in peripheral blood are

limited to G_0 cells leaves open the question of whether cells in other stages of the cycle may be more sensitive. Moreover, the main route of exposure was by skin contact, although inhalation exposure probably also occurred for the group using the powdered resin of higher molecular weight. Therefore, it should be borne in mind that extensive inhalation exposure might subject workers to higher doses of resin and thus increase the chance of chromosomal aberrations.

Intratracheal Sudan IV

Parent, R. A. & Dressler, I. (1979). Absorption and distribution of C.I. Solvent Red 24 in rats; intratracheal administration of ^{14}C labeled dye. *Drug chem. Toxicol.* **2**, 409.

Inhalation exposure to Sudan IV (C.I. Solvent Red 24; Scarlet Red; 1-[(2-methyl-4-[(2-methylphenyl)-azo]phenyl)azo]-2-naphthalenol) may occur during its manufacture of use in the colouring of plastics, petroleum spirit and lacquers. A previous study of the metabolism of Sudan IV (Ryan & Welling, *Fd Cosmet. Toxicol.* 1967, **5**, 755) involved intraperitoneal administration of the dye. The authors cited above used intratracheal administration in an attempt to model inhalation exposure. Absorption and distribution were studied in rats after intratracheal administration of Sudan IV with a ^{14}C label on the central-ring methyl group. The urine and faeces were collected over the first 96 hr and concentrations of radioactivity in the tissues were measured.

Roughly 60% of the administered activity was absorbed (i.e. was not present in the lung parenchyma after 96 hr). Of this quantity 98% appeared in the urine (19%) and faeces (79%). Neither free dye nor free metabolite (4-amino-2',3-dimethylazobenzene) could be detected in the urine or faeces but at least 1·65–2·1% of the total radioactivity in the urine was attributable to conjugates of 4-amino-2',3-dimethylazobenzene. Of the absorbed dye, only 1·13–2·89% of the activity was found in the tissues, and this was located mainly in the liver, adipose tissue and skin; in the male only, 1·85% of the dose was found among the contents of the intestinal tract. Residual activity of unabsorbed dye remained mainly in the lungs (37·51% in males, 41·97% in females) and there was little in the trachea. In a separate study a conjugate of 4-amino-2',3-dimethylazobenzene was detected in lung tissue, a finding that may have significance in view of the ability of the free aminoazo compound to produce neoplasms in experimental animals (*IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Man: Vol. 8. Some Aromatic Azo Compounds*, p. 61, IARC, Lyon, 1975).

COSMETICS, PHARMACEUTICALS AND HOUSEHOLD PRODUCTS

Mouthwash with a tingle

Mathias C. G. T., Chappler, R. R. & Maibach, H. I. (1980). Contact urticaria from cinnamic aldehyde. *Archs Derm.* **116**, 74.

Sensitization reactions in humans as a result of skin contact with phenylacetaldehyde, citral and cinnamic aldehyde have been reported for these substances singly although not for their application in simple mixtures with other constituents of natural fragrant oils (Opdyke, *Fd Cosmet. Toxicol.* 1976 **14**, 197). Some of the factors involved in contact urticaria caused by cinnamic aldehyde have now been elucidated.

A 30-yr-old woman complained of swelling of her lips and tongue after she had used a mouthwash containing 0·18% cinnamic aldehyde in a 10% denatured ethanol solvent containing also glycerin, saccharin, polysorbate 80, zinc chloride, menthol, clove oil and two dyes, D & C Red No. 6 and D & C Red No. 3. Open-patch testing of the ingredients of the mouthwash indicated that cinnamic aldehyde was responsible for the adverse reaction. In each of ten control subjects, cinnamic aldehyde (0·2% in 10% aqueous ethanol) produced a positive reaction when applied to the cubital fossa for 15 min. A more detailed examination of 0·2% cinnamic aldehyde in ethanol and 0·2 and 2% cinnamic aldehyde in petrolatum was carried out on a further 16 control subjects, to determine the relative reaction when applications were made to different sites. Application of the ethanolic solution to the cubital fossa caused reactions in all of the subjects, whereas application to the forearm induced reactions in only 13 out of the 16. The strength of the response was also greater on the cubital fossa, where a high incidence of erythema, often accompanied by local induration, occurred within 15–30 min. Application of 0·2% cinnamic aldehyde in petrolatum induced a response in only half of the subjects, but when the concentration was increased to 2% all of the subjects reacted, some quite strongly. When lower concentrations of cinnamic aldehyde (in 10% aqueous ethanol) were tested, 13 of the 16 subjects reacted to a 0·1% solution, but only two reacted to a 0·01% solution. At both concentrations the responses were weak and were only detectable upon close inspection of the test site. A challenge with the mouthwash in these subjects produced stinging and burning in all of them within 30 sec, and numbness or swelling of lips, cheeks or tongue in five within 15 min, but there was no objective evidence of mucosal erythema or oedema. A cross-reactivity study was carried out using 0·2% solutions of related cinnamates in 10% aqueous ethanol. Solutions of methyl cinnamic aldehyde and α-amyl aldehyde applied to the cubital fossa provoked no reaction, but one subject gave a mild reaction to cinnamic alcohol and five gave mild reactions to cinnamic acid.

It is evident from the results of this study that preparations containing more than 0·01% cinnamic aldehyde should be suspect urticants. However, the relevance of positive skin or mucosal responses to high concentrations (e.g. 2%) remains open to question. It is also pointed out that the tingling sensation produced in the mouth by cinnamic aldehyde is favoured by consumers.

Hair-dyed Salmonella again

Shahin, M. M., Bugaut, A. & Kalopissis, G. (1980). Structure–activity relationship within a series of *m*-diaminobenzene derivatives. *Mutation Res.* **78,** 25.

Certain of the compounds used in hair dyes are undoubtedly mutagenic in *Salmonella typhimurium*, but the relevance of such findings for man is still a contentious issue. The present studies, whilst confirming earlier work which reported 1,3-diaminobenzene (*m*-phenylenediamine) and 2,4-diaminotoluene (toluenediamine) to be positive in the Ames test (Ames *et al. Proc. natn. Acad. Sci. U.S.A.* 1975, **72,** 2423), indicate that mutagenic activity, in *S. typhimurium* anyway, resides only with the lower members of the homologous series.

The mutagenicity of 1,3-diaminobenzene and four 2,4-diaminoalkylbenzenes was determined in a standard Ames system with *S. typhimurium* strains TA1538, 100 and 98. Only unpurified 1,3-diaminobenzene gave evidence of mutagenic activity in the absence of metabolic activation. This response was weak, (defined as a repeatable 2·5-fold increase in histidine reversion in the bacteria), was limited to strain TA1538, and was eliminated by purification of the sample. With metabolic activation using an S-9 liver fraction from Aroclor-induced rats, 1,3-diaminobenzene purified by distillation was highly active in strains TA1538 and 98, giving a good dose-response, and was slightly active in TA100, a strain in which no other test material exhibited a positive result. Good dose-responses in TA1538 and 98 were also seen with purified 2,4-diaminotoluene, although at all the concentrations tested the unalkylated diamine produced the higher level of histidine reversion. With 2,4-diaminoethylbenzene, the increases in histidine reversion observed in the dose range 50–2500 μg/plate (higher concentrations tended to be less active possibly due to cytotoxicity) were lower than those seen with equivalent concentrations of 2,4-diaminotoluene. 2,4-Diaminoisopropylbenzene and 2,4-diamino-*n*-butylbenzene did not induce mutagenic changes in any of the *S. typhimurium* strains, even though a wide concentration range was tested.

[These findings may offer the cosmetic chemist the opportunity to overcome some of the present toxicological doubts over this particular group of hair dyes.]

Lipsticks licked?

Green, M. R. & Pastewka, J. V. (1980). Mutagenicity of some lipsticks and their dyes. *J. natn. Cancer Inst.* **64,** 665.

Thirty-seven dyes that are used in cosmetics were previously screened for mutagenicity in the Ames *Salmonella typhimurium* test (Muzzall & Cook, *Mutation Res.* 1979, **67,** 1). Only D & C Orange No. 17 was found to be mutagenic. Extracts of a number of lipsticks were also tested and only those containing D & C Orange No. 17 were mutagenic. The dyes used in lipstick are of particular concern since they are ingested to some extent. The paper cited above confirms the conclusions of Muzzall & Cook with respect to D & C Orange No. 17.

Mutagenicity tests with *S. typhimurium* TA98 were performed on 24 commercial lipsticks of various shades and colours, using a solution of each sample in dimethylsulphoxide. Nine lipsticks were mutagenic for TA98 without any microsomal activation and the response was dose-related; the addition of S-9 mix decreased or abolished this effect. Of the 20–30 substances listed on the labels of each lipstick, a total of 78 were selected for individual mutagenicity testing. Not all labels established the contents categorically, and colorants of 14 of the 24 lipsticks were in the "may contain" category, making identification of the mutagenic ingredient(s) more difficult. Of the eight most frequently included pigments, D & C Red Nos 3, 6, 8, 10 and 22 showed no mutagenicity. D & C Red No. 36 at 100 μg/plate produced an increase of mutations over control values in the presence of S-9 activation. D & C Orange Nos 5 and 17 proved mutagenic in the absence of S-9, which diminished or abolished their effect. The numbers of revertants found with D & C Orange No. 17 at 0·1 or 1 μg/plate were equivalent to the numbers found with some lipsticks at concentrations of 100 or 500 μg/plate. Thus D & C Orange No. 17 at a level of less than 1% in the lipstick could account for the observed mutagenicity. It is unlikely that D & C Orange No. 5 could account for the mutagenicity of lipsticks since the response with this dye was only seen at and above the 100-μg level. Of the eight lipsticks matched for ingredients other than dyes, only the two that contained D & C Orange No. 17 proved mutagenic.

The two nitro- groups on the aniline ring of D & C Orange No. 17 may account for its mutagenicity. In addition, 2,4-dinitroaniline, which has been reported to be mutagenic in the Ames test, is used in the synthesis of D & C Orange No. 17 and may be present in the final product either as a contaminant of the pigment or as a metabolite of the dye. On the basis of a dye content of 1% and applications of lipstick 1–5 times daily at 4–6 mg/application, a total of 50–100 mg D & C Orange No. 17 might be applied over 1 yr, but the amount ingested, absorbed, metabolized and eliminated is difficult to assess.

MEDICAL DEVICES

Methacrylate and the liver

Herndon, J. H., Morrison, W. A. & Pilcher, F. J. (1980). Effects of methyl methacrylate on liver function in patients undergoing total hip or total knee replacement. *Surgery Gynec. Obstet.* **150,** 177.

Two categories of inhalation exposure to methyl methacrylate (MMA) monomer can be recognized—the long-term industrial exposure of workers involved in the manufacture and processing of the monomer, and the briefer encounters of operating-theatre personnel with the higher localized concentrations likely to occur during the use of MMA in surgical pro-

cedures. The latter application has been associated in some patients with marked cardiovascular reactions (*ibid* 1975, **13**, 390) and the attention focused on this effect has led to a number of experimental studies. There seems to be little information, however, on liver function in patients given orthopaedic surgery involving the use of MMA polymer, and the reports that have appeared have been conflicting.

The authors cited above undertook a prospective study of groups of unselected patients undergoing hip and knee operations. Routine and specific laboratory tests for liver toxicity were carried out before the operation. Patients showing abnormal liver function at this stage were excluded from the study, leaving a total of 61 patients, in whom the tests, including estimations of serum γ-glutamyl transpeptidase (SGGT) and alkaline phosphatase (SAP) and of the relative levels of bone and liver alkaline phosphatase isoenzymes, were repeated 1, 3, 7 and 14 days and 3, 6 and 12 months after the operation. Of these patients, 45 had total hip (25) or total knee (20) replacements in which MMA polymer was used, while the other eight hip and eight knee operations did not involve MMA and were used as controls.

There were no statistically significant differences in enzyme determinations between the methacrylate-exposed patients and the controls, but during the first 14 days after operation the former groups showed some trend towards increased activities of SAP and SGGT. Rises in SAP were secondary to increases in the levels of isoenzymes from liver not bone. These transient enzyme changes occurred more frequently in the methacrylate-exposed patients who were anaesthetized with halothene than in those given enflurane or thiopental. In the combined control groups, increases in enzyme levels occurred only in patients anaesthetized with halothane. There appears, therefore, to be a tendency for MMA exposure combined with a halothane anaesthetic to increase the risk of interference with liver function, but whether the effects are additive and whether they are attributable directly to the two compounds or to their metabolic products are questions that require further investigation.

TOXICITY MECHANISMS

Dietary fibre and gastro-intestinal function

Kimura, T., Furuta, H., Matsumoto, Y. & Yoshida, A. (1980). Ameliorating effect of dietary fiber on toxicities of chemicals added to a diet in the rat. *J. Nutr.* **110**, 513.

The ability of dietary fibre to protect against the effects of toxic materials added to the diets of laboratory animals has been widely reported (*Cited in F.C.T.* 1979, **17**, 175). The authors of the paper cited above support the hypothesis that the protective property involves the prevention of exfoliation of the brush-border membrane, so that normal absorptive capacities of the small intestine, faecal quality, food consumption and growth can be maintained. Their study investigated the relationships between changes in body weight, food consumption, diarrhoea and villous function (as represented by the activities of enzymes located in the brush border) in rats fed various dietary regimes.

After 2 days of fasting, groups of male Wistar rats were fed, for 4 days, on diets containing either 10% polyethylene glycol 4000 (PEG), 5, 10 or 15% Tween 20 (polyoxyethylene sorbitan monolaurate) or 5% amaranth and with or without plant fibre, added at levels of 2·5, 5·0, 7·5 or 10·0% and derived from the roots of edible burdock (BDF) or soya bean (SDF). The introduction of PEG into the diet induced diarrhoea, decreased food consumption, reduced bodyweight gain, reduced the weight of the intestinal segment representing the second 15-cm length of jejunum distal to the pylorus and of its mucosa, and lowered segmental sucrase activity. In addition to these effects, Tween 20 and amaranth caused a reduction in liver weight and in segmental alkaline-phosphatase activity. The plant fibre supplements were effective in protecting against these effects. BDF was more effective than SDF in the case of Tween 20 and amaranth, but none of the PEG regimes included SDF.

Another experiment revealed that simple dietary restriction resulted in a decrease in weight gain, reduction in the weight of the selected intestinal segment and its mucosa, and lowered alkaline-phosphatase activity, although sucrase activity remained unaffected. Although supplementation of the basal diet with 10% BDF or 10% SDF alone resulted in relatively higher levels of food consumption and weight gain there were no substantial differences in liver weight, intestinal segment and mucosal weight or enzyme activities.

Since preliminary studies had shown that the diarrhoea caused by PEG and Tween 20 could be ameliorated by dietary fibre and that similar effects caused by magnesium salts could not, the action of PEG and Tween 20 did not appear to be due solely to the osmotic effect. Thus further credence is given to the suggestion that the protective action of dietary fibre is due to prevention of the damage to the small intestine that results in malabsorption, although the mechanism is, as yet, unclear.

CARCINOGENICITY AND MUTAGENICITY

Inter-species comparisons of carcinogenicity

Purchase, I. F. H. (1980). Inter-species comparisons of carcinogenicity. *Br. J. Cancer* **41**, 454.

In assessing the carcinogenic potential of compounds in man greater emphasis is generally placed on the results from long-term studies in mammals than on the results of short-term *in vitro* studies. Tumours generated in animal studies appear to be more relevant as a model of carcinogenicity in man but this assumption has not been thoroughly investigated. The review cited here compares carcinogenicity data from two species of mammal (in most cases mice and rats) as a step in understanding the usefulness of data from one species when predicting the carcinogenicity of a particular compound in another.

References and opinions were obtained from three sources, the National Cancer Institute Bioassay Programme, IARC Monographs (1972–1978) and US Public Health Service Document No. 149 and were checked to ensure that all studies conformed to defined standards such as adequacy of histological examination and minimum number of animals per group. It was found that of the 250 compounds studied, 98 (38%) were negative in both rats and mice, and 109 (44%) were positive in both species. A total of 43 compounds (17%) had different results according to the species, 21 (8%) being carcinogenic in mice only, 17 (7%) in rats only, and 5 (2%) having results differing from other species. When a comparison was made of the major target organs affected, only 64% of the chemicals were found to produce cancer at the same site in both species. This suggests that a chemical found positively carcinogenic in one species has only about an 85% chance of being positive in a second species.

Differences in the responses of the two species may be attributable in a few cases to differences in the route of administration, and species differences in the metabolism of a compound may well contribute to the seemingly contrary results. Accurate extrapolation to man therefore requires a thorough knowledge of the metabolism and mode of action of the chemical in the particular laboratory species and a knowledge of whether the main features also apply to man. However our understanding of the metabolism of individual chemicals is rarely thorough, particularly in man. Although there are some exceptions, it seems fairly reasonable to say that a compound that proves positive in all species tested and in the available *in vitro* tests is much more likely to be carcinogenic in man than one that is negative in all but one test. The authors conclude that data on the mode of action, metabolism and pharmacokinetics of a chemical, together with results for similar chemicals and data on interspecies consistency, will provide a much stronger basis for extrapolation than the assumption that carcinogenicity in one species indicates a carcinogenic hazard to man.

The usefulness of short-term tests in predicting carcinogenicity in animals is frequently expressed in terms of their specificity, sensitivity and predictive accuracy. Applying these calculations to the animal carcinogenicity studies reviewed the author found that the rat carcinogenicity studies had a specificity of 85·2% and a sensitivity of 86·5% as a predictor of carcinogenicity in the mouse—and the mouse studies had a specificity of 82·4% and a sensitivity of 86·5% as a predictor of carcinogenicity in the rat. The author also calculated, however, that if only 10% of the chemicals tested were carcinogenic, the predictive value of both rat and mouse results would be 27%.

[Although in isolation the latter figures may be misleading they do shed some light on the probable worth of similar figures for short-term tests.]

New carcinogenesis theory

Holliday, R. (1979). A new theory of carcinogenesis. *Br. J. Cancer* **40**, 513.

This paper extends the theories of Holliday & Pugh (*Science, N.Y.* 1975, **187**, 226) on possible biochemical mechanisms for the control of gene expression during development. These authors suggested that post-replicational methylation of DNA might provide the switching mechanism determining whether or not structural genes were transcribed. It is now suggested that repair of carcinogen-induced damage to DNA, while accurately replacing the base sequence, may not lead in all circumstances to a DNA strand with the correct site-specific methylation. Such an error might lead to aberrations in gene expression, since the 'switch' might be 'turned' the wrong way.

In justifying the necessity for an alternative to the simple somatic mutation theory of carcinogenesis, the author of the paper under review draws together a number of lines of evidence not easily explicable in terms of a mutational theory of the origin of malignancy. Attempts to compare the rates of transformation and mutation in cultured cells suggest a large and species-dependent discrepancy between the two. [It is of interest that rodent cells generally appear to have a much higher probability of spontaneous transformation *in vitro* than human cells, since the probability of malignancy *in vivo* is also claimed to be much higher in rodents than man.] In addition, cell- and nuclei-transplantation experiments suggest that the genes of at least some malignant-cell nuclei may be reprogrammed to normal genes when their environment is appropriately altered.

The author discusses in some detail possible biochemical processes whereby a stable and inheritable alteration in gene expression might result from changes in the methylation pattern of DNA. The central idea (for which there is increasing, although not entirely clear-cut, experimental support) is that the expression of a structural gene might be controlled by the degree of methylation of specific regions of the putative regulator gene controlling it. The methylation processes might be carried out by several methylase enzymes or by one.

This idea can be applied to produce a theory for the mechanism of carcinogenesis by examining the consequences of damage to the DNA, caused by carcinogenic chemicals or radiation, on the pattern of methylation in each strand. Repair processes, even if they accurately replace the base sequence in a strand, might not always remethylate. The consequent aber-

rations in gene expression could lead to malignancy. The probability of such a sequence of events would, clearly, be dependent on a number of factors, including the relative efficiencies of the repair and remethylation processes compared with the rate of DNA replication and the stage in the cell cycle at which the damage occurs, and the type of DNA repair mechanism involved (recombination repair would be expected to lead to more methylation errors than excision repair).

The paper concludes with a number of examples of processes that might be more or less neatly explained on the basis of this hypothesis. Xeroderma pigmentosum patients, in whom a high incidence of skin tumours occurs, show enhanced sister chromatid exchange and deficient excision repair of DNA, factors which would be predicted to enhance the probability of malignancy. Although ethionine would not be expected to alter the structure of DNA in any way, its presence inhibits DNA methylation, and it is suggested that ethionine may exert its carcinogenic activity by interfering with DNA methylation rather than by mutation. In initiated cells which develop into tumours only if subsequently treated with promotors, the initiation is 'remembered', since promotors are almost as effective when applied months after initiation. It is suggested that the memory effect is related to DNA methylation and that promotion is related to the control of gene expression in cells (in which abnormal switching of gene activity has already occurred), perhaps by preventing terminal differentiation and allowing continued proliferation of cancerous cells.

[At no point does the author exclude the possibility of a mutational origin of cancer and he avoids discussion of the question of viral oncogenesis. Nevertheless, as a statement of the 'epigenetic' case this paper takes a lot of beating. The verification of the theory proposed must await a good deal of experimental work.]

Allyl compounds as direct mutagens

Eder, E., Neudecker, T., Lutz, D. & Henschler, D. (1980). Mutagenic potential of allyl and allylic compounds. Structure–activity relationship as determined by alkylating and direct *in vitro* mutagenic properties. *Biochem. Pharmac.* **29**, 993.

Epoxides have been suggested as active metabolites of halogenated olefins (*Cited in F.C.T.* 1977, **15**, 85). This suggestion was backed by the results of a study of the mutagenic and alkylating properties of several such compounds reported by Bartsch *et al.* (*Arch. Tox.* 1979, **41**, 249), who concluded that the oxidation of the double bond by microsomal monooxygenases is a common pathway in the formation of biologically-active intermediates from many halogenated olefins. However, in the paper cited above, a different mechanism of action is proposed for a certain group of halogenated olefins, the allyl and allylic compounds. The mutagenic activity of 22 compounds containing an allyl group was tested on *Salmonella typhimurium* strain TA100. Four compounds, allyl methanesulphonate, iodide, bromide and chloride, in descending order of activity, were mutagenic without addition of rat-liver S-9 mix. All were also shown to be alkylating agents by the 4-(*p*-nitrobenzyl)pyridine test. Allyl isothiocyanate showed borderline activity in both tests.

Other allyl compounds with groups less readily split from the molecule (e.g. $-SH$, $-SR$, $-NH_2$, $-CN$) gave negative results in both tests, showing no mutagenicity in the presence or absence of S-9. For all of the compounds found to be mutagenic, a partial or total decrease in mutagenicity occurred if S-9 mix was added to the media. Thus there was no evidence that enzymatic epoxidation was a prerequisite for mutagenicity in the active compounds. It is proposed instead that the direct alkylating activity of these allyl compounds is explicable on the basis of their highly electrophilic character.

Ferns as pollution detectors

Klekowski, E. J., Jr (1978). Screening aquatic ecosystems for mutagens with fern bioassays. *Envir. Hlth Perspect.* **27**, 99.

Klekowski, E. & Levin, D. E. (1979). Mutagens in a river heavily polluted with paper recycling wastes. Results of field and laboratory mutagen assays. *Envir. Mutagen.* **1**, 209.

The pollution of surface waters as a result of industrial processes is still a considerable problem in some areas. Pulp mills have been shown to produce 9,10-epoxystearic acid (*Cited in F.C.T.* 1980, **18**, 554) and chlorinated guaiacols (*ibid* 1980, **18**, 447), which may be toxic to river animals, and waste waters from municipal treatment plants may contain mutagens (*ibid* 1980, **18**, 447). Possible methods of monitoring rivers and lakes for mutagenic pollutants are therefore of considerable interest.

The screening methods described in the first paper cited above were developed after researchers found a high incidence of mutational damage in a population of royal fern (*Osmunda regalis*) growing in a river that was heavily polluted with paper-processing wastes. Mutational damage was not found in nearby populations in a non-polluted environment. Because the life cycle of a fern involves an alternation of generations it provides several genetic endpoints that can be assessed for genetic damage. The diploid, sporophytic generation is the familiar 'leafy' stage in the fern's life cycle. Sporangia are formed on the leaves and from these haploid spores are released. The spores germinate and develop into haploid gametophytes which bear male and female gametangia in which gametes are produced by mitosis. The life cycle is completed by the fusion of these gametes to form zygotes which develop into sporophytes. Mutagenicity screening can be carried out using the gametophyte generation, which can be grown readily on nutrient agar. Phenotypic alterations can be observed, and if each gametophyte is self-fertilized to yield a completely homozygous zygote, screening can be carried out for mutational damage in the sporophyte genotype. For example, if the mutation is a recessive sporophytic lethal the zygote produced will abort.

Furthermore, the sporophytic state of the fern provides an organism ideal for the study of post-zygotic mutations. A single zygote will develop into a plant with an extensive network of leaves and rhizomes, and the growth of these is extremely regular and pat-

terned. This regularity can be used to date when various mutations have occurred by analysing the distribution of a given mutation in any one sporophyte. [The mutations must be post-zygotic in origin since all of the shoot and rhizome apices, and the leaves and their sporangia, trace their origin to the same zygote.]

Another method of detecting post-zygotic mutations is to observe meiosis in the spore mother cells contained in the sporangia on the leaves. Two-break chromosomal aberrations, such as paracentric inversion, reciprocal translocation and ring chromosome heterozygosity are rare in normal *O. regalis* populations and their presence indicates recent mutations. This method was used to detect mutagens in a river heavily polluted with paper recycling wastes. The fern population was monitored for 4 yr and the results of the study are described in the second paper cited above. The fern population consisted of about 100 plants along one bank of the river. At periods of high water these were submerged. In two of the four sampling seasons the fern population was completely submerged through the time prior to and during meiosis and when meiotic samples were taken from the sporangia. In each year, the incidence of chromosomal aberrations was higher than in control populations. There were some indications that the frequency of mutations was lowest when water levels were low in the 20 days prior to meiosis, and the premeiotic period of leaf development was considered to be the most sensitive to mutagenesis. The year after the study was completed, a waste-water treatment plant was put into operation; solid waste produced by this was extracted with various solvents and the extracts were tested for mutagenicity by the Ames test. Most waste samples showed some degree of mutagenicity, but all required metabolite activation. Four out of six samples were also positive in the soya-bean somatic crossing-over assay described by Vig (*Mutation Res.* 1975, **31**, 49).

[Such methods may be useful for monitoring the impact of water pollution on indigenous plant populations. Unfortunately *Osmunda regalis* is found only in restricted habitats. Klekowski (*Envir. Hlth Perspect.* 1978, **27**, 99) proposes that genetic studies on other, commoner ferns should be carried out, so that the methods developed for *O. regalis* can be applied in other aquatic environments.]

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LETTERS TO THE EDITOR

EVALUATION OF THE MUTAGENICITY OF SORBIC ACID–SODIUM NITRITE REACTION PRODUCTS

Sir,—In a recent paper, Robach *et al.* (*Fd Cosmet. Toxicol.* 1980, **18,** 237) reported interesting observations on the mutagenicity of sorbic acid–nitrite reaction products. Undoubtedly, these observations will contribute to the understanding of this problem. Unfortunately, in quoting our previous findings on the mutagenic activity of sorbic acid in the presence of nitrite or sulphite, the authors seem to interpret these incompletely or even incorrectly. As, furthermore, the reader was not given a literature reference to the original publication*, may we request the liberty of using this channel to clarify some essential aspects?

The mutagenic activity of sorbic acid in the presence of nitrite in an acidic environment was first reported by Japanese authors (Kada, *Rep. natn. Inst. Genet., Mishima*, 1974, **24,** 43; Hayatsu *et al. Mutation Res.* 1975, **30,** 417). One of the known highly mutagenic compounds obtained under these conditions has been identified as ethylnitrolic acid (Namiki & Kada, *Agric. biol. Chem.* 1975, **39,** 1335).

Applying the same technique—the so-called *Bacillus subtilis* rec-assay, in which the test organism is a mutant strain, *B. subtilis* M45, unable to repair DNA-damage (Kada & Sadaie, *Rep. natn. Inst. Genet., Mishima* 1975, **25,** 49)—we have confirmed the Japanese findings and have also shown the mutagenic compound(s) detected in this test to be a result of the reaction of sorbic acid and nitrous acid and therefore pH dependent (Khoudokormoff, *Mutation Res.* 1978, **53,** 208). Indeed, whereas over 85% of sorbic acid is undissociated at pH 4, the situation is reversed at a pH of about 5·5, when only 15% is present in the undissociated state (Rehm & Lukas, *Zentbl. Bakt. ParasitKde II*, 1963, **117,** 306). At a higher pH, both acids dissociate virtually completely into sorbate and nitrite ions, respectively, which do not react with each other.

Due to the narrow range of pH supporting the growth of Enterobacteriaceae, and especially of *Salmonella typhimurium*, both the Ames test and the assay with *Escherichia coli* strains have to be carried out in a medium buffered at pH 7·0 (Ames *et al. Mutation Res.* 1975, **31,** 347; Vogel & Bonner, *J. biol. Chem.* 1956, **218,** 97) and are therefore unsuitable for the detection of the mutagenic compound(s) formed in the acidic reaction mixture between sorbic acid and nitrous acid or sulphurous acid. Such mutagenic compounds, however, are readily detected in the *B. subtilis* rec-assay, since this test organism is able to grow in a fairly wide range of pH values, thus permitting the test to be carried out in an unbuffered medium. The text of Robach *et al.* (*loc. cit.*) regarding the reference to our observations on the failure of the Ames test to detect the mutagenic compound(s) should, therefore, read "... was negative in the Ames Salmonella assay as well as in the *E. coli* ... system *because of* the pH of the medium in which these tests are carried out" and not "... regardless of the pH of the reaction mixture".

We should also like to stress that, far from the suggestion made by Robach *et al.* (*loc. cit.*), the mutagenic activity of sorbic acid, as revealed in the *B. subtilis* rec-assay, is detectable well within the normal range of temperature, pH and sorbic acid concentration, namely 100–1000 ppm (Khoudokormoff, International Symposium on the Chemical Toxicology of Food, Milan, 8–10 June 1978, abstr. p. 102) and nitrite concentration occurring in the practical application of sorbic acid as an antimicrobial preservative for cheese, raw sausages, fruit juices, wine and other products.

The concentration of nitrite can never be completely controlled in food products when bacterial fermentation is involved; 50–250 ppm is a fair and realistic estimate of the actual situation in practice. Moreover, whereas the weak mutagenic activity of nitrous acid, in the absence of sorbic acid, fades within 24–30 hr, that of a sorbic acid–nitrous acid mixture, such as an edible cheese coating to which traces of nitrite and the usual concentration of sorbic acid have been added, persists for at least 10 wk at room temperature (personal observations).

The mutagenic activity of sorbic acid reaction products has since been independently confirmed in wine (Lafont & Lafont, *Méd. Nutr.* 1979, **15,** 195) and in curing brines (*Food Chemical News* 27 November 1978, **20** (37), 13). In both cases the pH, temperature and concentrations used were those normally encountered in the practical application of sorbic acid as a food preservative.

Finally, it has long been known that, for example, the antimicrobial activity of sorbate ions against *Aspergillus niger* is 100 times weaker than that of undissociated sorbic acid; similarly, *E. coli* is 300–400 times more resistant to sorbate than to sorbic acid (Rehm & Lukas, *loc. cit.*). Bearing in mind that the minimal inhibitory concentration of sorbic acid for most contaminants ranges between 100 and 2000 ppm (Lück, *Sorbinsäure: Chemie, Biochemie, Mikrobiologie und Technologie—II*, p. 39; B.

*An editorial error for which we offer our apologies to Dr Khoudokormoff, to Dr Robach and his colleagues and to our readers (Ed.).

Behr's Verlag, Hamburg, 1972), with an average of 1000 ppm at pH 5·5, one is left with a most intriguing contradiction in the conclusion of Robach *et al.* (*loc. cit.*) that "... the levels of sorbate and nitrite proposed for use in cured meats (0·26% sorbate and 40 ppm nitrite)... together with a pH of at least 5·5 and the presence of ascorbate do not pose a hazard in regard to the formation of reaction products in cured meat or the curing brine". Either 0·26% sorbate—i.e. 0·19% sorbic acid—does exert effective antimicrobial protection, which entails, as we have seen, an acidic pH of 5 or less (and hence the risk of a mutagenic reaction), or the pH of the curing brines is indeed 5·5 or more, which infers that at least 85% of the sorbate added is, in fact, in the dissociated ionic form and hence *inactive*, as not only the mutagenic activity but also antimicrobial protective effect of sorbic acid stops short at a pH of 5·5–6·0.

B. KHOUDOKORMOFF,
Medical Bacteriological Laboratory, Research & Development, Gist-Brocades, P.O. Box 1, Delft, The Netherlands

Sir,—We appreciate this opportunity to respond to Dr Khoudokormoff's comments on our paper.

The literature reference to Dr Khoudokormoff's 1978 publication (Khoudokormoff, *Mutation Res.* 1978, **53**, 208) was inadvertently left out of the list of references. It appeared in our original manuscript and apparently was deleted as the paper went to press. In the second paragraph of his letter, Dr Khoudokormoff states that ethylnitrolic acid (ENA) is highly mutagenic; this is simply not so. Our report, as well as an extensive unpublished USDA study, shows that ENA is a weak mutagen. Its activity is low compared to many other materials and very low compared to other active nitro compounds.

Dr Khoudokormoff's observation in the third paragraph that the reaction products result from reaction between sorbic acid and nitrous acid is true. In the same paragraph, however, it is inferred that the *Bacillus subtilis* recombination assay detects mutagenicity. The *B. subtilis* rec-assay is an indirect assay for DNA damage and not an assay for mutagenicity, as claimed by Dr Khoudokormoff. The point of our work was to show that under commercial bacon-curing conditions sorbate and nitrite would not react to form any mutagenic products.

In paragraph 4, Dr Khoudokormoff states that the Ames test is unsuitable for the detection of mutagenic compounds formed under acidic conditions. It is clearly not true that the Ames/Salmonella test is unsuitable for the detection of mutagenic compounds produced under unusual conditions of reaction between sorbic and nitrous acids. As described in our paper, mutagenic activity was readily detected in Hayatsu-type reaction mixtures. Furthermore, two reaction products clearly possess activity in the Ames/Salmonella assay. Ethylnitrolic acid is mutagenic towards strain TA100, and 1,4-dinitro-2-methylpyrrole is moderately active towards strains TA98 and TA100 (Osawa *et al. Biochem. Biophys. Res. Commun.* 1980, **95**, 835).

Dr Khoudokormoff seems to be confused on the question of pH effects. One issue is whether mutagenic products *formed* under unusual reaction conditions and acid pH can be detected by the Ames/Salmonella assay. This point is discussed above. A second issue is whether mutagenic products that are formed only *express* genotoxic activity at low pH. With the exception of nitrous acid, there is no clear evidence that any such activity exists in nitrous acid–sorbic acid solutions. The biological significance of such a type of activity to mammals is also not clear.

Two points in the fifth paragraph need to be clarified. The first is that Dr Khoudokormoff presents no evidence in his letter that acidic solutions *per se* are not positive in the *B. subtilis* rec-assay. The second point is that it is well recognized that the genotoxic activity of nitrous acid is pH dependent. The attempt to draw conclusions by comparing nitrous acid solutions with treated edible cheese coatings indicates a significant lack of appreciation for proper experimental controls. Such conclusions should not be seriously considered as evidence for the formation of mutagenic products under natural conditions.

The reference in the sixth paragraph to *Food Chemical News* of 27 November 1978 relates to an unpublished report that revealed the formation of mutagenic compounds in a bacon-curing brine. However, this work was done at low pH levels (5·2 or below), with an excess of nitrite and grossly inadequate levels of ascorbate or erythorbate (nitrite traps). These conditions do not exist in the cured-meat industry.

The final paragraph, in which Dr Khoudokormoff refers to a "most intriguing contradiction", is neither intriguing nor contradictory. Many published articles† have demonstrated conclusively that

†Huhtanen & Feinberg, *J. Fd Sci.* 1980, **45**, 453; Ivey & Robach, *ibid* 1978, **43**, 1782; Ivey *et al. J. Fd Prot.* 1978, **41**, 621; Robach, *Appl. envir. Microbiol.* 1979, **38**, 846; Robach *et al. ibid* 1978, **36**, 210; Sofos *et al. J. Fd Sci.* 1979, **44**, 1267 & 1662; *idem, ibid* 1980, **45**, 1285; Tanaka *et al. Rep. Fd Res. Inst., Tokyo* 1977, p. 36; Tompkin *et al. Appl. Microbiol.* 1974, **28**, 262; US Department of Agriculture, FSQS Four Plant Study, 23 July 1979.

sorbate is an effective compound against the growth and toxin production of *Clostridium botulinum* in meat products. Our work (Robach *et al. Fd Cosmet. Toxicol.* 1980, **18,** 237) clearly demonstrates that under the conditions of meat curing (pH above 5·5; 40 ppm NO_2^- and 2600 ppm sorbate; 550 ppm sodium ascorbate) no mutagenic products are formed.

M. C. Robach, V. G. DiFate and L. D. Kier
Monsanto Industrial Chemicals Co.,
800 N. Lindbergh Boulevard,
St. Louis, MO 63166, USA

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MEETING ANNOUNCEMENTS

AMIEV CONGRESS

AMIEV is an international medical association for the study of the influence of living conditions on health. The eighth AMIEV Congress will be held from 1–5 June 1981, in Tampere in the south of Finland. The main themes of the Congress will be "Nutrition and Health", "Accidents and Progress of Technology" and "Humanism and Medical Responsibility". Further details may be obtained from VIIIth International Congress of AMIEV, Department of Public Health, University of Tampere, P.O. Box 607, 33101 Tampere 10, Finland.

CHEMICAL CARCINOGENS IN THE WORKPLACE

A two-day conference entitled "Identification and Control of Chemical Carcinogens in the Workplace" is to be held on 30 June/1 July 1981 at the Sudbury Conference Centre, London EC1. The conference is being organized by Oyez International Business Communications Ltd. The topics to be covered include developing concepts in chemical carcinogenesis, laboratory approaches to assessing occupational cancers, short-term tests and the epidemiological approach. The progress being made in the EEC and the USA and the views of the TUC and the Health and Safety Executive in the UK will be discussed as will a chemical industry approach to controlling carcinogens. Further information may be obtained from Fiona Spindlove, Scientific & Technical Studies, Oyez IBC Ltd, Norwich House, Norwich Street, London EC4A 1AB (telephone no. 01-242 2481).

CANCER RESEARCH TRAINING COURSE

A training course in cancer research will be held at the German Cancer Research Center, Heidelberg from 31 August to 11 September 1981. The course is being organized by the German Cancer Research Center under the auspices of the Programme on Experimental Oncology of the International Union Against Cancer. It is primarily intended for post-graduate students under the age of 30, who wish to specialize in cancer research. The subjects covered will include the molecular biology of tumour viruses, methods of tissue culture, tumour immunology, cytology, pharmacology of carcinogens, animal husbandry and inbreeding, interferon, and clinical aspects of cancer. There will be no entrance fee and living expenses will be provided although students are expected to cover their travelling expenses. Applications including specified academic details should be sent before 1 May 1981 to Prof Dr Gerhard Sauer, German Cancer Research Center, Im Neuenheimer Feld, 6900 Heidelberg, Federal Republic of Germany.

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CORRIGENDA

Volume 18 (1980)

p. 736, line 40: *For* Brooks *read* Brookes.

Volume 19 (1981)

p. 126, line 44: *For* Bau-Hoï *read* Buu-Hoï.
p. 133, line 33: *For* Eden *read* Eben.
p. 135, line 15: *For* 148 *read* 149.

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FORTHCOMING PAPERS

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

Comparative mutagenicity of two triarylmethane food dyes in Salmonella, Saccharomyces and Drosophila. By D. S. Angus, R. S. U. Baker, A. M. Bonin, D. Callen and A. M. Clark.

Absorption, distribution and excretion of [^{14}C]carmoisine in mice after oral and intravenous administration. By C. L. Galli, M. Marinovich and L. G. Costa.

Inhibition of *N*-nitrosamine formation by soya products. By T. Kurechi, K. Kikugawa, S. Fukuda and M. Hasunuma.

The influence of milk in the diet on the toxicity of orally ingested lead in rats. By. R. R. Bell and J. T. Spickett.

Pesticide residues in human milk. By J.-C. Dillon, G. B. Martin and H. T. O'Brien.

The toxicity of alfalfa saponins in rats. By M. R. Malinow, W. P. McNulty, P. McLaughlin, C. Stafford, A. K. Burns, A. L. Livingston and G. O. Kohler.

Hepato- and cardiotoxicity of *Fusarium verticillioides* (*F. moniliforme*) isolates from Southern African maize. By N. P. J. Kriek, W. F. O. Marasas and P. G. Thiel.

Disposition of [^{14}C]caprolactam in the rat. By P. D. Unger, A. J. Salerno and M. A. Friedman.

Acute structural changes in renal tubular epithelium following administration of nitrilotriacetate. By J. A. Merski.

The *in vitro* assessment of severe eye irritants. By A. B. G. Burton, M. York and R. S. Lawrence.

Reductive destruction of *N*-nitrosodimethylamine as an approach to hazard control in the carcinogenesis laboratory. By G. Lunn, E. B. Sansone and L. K. Keefer.

Detection of nitrosamines in a commonly used chewing tobacco. By S. V. Bhide, A. I. Pratap, N. M. Shivapurkar, A. T. Sipahimalani and M. S. Chaddha. (Short paper).

Chromatopolarography of *N*-nitrosamines including determination of *N*-nitrosodiethanolamine in cosmetic products. By S. K. Vohra and G. W. Harrington. (Short paper).

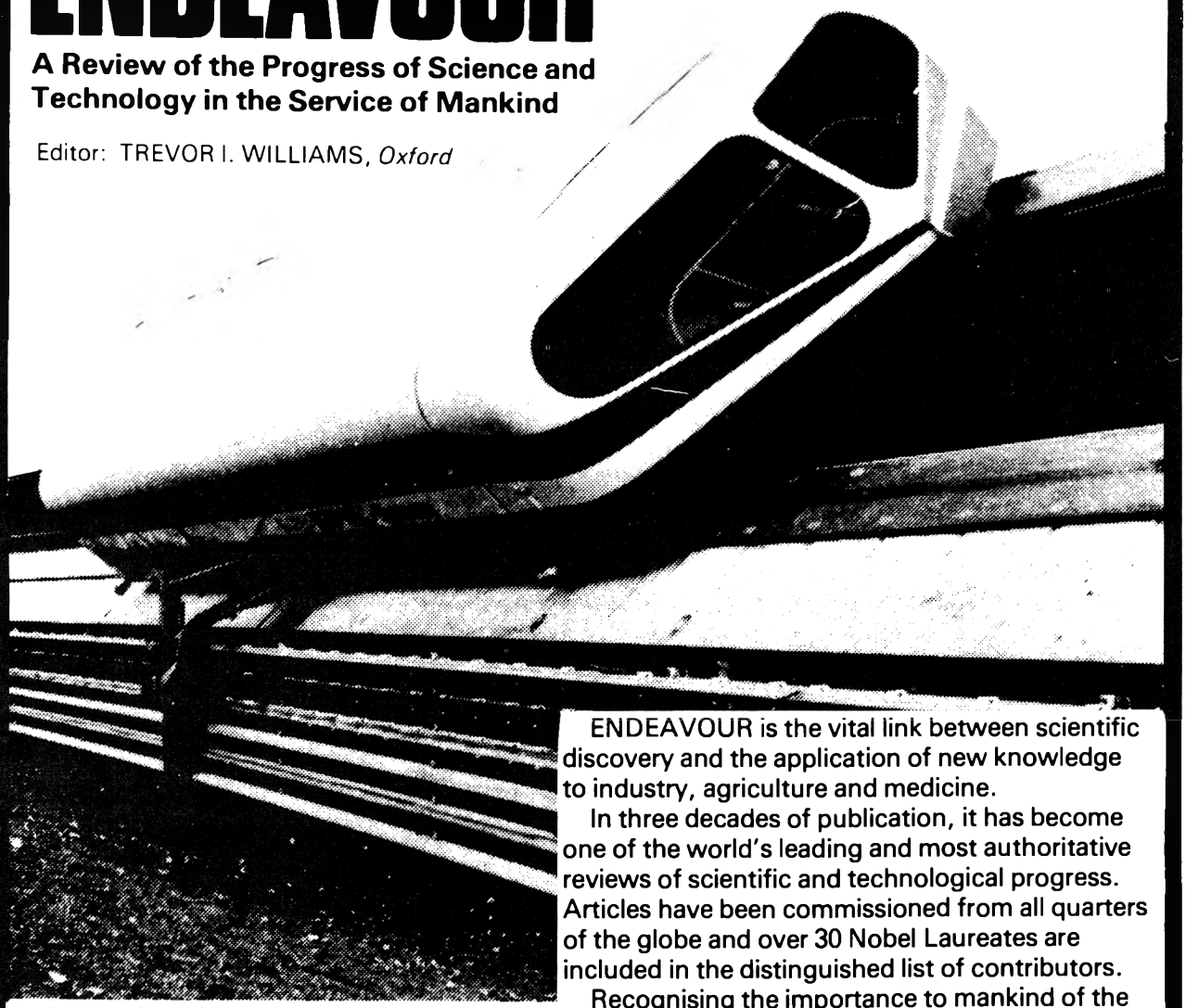
Analysis of volatile *N*-nitrosamines in commercial drugs. By M. Castegnaro, B. Pignatelli and E. A. Walker. (Short paper).

THE VITAL LINK BETWEEN SCIENCE AND INDUSTRY.....

ENDEAVOUR

A Review of the Progress of Science and Technology in the Service of Mankind

Editor: TREVOR I. WILLIAMS, *Oxford*



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[*Contents continued*]

Aims and Scope

The Journal publishes original papers and reviews relating to the interests of the British Industrial Biological Research Association. This is a wide-ranging field covering all aspects of toxicology but with particular reference to food and cosmetics. The Journal aims to be informative to all who generate or make use of toxicological data.

Instructions to Authors

General. Original papers, reviews and letters to the editor may be submitted for publication. Manuscripts from the USA and Canada should be sent to the American Editor, Dr L. Golberg. All others must be sent to the Assistant Editor or to the appropriate Regional Editor. Letters must be signed before they can be considered for publication.

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

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