Volume 14 Number 3 June 1976

Food and Cosmetics Toxicology

An International Journal published for the
British Industrial Biological Research Association

CONTENTS

Continued on inside back cover

ISSN 0015–6264

FCTXAV 14(3) 163–232 (1976)

Pergamon Press OXFORD LONDON NEW YORK PARIS

FOOD AND COSMETICS TOXICOLOGY

An International Journal published for the British Industrial Biological Research Association

Publishing Offices

Pergamon Press Limited, Headington Hill Hall, Oxford OX3 0BW, England
Pergamon Press Inc., Maxwell House, Fairview Park, Elmsford, New York 10523, USA

Advertising Office

Pergamon Press Limited, Headington Hill Hall, Oxford OX3 0BW, England

Published bi-monthly

Annual Subscription Rates (1976)

For Libraries, Research Establishments and all other multiple-reader institutions $110
Specially reduced rates for individuals: In the interests of maximizing the dissemination of the research results published in this important international journal we have established a two-tier price structure. Individuals, whose institution takes out a library subscription, may purchase a second or additional subscription for their personal use at the much reduced rate of US $25.00 per annum.
For members of BIBRA £4

Microform Subscriptions and Back Issues

Current subscriptions on microfilm, and back files on microfilm as well as back issues in the regular editions of all previously published volumes are available. Prices on request.

All subscription enquiries other than those above should be addressed to:

The Subscriptions Fulfillment Manager, Pergamon Press Limited, Headington Hill Hall, Oxford OX3 0BW

PERGAMON PRESS LIMITED

HEADINGTON HILL HALL
OXFORD OX3 0BW, ENGLAND

MAXWELL HOUSE, FAIRVIEW PARK
ELMSFORD, NEW YORK 10523, USA

Fd Cosmet. Toxicol. Vol. 14, p. i. Pergamon Press 1976. Printed in Great Britain

INFORMATION SECTION

ARTICLES OF GENERAL INTEREST*

TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS*

*These items have been contributed by the staff of the British Industrial Biological Research Association. Comments on them will be welcomed and should be sent to the Assistant Editor at BIBRA.

Fd Cosmet. Toxicol. Vol. 14, pp. 163–165. Pergamon Press 1976. Printed in Great Britain

Research Section

FAILURE OF FD & C RED NO. 2 TO PRODUCE DOMINANT LETHAL EFFECTS IN THE MOUSE

D. W. Arnold, G. L. Kennedy, Jr., M. L. Keplinger and J. C. Calandra

Industrial BIO-TEST Laboratories, Inc., 1810 Frontage Road, Northbrook, Illinois 60062, USA

(*Received* 20 *November* 1975)

Abstract—Groups of 12 male albino mice were given a single ip injection of either 250 or 500 mg FD & C Red No. 2/kg body weight. Each male was subsequently mated with three females each week for six consecutive weeks. Genetic damage, indicated by an increase in dominant lethal mutations observed *in utero*, was not produced.

INTRODUCTION

The colour additive amaranth (FD & C Red No. 2) is the trisodium salt of 1-(4-sulpho-1-naphthylazo)-2-naphthol-3,6-disulphonic acid. The acceptable daily intake (ADI) had been established for man at up to 1·5 mg/kg by the Joint FAO/WHO Expert Committee on Food Additives in 1966, but the same committee recommended a temporary ADI in 1972 of up to 0·75 mg/kg, which suggested that the toxicological evaluation of the compound should be expanded.

Reproduction studies (Shtenberg & Gavrilenko, 1970) indicated a possible embryotoxic response in the rat. No evidence of teratogenicity was obtained in the rat by Collins & McLaughlin (1972) but increased embryolethality was reported. A second study by Shtenberg & Gavrilenko (1972) indicated that FD & C Red No. 2 was foetolethal at a dose as low as 1·5 mg/kg body weight. On the other hand, no evidence for either teratogenicity or embryotoxicity in the rat was reported by Keplinger, Wright, Plank & Calandra (1974), Khera, Przybylski & McKinley (1974) or Larsson (1975). Further, a collaborative teratological study involving both government and industry (Collins, Holson, Kennedy, Gaylor & Ruggles, 1975) failed to produce any increase in early foetal death that could be related to ingestion of the dye.

This study was undertaken to see whether genetic damage, as measured by an increase in early foetal death, could be produced in the mouse.

EXPERIMENTAL

Material. A composite sample made from equal portions of dye supplied by each of the US manufacturers and certified by the FDA as FD & C Red No. 2, Batch CCIC-1 (89·2% Red No. 2, 7·03% volatile matter, 3·1% NaCl, 1·0% Na_2SO_4 and 0·07% R-salt) was obtained from the Inter-Industry Color Committee.

Animals and diet. The animals used were CD-1 random-bred albino mice purchased from Charles River Breeding Laboratories, Wilmington, Mass. All animals were 80 days old when placed in the experiment. They were maintained on a standard pelleted feed, obtained from Ralston Purina, St. Louis, Mo., with water provided *ad lib.*

Experimental design

Range-finding study. Male mice were divided into five groups each of four animals in order to determine appropriate exposure levels for the main study. Animals were treated with a single ip injection of FD & C Red No. 2 at doses ranging from 30 to 3000 mg/kg body weight and were observed for any pharmacotoxic response hourly for the first 8 hr and then once daily for each of the next 14 days. At this point, an LD_{50} was calculated.

Dominant lethal study. Male mice were divided into three groups each of 12 animals and were treated by ip injection with a 5% aqueous solution of FD & C Red No. 2 at dose levels of 250 or 500 mg/kg body weight. These exposure levels were selected from results of the preliminary range-finding study. Concurrent control animals were treated with distilled water in volumes equivalent to that received by the mice on the higher test level. Immediately after treatment, each male was housed individually with three untreated nulliparous females. Females were changed weekly, caged by group and replaced by fresh females for a period of six consecutive weeks.

Females were killed 1 wk after their removal from the breeding cages and were examined for signs of pregnancy. The numbers of implantation sites were divided into embryos and resorption sites, which were differentiated into early (deciduomas) and late. From these data, mating indices and mutation rates were calculated according to the following definitions:

$$\text{Mating index} = \frac{\text{no. of females pregnant}}{\text{no. of females mated}} \times 100$$

$$\text{Pre-implantation loss} = \frac{\text{no. of corpora lutea (calc.)} - \text{no. of implantation sites}}{\text{no. of corpora lutea (calc.)}} \times 100$$

$$\text{Percentage of deciduomas} = \frac{\text{no. of early embryonic deaths (deciduomas)}}{\text{total no. of implantation sites}} \times 100$$

Embryonic index =

$$100 - \left(\frac{\text{no. of embryos/female test}}{\text{no. of embryos/female control}} \times 100 \right)$$

In calculating the total number of corpora lutea, the 'historical number' of corpora lutea/female was used. This value was obtained from corpora-luteal counts obtained from untreated male–female pairings and histological examination of the ovaries from the pregnant females. This procedure was used to overcome the difficulty inherent in counting corpora lutea accurately in mice and to allow an estimation of pre-implantation losses in these animals. Statistical evaluations compared results obtained in the test groups with those of the parallel control animals. Mating indices and pre-implantation losses were evaluated using the Chi-square test and deciduomas were evaluated using Student's *t* test (Krüger, 1970).

RESULTS AND DISCUSSION

In the preliminary acute studies, all mice treated with 3000 mg FD & C Red No. 2/kg died in the 12 hr following treatment. Generalized hypoactivity,

Table 1. *Mean values for reproduction data in untreated female mice mated with males given an ip injection of FD & C Red No. 2 in a dose of 0–500 mg/kg*

Dose level (mg/kg)	Test wk no.	No. of females pregnant	Implantation sites*	Resorption sites*		Embryos*
				Early	Late	
0	1	20	12·4	0·8	0·1	11·4
	2	23	13·6	0·7	0·3	12·7
	3	24	12·7	0·4	0·1	12·2
	4	22	12·4	0·8	0·1	11·4
	5	21	11·8	0·6	0·0	11·2
	6	23	12·9	0·9	0·2	11·7
250	1	23	12·5	0·9	0·1	11·6
	2	27	12·0	1·0	0·0	11·0
	3	30	11·9	0·6	0·1	11·2
	4	27	12·4	0·6	0·1	11·7
	5	18	11·7	0·4	0·1	11·2
	6	20	12·0	0·6	0·1	11·4
500	1	35	12·5	0·8	0·1	11·6
	2	25	11·2	0·6	0·1	10·6
	3	20	11·6	0·5	0·0	11·2
	4	26	12·1	0·6	0·0	11·5
	5	25	12·2	0·3	0·0	11·9
	6	20	12·8	0·8	0·1	12·0

*Mean no./pregnancy.

Table 2. *Mating indices and mutation rates in untreated female mice mated with males given ip injection of FD & C Red No. 2 in a dose of 0–500 mg/kg*

Dose level (mg/kg)	Test wk no.	Mating index†	Pre-implantation loss† (%)	Deciduomas† (%)	Embryonic index†
0	1	55·6	8·2	6·4	—
	2	64·0	0·0	5·1	—
	3	66·7	5·9	3·6	—
	4	61·1	8·1	6·2	—
	5	58·3	12·7	5·2	—
	6	64·0	4·2	7·1	—
250	1	64·0	7·1	6·9	− 1·8
	2	75·0	11·0*	8·0	13·4
	3	83·3*	11·9*	4·0	8·2
	4	75·0	8·0	4·8	− 2·6
	5	50·0	13·2	3·3	0·0
	6	55·6	11·1*	4·6	2·6
500	1	97·2*	7·3	6·6	− 1·8
	2	69·4	17·2*	5·0	16·5
	3	55·6	14·1*	4·3	8·2
	4	72·2	10·3	5·1	− 0·9
	5	69·4	9·8	2·3	− 6·2
	6	55·6	5·2	6·2	− 2·6

†For definitions, see under Experimental.

Values are derived from the data given in Table 1 and those marked with an asterisk differ significantly ($P < 0·01$) from the corresponding control value.

accompanied by discoloration (deep red to purple) of the testes, ears and extremities, was observed prior to death. At a dose level of 1000 mg/kg, two of the four treated animals died within 24 hr of dosing. Again, reduced activity was observed along with discoloration, which remained apparent until death in the two mice, but which was not apparent 24–30 hr after treatment in the survivors. Minimal hypoactivity and slight colour change was observed in animals treated with 300 mg/kg; this was not apparent 24 hr after dosing. No signs of a response to the chemical were seen at a dose of either 30 or 100 mg/kg. From these results, an approximate LD_{50} of 1000 mg/kg was established and 250 and 500 mg/kg were selected as dose levels for the main study.

In the dominant lethal study, no signs of a toxic response to FD & C Red No. 2 were seen among males given either dose, and no deaths occurred. In the first 24 hr after treatment, animals treated with 500 but not with 250 mg/kg displayed slight colour changes in the extremities.

The autopsies on females that had mated revealed no differences between test and control groups in the numbers of implantations, resorptions (deciduomas) or embryos (Table 1). The mating abilities of treated animals were comparable to those of the control group although two values, those for the 250-mg/kg animals during mating wk 3 and for the 500-mg/kg animals during mating wk 1, were significantly greater than those for the control group (Table 2). The direction of the change (an increase) and the rather large background deviation seen in control populations for this strain of mouse (50–90%) makes the significance of the finding doubtful, however.

The percentages of deciduomas among test animals were essentially the same as those in the controls (Table 2). Values for the embryonic index, which serves as an indirect measure of pre-implantation losses, and the calculated pre-implantation losses are also presented in Table 2. Pre-implantation loss ranged from 0 to 12·7% among the control animals. Statistically significant increases in pre-implantation loss were observed in the 250-mg/kg group during mating wk 2, 3 and 6 and in the 500-mg/kg group during wk 2 and 3. In all cases, the differences resulted from an unusually low background rate (0–5·9%), all values for the test groups being within the range (0–20%) for this parameter that has been established as normal in this strain of mouse using larger populations. It was considered, therefore, that the statistical differences detected reflected biological variation rather than a treatment-related effect.

It is concluded that FD & C Red No. 2, given as a single ip injection at maximally tolerated levels (approximately one half of the calculated LD_{50}) does not produce an increase in early foetal death (dominant lethals) in the mouse.

REFERENCES

Collins, T. F. X. & McLaughlin, J. (1972). Teratology studies on food colourings. Part I. Embryotoxicity of amaranth (FD & C Red No. 2) in rats. *Fd Cosmet. Toxicol.* **10,** 619.

Collins, T. F. X., Holson, J. F., Jr., Kennedy, G. L., Jr., Gaylor, D. W. & Ruggles, D. I. (1975). Teratological evaluation of FD & C Red No. 2—A collaborative government-industry study. In press.

Joint FAO/WHO Expert Committee on Food Additives (1966). Specifications for Identity and Purity and Toxicological Evaluation of Food Colours. WHO/Food Add./66.25. *F.A.O. Nutr. Mtg Rep. Ser.* no. 38B, p. 22.

Keplinger, M. L., Wright, P. L., Plank, J. B. & Calandra, J. C. (1974). Teratologic studies with FD & C Red No. 2 in rats and rabbits. *Toxic. appl. Pharmac.* **28,** 209.

Khera, K. S., Przybylski, W. & McKinley, W. P. (1974). Implantation and embryonic survival in rats treated with amaranth during gestation. *Fd. Cosmet. Toxicol.* **12,** 507.

Krüger, J. (1970). Statistical methods in mutation research. In *Chemical Mutagenesis in Mammals and Man.* Edited by F. Vogel and G. Röhrborn. Springer-Verlag, Berlin, p. 460.

Larsson, K. S. (1975). A teratologic study with the dyes amaranth and Ponceau 4R in mice. *Toxicology* **4,** 75.

Shtenberg, A. I. & Gavrilenko, Ye. V. (1970). Influence of the food dye amaranth upon the reproductive function and development of progeny in tests on albino rats. *Vop. Pitan.* **29** (2), 66.

Shtenberg, A. I. & Gavrilenko, Ye. V. (1972). The gonadotoxic and embryotoxic effect of the food dye amaranth. *Vop. Pitan.* **31** (5), 28.

Fd Cosmet. Toxicol. Vol. 14, pp. 167–170. Pergamon Press 1976. Printed in Great Britain

DIMETHYLNITROSAMINE AND NITROSOPYRROLIDINE IN FUMES PRODUCED DURING THE FRYING OF BACON

N. P. SEN, S. SEAMAN and W. F. MILES

Food Research Division, Food Directorate, Health Protection Branch, Ottawa, Canada K1A OL2

(*Received* 15 *December* 1975)

Abstract—A study was undertaken to determine the amounts of volatile nitrosamines given off in the fumes during the normal cooking of cured bacon. The results show that, on average, about 70% of the total dimethylnitrosamine and 50% of the total nitrosopyrrolidine produced during cooking volatilized in the fumes. The treatment of samples of bacon with ascorbyl palmitate just before cooking markedly reduced the concentrations of the two nitrosamines in the fumes. The results were confirmed by combined gas–liquid chromatographic and high-resolution mass spectrometric analysis.

INTRODUCTION

N-Nitrosamines are a group of potent chemical carcinogens (Druckrey, Preussmann, Ivankovic & Schmähl, 1967; Lijinsky, Tomatis & Wenyon, 1969; Magee & Barnes, 1956). Recent studies have demonstrated traces of certain nitrosamines in some foodstuffs, especially nitrite-cured meat products (Crosby, Foreman, Palframan & Sawyer, 1972; Sen, 1972; Wasserman, Fiddler, Doerr, Osman & Dooley, 1972). Among the cured-meat products studied, fried bacon has been shown consistently to contain dimethylnitrosamine (DMN) and nitrosopyrrolidine (NPY), the latter being present in higher concentrations than the former (Fazio, White, Dusold & Howard, 1973; Pensabene, Fiddler, Gates, Fagan & Wasserman, 1974; Sen, Donaldson, Iyengar & Panalaks, 1973; Telling, Bryce, Hoar, Osborne & Welti, 1974). Raw bacon is usually free of nitrosamines, most of which are formed during cooking. The concentration of nitrosamines in the cooked product is proportional to that of the nitrite used in preparing the bacon (Gough & Walters, 1975; Sen, Iyengar, Donaldson & Panalaks, 1974b).

Since both DMN and NPY are volatile compounds it was thought that considerable amounts of these compounds would be lost into the air during frying. In this paper we present data showing that the fumes produced during the frying of bacon do indeed contain significant amounts of both DMN and NPY. Some data on the inhibitory effect of ascorbyl palmitate on the concentrations of DMN and NPY in the bacon fumes are also presented.

EXPERIMENTAL

Materials. The bacon samples were purchased from the local supermarkets. The samples were cooked and analysed either immediately or after storage for 1–3 days in a refrigerator. Ascorbyl palmitate was purchased from ICN-K&K Laboratories Inc.

Cooking and fume collection. Samples of about 100 g were fried for 13 min in an electric frying pan at a temperature setting of 340–350°F (the setting recommended by the pan manufacturer). In a few cases, the samples were cooked for 8–10 min at a setting of 400°F. The cooking was carried out with the lid placed on the frying pan. The fumes produced during frying were sucked through a hole at the centre of the lid and passed in succession via tygon tubing to a cold trap and a water aspirator. The cold trap consisted of a tall cylindrical washing bottle in which the long inlet tube was joined at the end with a fritted glass cylinder (similar to the Corning gas washing bottle, catalogue no. 441460, Corning International Corp., Corning, New York). The washing bottle was filled with 200 ml of the appropriate trapping liquid (see below) and the entire bottle was immersed in an ice-water mixture. The trapping liquid was allowed to cool for 15 min before cooking and fume collection was begun. Three different types of trapping liquids were used, namely methylene chloride, 1 N- or 3 N-potassium hydroxide and an aqueous solution containing 0·25% gallic acid and 0·05% piperazine hydrate. The suction rate was such that it was capable of emptying 2 litres water/min from a filled jar. During the cooking period the lid was raised for a few seconds every 2 min and the bacon strips were turned over for uniform cooking.

Treatment with ascorbyl palmitate. Ascorbyl palmitate was sprinkled from a salt shaker on to both sides of the bacon strips (approximately 160–240 mg/100 g) and the samples were cooked and the fumes collected as described above.

Analysis of nitrosamines. Enough solid potassium hydroxide was added to the liquid in the cold trap to make it 3 N in strength, and the solution was distilled under vacuum in a rotary evaporator. In the case of the methylene chloride trap, 200 ml 3 N-potassium hydroxide solution was used instead of the solid reagent. The nitrosamines in the distillate were extracted into methylene chloride, cleaned up on a basic alumina column and analysed by gas–liquid chromatography (GLC) using a Coulson electrolytic conductivity detector (pyrolytic mode), thin-layer chromatography (TLC) and GLC–high-resolution mass spectrometry as described previously (Sen *et al.* 1974b; Sen, Donaldson, Seaman, Iyengar & Miles,

Table 1. *DMN and NPY in cooked bacon and in the fumes*

Brand	Sample	DMN Found* (μg)	DMN % of total	NPY Found* (μg)	NPY % of total
A	Cooked fat + lean	0·2†	12	1·8†	18
	Fumes	1·7†	88	8·5†	82
B	Cooked fat + lean	0·3	28	2	53
	Fumes	0·7	72	1·8	47
C	Cooked fat + lean	0·4†	13	3·2†	45
	Fumes	2·8†	87	4†	55
C	Cooked lean + fat	1·8†	27	12·1†	37
	Fumes	4·9‡	73	20·4‡	63
C	Fumes	1·4†		11·2†	
D	Cooked fat + lean	0·4	40	2	52
	Fumes	0·6†	60	1·8†	48
D	Fumes	0·3†		2·3†	
D	Cooked lean + fat	0·2	8	4	50
	Fumes	2·2	92	4	50
E	Fumes	0·7		3·5	
F	Cooked lean + fat	0·5	72	2	72
	Fumes	0·2	28	0·8	28
G	Cooked lean + fat	0·6†	34	4·4†	67
	Fumes	1·2†	66	2·2†	33

DMN = Dimethylnitrosamine NPY = Nitrosopyrrolidine

*Bacon samples weighing 100 g were cooked in each case.

†Confirmed by GLC–high-resolution mass spectrometry by *m/e* monitoring for NO^+ ion at a resolution of 5000.

‡Confirmed by GLC–high-resolution mass spectrometry by *m/e* monitoring for both NO^+ ion (5000 resolution) and the molecular ion, M^+ (10,000 resolution).

1976). The cooked-out fat and the cooked lean were blended together and analysed by the same method, except that the samples were taken through a preliminary extraction step (with methylene chloride) before distillation (Sen, Donaldson, Charbonneau & Miles, 1974a).

RESULTS

The data in Table 1 show that considerable amounts of both DMN and NPY were present in the fumes. Since DMN is more volatile than NPY the percentage loss (of the total) of DMN (average 70%) in the fumes was greater than the corresponding figure for NPY (average 50%). These results are very similar to those reported recently by Gough & Walters (1975). Treatment of bacon with ascorbyl palmitate markedly reduced the concentration of both DMN and NPY in the fumes (Table 2). Since we had already shown (Sen *et al.* 1976) that similar treatments also inhibited the formation of NPY in the

Table 2. *Inhibition of nitrosamine formation in ascorbyl palmitate-treated bacon fumes*

Brand	Sample	DMN μg*	DMN % inhibition	NPY μg*	NPY % inhibition
A	Untreated	1·7†		8·5†	
	Treated	0·5†	73	1·4†	83
B	Untreated	0·7		1·8	
	Treated	0·2	71	0·2	88
C	Untreated	4·5		5	
	Treated	0·6	86	1	80
C	Untreated	4·9‡		20·4‡	
	Treated	1·6†	67	3·3†	84
E	Untreated	0·7		3·5	
	Treated	0·2	71	1	71
F	Untreated	0·2		0·8	
	Treated	trace	>90	0·2	75

DMN = Dimethylnitrosamine NPY = Nitrosopyrrolidine

*Bacon samples weighing 100 g were cooked in each case.

†Confirmed by GLC–high-resolution mass spectrometry by *m/e* monitoring for NO^+ ion at a resolution of 5000.

‡Confirmed by GLC–high-resolution mass spectrometry by *m/e* monitoring for both NO^+ ion (5000 resolution) and the molecular ion, M^+ (10,000 resolution).

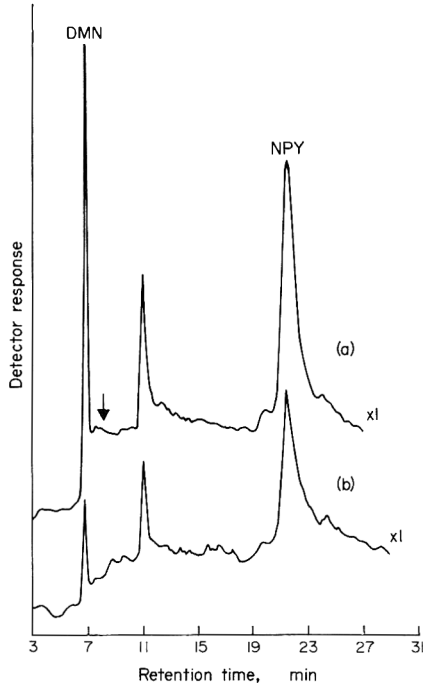

Fig. 1. GLC diagrams using a Coulson electrolytic conductivity detector (pyrolytic mode): (a) 4·8 μl/ml of final extract from bacon fumes collected in the piperazine/gallic acid trap containing 5 mg added diethylamine hydrochloride; (b) 5·2 μl/ml of final extract obtained from the cooked-out fat and lean from the same bacon. The arrow indicates the retention time of DEN, which was not formed in the trap. Conditions: 6 ft × 1/8 in. o.d. stainless-steel column packed with 25% Carbowax 20 M and 2% sodium hydroxide on 60–80 mesh Chromosorb P; He flow, 25 ml/min; column temperature, 140°C for 3 min, then programmed to 180°C at the rate of 10°C/min; Coulson furnace temperature (pyrolytic mode), 360–380°C.

cooked-out fat and the cooked lean, these cooked samples were not analysed in this study.

In addition to DMN and NPY, a few samples of bacon fumes contained traces of diethylnitrosamine (DEN). Since both the concentration of DEN and the frequency of its occurrence were lower than that of the other two nitrosamines, it was not studied in detail.

Figures 1 and 2 give examples of the diagrams obtained by GLC (Coulson detector) and GLC–high-resolution mass spectrometric analysis, respectively.

DISCUSSION

The results presented in this paper clearly demonstrate that traces of nitrosamines are present in the fumes produced during the frying of bacon, but it is not clear whether such minute levels of nitrosamines would pose any toxicological hazard to man. Since both DMN and NPY are strong carcinogens, the findings cannot be overlooked. Under unusual circumstances, such as when a person is constantly exposed to such fumes, problems may arise.

To investigate the remote possibility that the nitrosamines were formed in the collection traps, some of the samples were analysed using the three different types of trapping liquid described under Experimental. The results obtained in these instances were very similar even though the composition and pH (strongly alkaline to acidic) of the various trapping liquids differed considerably. If the nitrosamines were formed in the trap, the pH should have had a marked influence on the yields, and widely differing results should have been obtained. Moreover, in several experiments, the fumes were collected after addition of 5 mg diethylamine hydrochloride to the trap to see whether any DEN was formed under the conditions

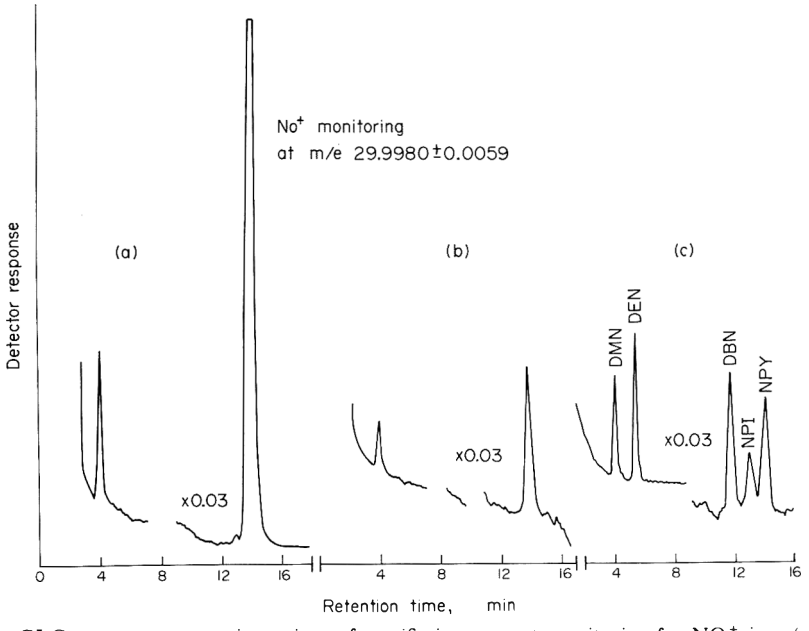

Fig. 2. GLC–mass spectrometric tracings of specific ion current monitoring for NO^+ ion: (a) 4·1 μl/ml of final extract from bacon fumes; (b) 4·2 μl/ml of extract of bacon fumes from the same sample cooked after treatment with ascorbyl palmitate; (c) nitrosamine standards, 4–6 ng of each (DBN = di-*n*-butylnitrosamine; NPI = nitrosopiperidine). A Varian Mat (model 311A) high-resolution mass spectrometer was used; for details, see Sen *et al.* (1976).

used. As can be seen from Fig. 1, no detectable DEN was formed, even though the fumes contained both DMN and NPY. Therefore, it is highly unlikely that any significant level of nitrosamine was formed in the traps used in this experiment. It should be pointed out that piperazine and gallic acid were chosen as constituents of one of the traps because of their well-known capacity to destroy nitrite (or other nitrosating agents), thereby preventing any formation of DMN and NPY in the cold traps.

The levels of nitrosamines found in this study in the cooked-out fat and cooked lean were very similar to those normally found in these products when cooked in an open frying pan without any applied suction. Therefore, it is reasonable to conclude that the nitrosamines collected in the traps originated from the fumes, and did not come from the nitrosamines in the cooked products as a result of the mild suction applied during collection of the fumes.

As mentioned above, treatment of bacon with ascorbyl palmitate just prior to cooking significantly reduced the concentration of NPY in the cooked product. Since the concentration of DMN in cooked bacon is normally very low (1–5 ppb; $b = 10^9$), it was not possible at that time to determine, with certainty, the inhibitory effect, if any, of ascorbyl palmitate on the level of DMN. The results of this study clearly demonstrate that such treatment also inhibits the formation of DMN. Since DMN is very volatile and less soluble in the fat than NPY, one sees only a low residual level in the cooked products. Our results on the bacon fumes also support our previous conclusion that the major part of the NPY in cooked bacon is formed by nitrosation reactions occurring during cooking and not by the decarboxylation of preformed nitrosoproline already present in the raw bacon. If the latter were the case, one would not be able to inhibit the formation of NPY by treatment with ascorbyl palmitate.

There is a possibility that traces of nitrosamines may be present in the fumes or vapours produced during the cooking of other cured meat products. Further studies are needed to explore this possibility. There is also a need for further research to determine whether ascorbyl palmitate can be used as an additive in bacon. This would definitely reduce the concentration of nitrosamines in the cooked products as well as in the fumes produced during cooking.

REFERENCES

Crosby, N. T., Foreman, J. K., Palframan, J. F. & Sawyer, R. (1972). Estimation of steam-volatile *N*-nitrosamines in foods at the 1 μg/kg level. *Nature, Lond.* **238,** 342.

Druckrey, H., Preussmann, R., Ivankovic, S. u. Schmähl, D. (1967). Organotrope carcinogene Wirkungen bei 65 verschiedenen N-Nitroso-Verbindungen an BD-Ratten. *Z. Krebsforsch.* **69,** 103.

Fazio, T. H., White, R. H., Dusold, L. R. & Howard, J. W. (1973). Nitrosopyrrolidine in cooked bacon. *J. Ass. off. analyt. Chem.* **56,** 919.

Gough, T. A. & Walters, C. L. (1975). Volatile nitrosamines in fried bacon. Presented at the IV International Working Conference on the Analysis and Formation of N-Nitroso Compounds, Sept 30–Oct 3, 1975, Tallinn, Estonia, USSR.

Lijinsky, W., Tomatis, L. & Wenyon, C. E. M. (1969). Lung tumors in rats treated with *N*-nitrosoheptamethyleneimine and *N*-nitrosooctamethyleneimine. *Proc. Soc. exp. Biol. Med.* **130,** 945.

Magee, P. N. & Barnes, J. M. (1956). The production of malignant primary hepatic tumours in the rat by feeding dimethylnitrosamine. *Br. J. Cancer,* **10,** 114.

Pensabene, J. W., Fiddler, W., Gates, R. A., Fagan, J. C. & Wasserman, A. E. (1974). Effect of frying and other cooking conditions on nitrosopyrrolidine formation in bacon. *J. Fd Sci.* **39,** 314.

Sen, N. P. (1972). The evidence for the presence of dimethylnitrosamine in meat products. *Fd Cosmet. Toxicol.* **10,** 219.

Sen, N. P., Donaldson, B., Charbonneau, C. & Miles, W. F. (1974a). Effect of additives on the formation of nitrosamines in meat curing mixtures containing spices and nitrite. *J. agric. Fd Chem.* **22,** 1125.

Sen, N. P., Donaldson, B., Iyengar, J. R. & Panalaks, T. (1973). Nitrosopyrrolidine and dimethylnitrosamine in bacon. *Nature, Lond.* **241,** 473.

Sen, N. P., Donaldson, B., Seaman, S., Iyengar, J. R. & Miles, W. F. (1976). Inhibition of nitrosamine formation in fried bacon by propyl gallate and L-ascorbyl palmitate. *J. agric. Fd Chem.* **24,** 397.

Sen, N. P., Iyengar, J. R., Donaldson, B. A. & Panalaks, T. (1974b). Effect of sodium nitrite concentration on the formation of nitrosopyrrolidine and dimethylnitrosamine in fried bacon. *J. agric. Fd Chem.* **22,** 540.

Telling, G. M., Bryce, T. A., Hoar, D., Osborne, D. & Welti, D. (1974). Progress in the analysis of volatile *N*-nitroso compounds. In *N-Nitroso Compounds in the Environment.* Edited by P. Bogovski and E. A. Walker, IARC Scient. Publ. no. 9, p. 12. International Agency for Research on Cancer, Lyon.

Wasserman, A. E., Fiddler, W., Doerr, R. C., Osman, S. F. & Dooley, C. J. (1972). Dimethylnitrosamine in frankfurters. *Fd Cosmet. Toxicol.* **10,** 681.

Fd Cosmet. Toxicol. Vol. 14, pp. 171–174. Pergamon Press 1976. Printed in Great Britain

STUDIES ON AFLATOXIN B_1 TOXICITY IN FEMALE RATS PRETREATED WITH AN ORAL CONTRACEPTIVE AGENT

M. U. K. MGBODILE

Department of Biochemistry and Nutrition, Meharry Medical College, Nashville, Tennessee 37208

and

M. HOLSCHER

Department of Pathology, School of Medicine, Vanderbilt University, Nashville, Tennessee 37232, USA

(*Received* 17 *October* 1975)

Abstract—The effect of short-term ingestion of an oral contraceptive agent on aflatoxin B_1 hepatotoxicity in female rats was investigated. Oral administration of 100 μg Ovral-28 for 16 consecutive days prior to ip administration of a single dose of aflatoxin B_1 diminished the hepatic necrosis observed in rats that received aflatoxin alone. Pretreatment with Ovral-28 also prevented completely the decrease in the level of hepatic reduced glutathione and partially reversed the decrease in the activity of the hepatic drug-metabolizing enzyme system caused by aflatoxin B_1.

INTRODUCTION

In recent years, several investigators have produced data showing that the microsomal mixed-function oxidase system catalyses the metabolic conversion of aflatoxin B_1 to an epoxide derivative which is thought to be responsible for producing the hepatic damage seen in animals exposed to the toxin (Garner, Miller & Miller, 1972; Schoental, 1970; Swenson, Miller & Miller, 1974). Dietary modification of the activity of this enzyme system may be responsible for the variation in responses seen when aflatoxin B_1 is administered to nutritionally deficient animals. For example, weanling rats on a low-protein diet which produced quantitative and qualitative changes in the drug metabolizing enzymes (Mgbodile & Campbell, 1972) showed marked susceptibility to the hepatotoxic effect of aflatoxin B_1 (Madhavan & Gopalan, 1965), whereas a high-protein diet appeared to induce the development of hepatomas (Madhavan & Gopalan, 1968). Marginal deficiency of dietary lipotropes potentiated the development of tumours, while rats of the same strain receiving a diet severely deficient in lipotropes were spared from aflatoxin-induced carcinogenesis (Rogers & Newberne, 1969 & 1971). Furthermore, pregnancy has been reported to increase the susceptibility of rats to the toxic effect of aflatoxin B_1 (Butler & Wigglesworth, 1966).

Increased foetal deaths (Le Breton, Frayssinet, Lafarge & DeRecondo, 1964) and foetal growth retardation (Butler & Wigglesworth, 1966; Le Breton *et al.* 1964) have been reported in rats given a single large dose or multiple small doses of aflatoxin B_1 in late pregnancy. No direct relationship was established, however, between aflatoxin foetotoxicity and the high levels of maternal oestrogen and progesterone encountered in those studies. The altered hormonal state associated with pregnancy may be responsible for the toxicity of aflatoxin B_1 in pregnant animals. In the present study, the effect of prior administration of oral contraceptive steroids on the hepatotoxicity of aflatoxin B_1 in female rats has been examined.

EXPERIMENTAL

Materials. Ovral-28, the oral contraceptive agent used in this study, contained 0·5 mg norgestrel and 0·05 mg ethinyloestradiol, and was a generous gift from the Family Planning Clinic of Lentz Public Health Center, Nashville, Tenn. Aflatoxin B_1 was purchased from Calbiochem, Atlanta, Georgia.

Animals and treatment. For each of the three experiments described below, virgin female Sprague–Dawley rats weighing 150–180 g were divided into four groups of five or seven rats (see tables of results). They were fed a semi-purified diet (Weatherholtz, Campbell & Webb, 1969) *ad lib.* and had free access to tap-water. Two of the four groups in each study received a daily oral dose of 100 μg Ovral-28 in 0·2 ml corn oil on 16 consecutive days, while the other two were given only 0·2 ml corn oil daily for the same period. One of the groups given Ovral-28 and one given corn oil alone received a single ip dose of 3 mg aflatoxin B_1/kg dissolved in 0·2 ml dimethylsulphoxide, 24 hr after the last dose of the contraceptive agent or corn oil, at which time the other two (control) groups received an ip dose of 0·2 ml dimethylsulphoxide only.

Experimental procedures

Effect of Ovral-28 pretreatment on aflatoxin B_1 lethality. In four groups of rats treated as described, the deaths occurring up to 48 hr after the single-dose treatment were recorded.

Effect of Ovral-28 pretreatment on aflatoxin B_1-induced hepatic necrosis and on reduced glutathione levels. The severity of hepatocellular damage in female rats given aflatoxin alone was compared with that

in animals given a daily dose of 100 μg Ovral-28 for 16 days before the aflatoxin treatment. Animals were treated as described above and all four groups were killed 48 hr after aflatoxin or dimethylsulphoxide administration, liver slices being preserved in 10% buffered formalin for histopathological studies.

The effect of administration of aflatoxin B_1 on the levels of tissue glutathione in rats pretreated with Ovral-28 was also studied, to establish the relationship between the levels of liver- and blood-glutathione and the severity of hepatic damage. The levels of reduced glutathione in the liver 6 and 48 hr after aflatoxin administration were determined by the method previously described by Beutler, Duron & Kelly (1963). Blood levels of reduced glutathione were determined 48 hr after a single ip dose of aflatoxin.

Hepatic microsomal mixed-function oxidase activity and cytochrome P-450 content. The effects of Ovral-28 pretreatment on aflatoxin B_1-induced changes in the activity of the hepatic microsomal mixed-function oxidase system and in the cytochrome *P*-450 content were studied using hepatic microsomal fractions isolated by the method of Cinti, Ritchie & Schenkman (1972) from rats treated as before and killed 48 hr after the aflatoxin or dimethylsulphoxide injection. Microsomal protein concentrations were determined by the biuret method (Layne, 1957). The activity of the mixed-function oxidase system was measured using benzphetamine as the substrate (Norman, Vaughn & Neal, 1973) and the microsomal cytochrome *P*-450 was determined quantitatively by the method of Omura & Sato (1964) using a molar extinction coefficient of 91 $mmol^{-1}$ cm^{-1}. Data were analysed statistically using Student's *t* test.

RESULTS

The extent to which pretreatment of female rats with the oral contraceptive agent affected aflatoxin B_1 toxicity, as measured by the number of deaths in the 48 hr following administration of a single dose of 3 mg aflatoxin/kg, is summarized in Table 1. A high death rate (43%) followed aflatoxin administration to rats not pretreated with the contraceptive steroids. All animals pretreated with the contraceptive preparation and then given a single dose of aflatoxin and all those given dimethylsulphoxide alone or the hormonal steroids and dimethylsulphoxide survived.

Table 1. *Effect of pretreatment with the oral contraceptive, Ovral-28, on the death rate in female rats treated with aflatoxin B_1*

Treatment*		No. of rats/group	Deaths within 14 days (%)
Daily oral doses (16)	Single ip dose		
Corn oil	DMSO†	5	0
	AFB_1	7	43
Ovral-28	DMSO†	7	0
	AFB_1	7	0

DMSO = Dimethylsulphoxide AFB_1 = Aflatoxin B_1
*Daily oral doses consisted of 0·2 ml corn oil alone or 100 μg Ovral-28 (norgestrel and ethinyloestradiol; single ip doses were of 0·2 ml DMSO alone (controls) or 3 mg AFB_1/kg in 0·2 ml DMSO.
†Control groups.

Figures 1–4 present the results of histopathological examinations of the livers of animals that received various treatments. The livers of the animals given dimethylsulphoxide alone (Fig. 1) appeared histologically normal, as did those of the group given the contraceptive preparation alone (Fig. 2). Livers of rats pretreated with the contraceptive agent and then given a single dose of aflatoxin showed minimal necrosis around the periportal area (Fig. 3). The livers of animals receiving aflatoxin alone, on the other hand, developed large zones of necrosis in the periportal area and extensive fatty infiltration was clearly evident (Fig. 4).

Table 2 summarizes the effect of aflatoxin B_1 on the levels of reduced glutathione in liver and blood. Compared with the levels in dimethylsulphoxide-treated controls, aflatoxin elicited 51 and 38% decreases in the levels of hepatic glutathione 6 and 48 hr, respectively, after the administration of the toxin. In contrast, pretreatment with the oral contraceptive agent completely prevented this decrease in liver-glutathione level in rats subsequently given aflatoxin. In animals given the synthetic steroids alone,

Table 2. *Effect of aflatoxin B_1 on hepatic and blood levels of glutathione in female rats pretreated with Ovral-28*

Treatment†		GSH levels		
		In liver (μmols/g) at		In blood (μmol/g) at
Daily oral doses (16)	Single ip dose	6 hr‡	48 hr‡	48 hr‡
Corn oil	DMSO§	4·56 ± 0·02*	4·59 ± 0·49*	1·11 ± 0·06
	AFB	2·24 ± 0·03	2·84 ± 0·09	1·30 ± 0·07
Ovral-28	DMSO§	5·18 ± 0·09*	5·01 ± 0·24*	1·16 ± 0·07
	AFB_1	4·96 ± 0·09*	4·50 ± 0·59*	1·31 ± 0·08

DMSO = Dimethylsulphoxide AFB_1 = Aflatoxin B_1
†For doses see footnote to Table 1 or Experimental section.
‡Time between aflatoxin administration and killing of rats.
§Control groups.
Values are means ± SD for groups of five rats and those marked with an asterisk differ significantly ($P < 0·05$) from those in rats given aflatoxin without prior administration of Ovral-28.

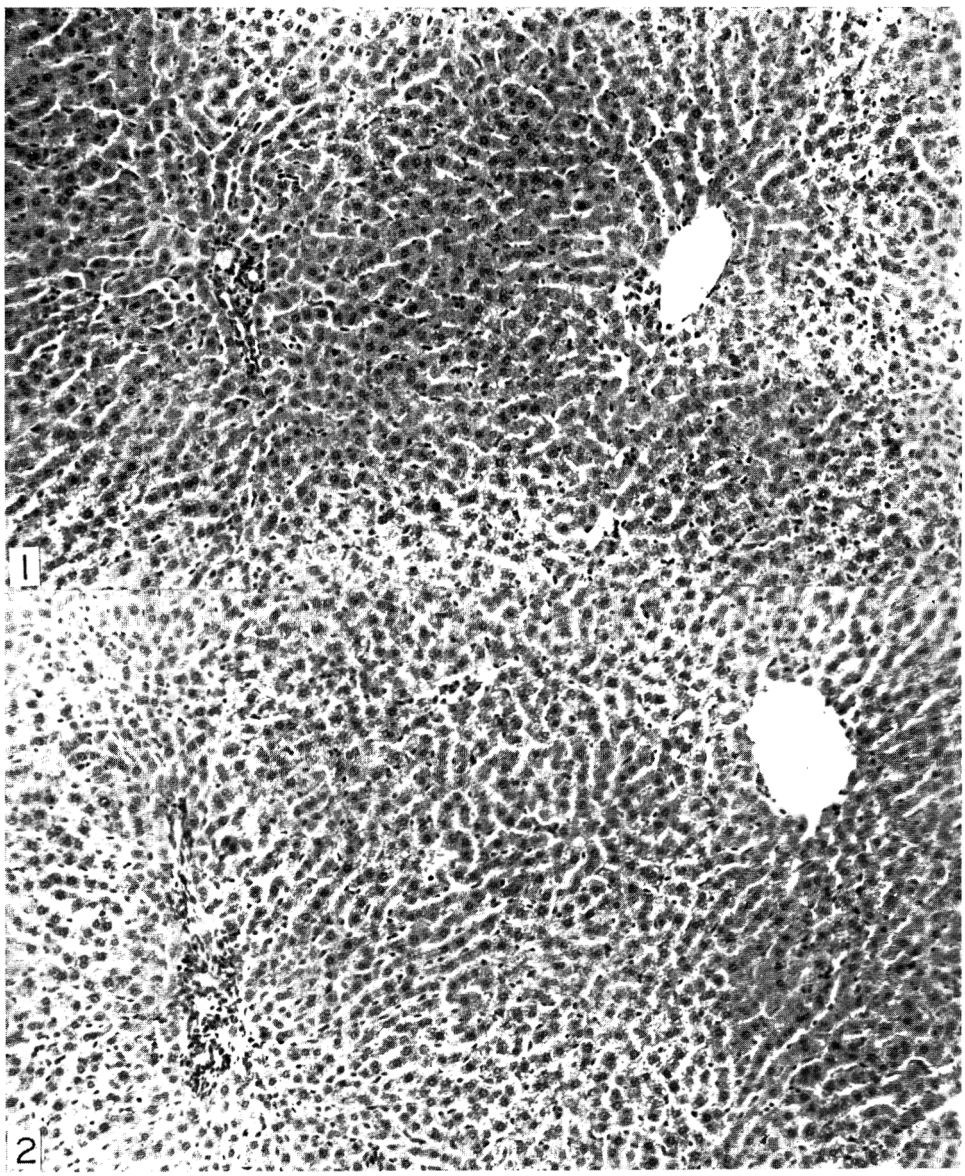

Fig. 1. Liver tissue from control rats given only 0·2 ml dimethylsulphoxide. Haematoxylin and eosin × 300.

Fig. 2. Hepatic parenchymal cells of normal appearance in liver tissue from rats given Ovral-28 (100 μg/animal) in corn oil orally for 16 days followed 24 hr after the last dose by a single ip dose of dimethysulphoxide (0·2 ml), the rats being killed 48 hr later. Haematoxylin and eosin × 300.

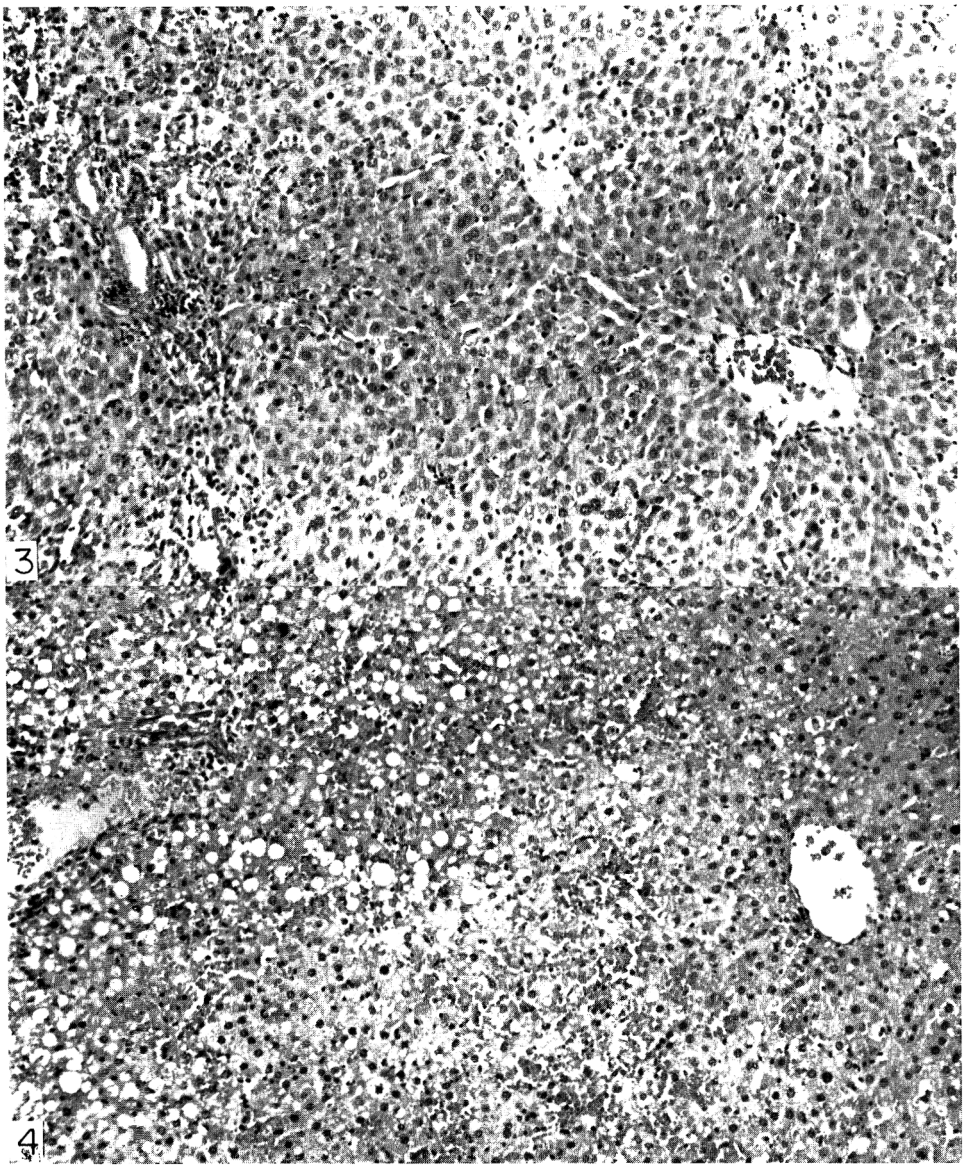

Fig. 3. Minimal necrosis in the periportal area of liver tissue from rats pretreated with Ovral-28 for 16 days, given a single ip dose of aflatoxin B_1 (3 mg/kg) 24 hr later and then killed after a further 48 hr. Haematoxylin and eosin × 300.

Fig. 4. Large zone of necrosis surrounding the periportal area and massive fatty infiltration in the liver tissue of a rat killed 48 hr after administration of a single ip dose of 3 mg aflatoxin B_1/kg. Haematoxylin and eosin × 300.

Table 3. *Effect of Ovral-28 pretreatment of rats on aflatoxin B_1-induced changes in hepatic cytochrome* P-450 *content and* in vitro *benzphetamine metabolism*

Treatment†			
Daily oral doses (16)	Single ip dose	Cytochrome *P*-450 (nmols/mg protein)	Benzphetamine metabolism (nmols HCHO formed/mg protein/min)
Corn oil	DMSO‡	0·48 ± 0·04*	3·64 ± 0·03*
	AFB_1	0·29 ± 0·03	1·52 ± 0·06
Ovral-28	DMSO‡	0·49 ± 0·01*	3·82 ± 0·02*
	AFB_1	0·40 ± 0·02*	2·89 ± 0·03*

DMSO = Dimethylsulphoxide AFB_1 = Aflatoxin B_1

†For doses see footnote to Table 1 or Experimental section.

‡Control groups.

Values are mean ± SD for groups of five rats and those marked with an asterisk differ significantly ($P < 0·05$) from those in rats given aflatoxin without prior administration of Ovral-28.

the hepatic glutathione level was slightly, but not significantly, higher than that in the dimethylsulphoxide-treated control rats. Administration of aflatoxin and/or Ovral-28 did not appear to affect the levels of blood glutathione.

The results of studies on the effects of aflatoxin B_1 on the hepatic cytochrome *P*-450 content and the rate of metabolism of benzphetamine by the hepatic microsomal mixed-function oxidase system are presented in Table 3. A comparison of results following aflatoxin treatment with those for controls given dimethylsulphoxide alone shows that administration of aflatoxin to untreated rats produced a 58% decrease in the specific activity of benzphetamine *N*-demethylase and a 40% diminution in the amount of cytochrome *P*-450 detectable as a carbon monoxide complex. Pretreatment of rats with the oral contraceptive agent partly counteracted the effect of aflatoxin on cytochrome *P*-450 and partly protected against loss of specific activity in the benzphetamine *N*-demethylase enzyme system.

DISCUSSION

The results of the study reported here indicate that Ovral-28, which is widely prescribed as an oral contraceptive agent, is capable of decreasing the hepatotoxic action of aflatoxin B_1 in female rats. In a trial experiment (using five rats in each group) 40% mortality was observed in the group that received aflatoxin alone. All the rats in the dimethylsulphoxide- and Ovral-treated control groups as well as those in the group that received Ovral-28 prior to aflatoxin administration survived. A repeat of the toxicity study using five rats in the dimethylsulphoxide control group and seven rats in each of the test groups resulted in the 43% death rate shown in Table 1 for the group that received aflatoxin B_1 alone.

The capacity of Ovral-28 to protect against aflatoxin B_1-induced liver damage may be related to its ability to alter the activity of the hepatic drug-metabolizing enzymes and/or its ability to prevent the aflatoxin B_1-induced decrease in the level of hepatic reduced glutathione. The increase in metabolism of benzphetamine, *in vitro*, by hepatic microsomes from female rats administered four cycles of Ovral-28 is in agreement with previous data obtained by Jori, Bianchetti & Prestini (1969), who showed (using the 9000 g supernatant fraction) that oral administration of medroxyprogesterone alone or combined with ethinyloestradiol for 30 days increased the metabolism of *p*-nitroanisole, aminopyrine and aniline by the hepatic drug-metabolizing enzyme system. Juchau & Fouts (1966) have also reported increases in the metabolism of hexobarbital and zoxazolamine *in vitro* 24 hr after prolonged administration of high doses of norethynodrel.

The effect of the synthetic oestrogenic and progestational steroids on the specific activity of the enzyme(s) involved in the metabolism of aflatoxin B_1 was not investigated *per se* in the present study. It is reasonable to assume, however, from the data presented in Table 3 that short-term administration of Ovral-28 altered the pattern of metabolism of aflatoxin B_1 by the liver. In rats, aflatoxin B_1 is predominantly metabolized by the demethylation of the methoxy side chain to aflatoxin P_1, which is not toxic, and by ring hydroxylation (Edwards, 1970; Portman, Plowman & Campbell, 1968; Wogan, 1966). Other recent studies have also demonstrated the metabolic conversion of aflatoxin B_1 to an epoxide derivative by the microsomal mixed-function oxidase system (Garner *et al.* 1971; Schoental, 1970; Swenson *et al.* 1974).

Stimulation of the conversion of aflatoxin B_1 to its 4-hydroxy and phenolic derivatives by synthetic steroid hormones would be expected to facilitate elimination of such derivatives through the bile as taurocholic acid conjugates and glucuronides (Bassir & Osiyemi, 1967). Very recent studies by Kaplowitz, Kuhlenkamp & Clifton (1975) showed that the activities of hepatic glutathione *S*-transferases were significantly induced in male and female rats pretreated with phenobarbital and polycyclic hydrocarbons. It is also possible that the progestational synthetic steroid in Ovral-28 may stimulate the rate of detoxication of aflatoxin B_1-2,3-oxide by glutathione transferases.

Acknowledgements—The support provided by Grant no. 467 from the Nutrition Foundation. PHS Grant ES 00267 and NSF Grant GB-44286 is gratefully acknowledged.

REFERENCES

Bassir, O. & Osiyemi, F. (1967). Biliary excretion of aflatoxin in the rat after a single dose. *Nature, Lond.* **215,** 882.

Beutler, E., Duron, O. & Kelly, B. M. (1963). Improved method for the determination of blood glutathione. *J. Lab. clin. Med.* **61,** 882.

Butler, W. H. & Wigglesworth, J. S. (1966). The effects of aflatoxin B_1 on the pregnant rat. *Br. J. exp. Path.* **47,** 242.

Cinti, D. L., Ritchie, A. & Schenkman, J. B. (1972). Hepatic organelle interaction. II. Effect of triboxylic acid cycle intermediates on *N*-demethylation and hydroxylation reactions in rat liver. *Molec. Pharmacol.* **8,** 339.

Edwards, G. (1970). Aflatoxin: Structure-activity Studies and a Site of Action in the Hepatocyte Nucleus. Ph.D. Thesis, Massachusetts Institute of Technology, Cambridge, Mass.

Garner, R. C., Miller, E. C. & Miller, A. (1972). Liver microsomal metabolism of aflatoxin B_1 to a reactive derivative toxic to *Salmonella typhimurium* TA 1530. *Cancer Res.* **32,** 2058.

Jori, A., Biachetti, A. & Prestini, P. E. (1969). Effect of contraceptive agents on drug metabolism. *Eur. J. Pharmac.* **7,** 196.

Juchau, M. R. & Fouts, J. R. (1966). Effects of norethynodrel and progesterone on hepatic microsomal drug-metabolizing enzyme systems. *Biochem. Pharmac.* **15,** 891.

Kaplowitz, N., Kuhlenkamp, J. & Clifton, G. (1975). Drug induction of hepatic glutathione *S*-transferases in male and female rats. *Biochem. J.* **146,** 351.

Layne, E. (1957). Spectrophotometric and turbidimetric methods for measuring proteins. In *Methods in Enzymology*, III (S. P. Colowick and N. O. Kaplan, editors) p. 447. Academic Press, New York.

Le Breton, E., Frayssinet, C., Lafarge, C. & de Recondo, A. M. (1964) Aflatoxine—Mécanisme de l'action. *Fd Cosmet. Toxicol.* **2,** 675.

Madhavan, T. V. & Gopalan, C. (1965). Effect of dietary protein on aflatoxin liver injury in weanling rats. *Archs Path.* **80,** 123.

Madhavan, T. V. & Gopalan, C. (1968). The effect of dietary protein on carcinogenesis of aflatoxin. *Archs Path.* **85,** 133.

Mgbodile, M. U. K. & Campbell, T. C. (1972). Effect of protein deprivation of male weanling rats on the kinetics of hepatic microsomal enzyme activity. *J. Nutr.* **102,** 53.

Norman, B. J., Vaughn, W. K. & Neal, R. A. (1973). Studies of the mechanisms of metabolism of diethyl *p*-nitrophenyl phosphorothionate (parathion) by rabbit liver microsomes. *Biochem. Pharmac.* **22,** 1091.

Omura, T. & Sato, R. (1964). The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J. biol. Chem.* **239,** 2370.

Portman, R. S., Plowman, K. M. & Campbell, T. C. (1968). Aflatoxin metabolism by liver microsomal preparations of two different species. *Biochem. biophys. Res. Commun.* **33,** 711.

Rogers, A. E. & Newberne, P. M. (1969). Aflatoxin B_1 carcinogenesis in lipotrope-deficient rats. *Cancer Res.* **29,** 1965.

Rogers, A. E. & Newberne, P. M. (1971). Diet and aflatoxin B_1 toxicity in rats. *Toxic. appl. Pharmac.* **20,** 113.

Schoental, R. (1970). Hepatotoxic activity of retrosine, senkerkine and hydroxysenkirkine in newborn rats, and the role of epoxides in carcinogenesis by pyrrolizidine alkaloids and aflatoxins. *Nature, Lond.* **227,** 401.

Swenson, D. H., Miller, E. C. & Miller, J. A. (1974) Aflatoxin B_1-2,3-oxide: Evidence for its formation in rat liver *in vivo* and by human liver microsomes *in vitro*. *Biochem. biophys. Res. Commun.* **60,** 1036.

Weatherholtz, W. M., Campbell, T. C. & Webb, R. E. (1969). Effect of dietary protein levels on the toxicity and metabolism of heptachlor. *J. Nutr.* **98,** 90.

Wogan, G. N. (1966). Chemical nature and biological effects of the aflatoxins. *Bact. Rev.* **30,** 460.

Fd Cosmet. Toxicol. Vol. 14, pp. 175–178. Pergamon Press 1976. Printed in Great Britain

EFFECTS OF PRENATAL ADMINISTRATION OF CITRININ AND VIRIDITOXIN TO MICE

R. D. HOOD, A. W. HAYES* and J. G. SCAMMELL

Departments of Biology and Microbiology, The University of Alabama, University, Alabama 35486, USA

(*Received* 10 *November* 1975)

Abstract—Citrinin and viriditoxin, toxic fungal metabolites, were administered ip to mice at dose levels of 0 (control), 10, 20, 30 or 40 mg/kg and 0, 2, 3 or 3·5 mg/kg, respectively, on one of days 7–10 of pregnancy. The highest doses of citrinin decreased foetal weight gain when given on any day of gestation and decreased prenatal survival when administered on gestation days 7, 8 or 10. No significant increase in foetal abnormalities was associated with exposure to either toxin, and viriditoxin had little effect on prenatal survival or growth. The highest doses of citrinin were sometimes fatal to the dams and those of viriditoxin were often fatal.

INTRODUCTION

Mycotoxins are potentially harmful to man and domestic animals because of the adverse effects they induce in biological systems and because of the widespread occurrence of the fungi that produce them. Penicillia and Aspergilli are especially important as contaminating organisms, since certain species grow on agricultural commodities under widely different conditions of moisture and temperature. Aflatoxin B_1, the most notorious of the mycotoxins, is carcinogenic in non-human primates and strong evidence supports the claim that hepatic cancer in certain human populations is the result of ingestion of this compound (Wilson & Hayes, 1973). Furthermore, relationships between the consumption of other mycotoxins and the development of diseases of unknown aetiology should not be ignored. Such problems concerning mycotoxin-producing fungi are not limited to certain geographic regions, but are world-wide.

Approximately 100 fungi that grow on standing crops or stored feeds are known to produce toxic substances. Two such compounds are citrinin and viriditoxin. Citrinin, elaborated by species of Aspergillus and Pencillium, has been detected along with ochratoxin A as a natural contaminant of barley involved in a porcine nephropathy termed "mould nephrosis" in Denmark (Krog, Hasselager & Friis, 1970). Renal lesions consisting of degeneration and necrosis of the tubular epithelium, most prominent in the thick segment of Henle and in the distal convoluted tubules, have been reported also in dogs exposed to citrinin (Carlton, Sansing, Szczech & Tuite, 1974). Viriditoxin, although not yet reported as a chemical agent involved in a field outbreak, is produced by *A. viridinutans*, a member of the *A. fumigatus* series (Lillehoj & Ciegler, 1972). Several mycotic infections in animals and man have been associated with pathogens of the *A. fumigatus* series (Raper & Fennell, 1965). However, it is likely that, with few exceptions, mycotoxicoses are not being recognized by clinicians or livestockmen. Many of the effects of known mycotoxicoses are not pronounced but some, such as reduced weight gain, reduced effectiveness of acquired immunity and genetic changes (Pier, 1973), are of economic significance in the production of farm animals. Except for aflatoxin, little information is available on the pathogenesis, diagnosis or clinical effects of acute or prolonged intake of these toxins. A limited number of studies concerned with the teratogenic effects of mycotoxins has been published, including for example those by Elis & DiPaolo (1967), Hayes, Hood & Lee (1974), Hood, Innes & Hayes (1973) and Stanford, Hood & Hayes (1975). This report, involving citrinin and viriditoxin, is one on a series of studies dealing with the effects of mycotoxins on developing mammals.

EXPERIMENTAL

Materials. Citrinin was supplied by Dr. N. D. Davis, Auburn University, Auburn, Ala. Viriditoxin, obtained from Dr. A. Ciegler, USDA, Peroria, Ill., was recrystallized from cold benzene to give a single spot in thin-layer chromatograms (Lillehoj & Ciegler, 1972). Purity was established by thin-layer chromatography, melting point and infra-red spectrum.

Animals and diet. Random-bred albino Swiss-Webster mice of the CD-1 strain (Charles River Mouse Farms, Wilmington, Mass.) were maintained on a diet of Wayne Lab Blox (Allied Mills, Inc., Chicago, Ill.) and water *ad lib.*

Experimental procedure. Animals were mated overnight. The day on which a copulation plug was detected was designated day 1 of gestation. The mycotoxins were dissolved in propylene glycol and administered as single ip injections on one of days 7–10 of gestation at a dose level of 10, 20, 30 or 40 mg/kg for citrinin and 2, 3 or 3·5 mg/kg for viriditoxin. Controls consisted of females injected only with an equivalent volume (0·1 ml) of solvent and a group of untreated females. On day 18 of gestation, the females were killed with an overdose of ether. The uterine horns were exposed and the numbers and locations of resorptions and live or dead foetuses were noted. Foetuses were then removed, weighed and examined under magnification for gross external malformations. One-third of the foetuses from each litter

*Present address: Department of Pharmacology and Toxicology, University of Mississippi Medical Center, 2500 North State Street, Jackson, Mississippi 39216, USA.

were fixed in ethyl alcohol. These foetuses were cleared and stained with Alizarin Red S by the method of Crary (1962) and inspected for skeletal defects. Additional foetuses from each litter were examined for visceral malformations by a combination of the methods of Staples (1974) and Wilson (1965).

Statistical analysis. Data on prenatal survival were analysed for statistical significance by Chi-square (Langley, 1970). The procedure of Gabriel (Sokal & Rohlf, 1969) was used for the analysis of data on foetal weight.

RESULTS

Results of the citrinin study are summarized in Table 1. The highest dose levels administered (30 and 40 mg/kg) resulted in six maternal deaths, five occurring at 40 mg/kg. Only the highest doses had a significant effect on any of the prenatal parameters studied. With the 40 mg/kg doses, survival of foetuses to gestation day 18 was decreased significantly by treatment on days 7, 8 or 10, and foetal growth rates, as reflected in the average weight at examination, were adversely affected by treatment on any day. Similar effects were associated with the 30 mg/kg doses.

Two foetuses exhibiting exencephaly were seen in citrinin-treated litters, but a similar foetus was seen also in one of the solvent-treated groups and another such defect was noted in a foetus from the untreated control group. The only internal soft-tissue malformation seen in a citrinin-treated mouse was cleft palate. Fused ribs and malformed vertebrae were observed in two offspring from separate litters from mothers given 40 mg citrinin/kg on day 10.

Treatment of pregnant mice with 3 or 3·5 mg viriditoxin/kg resulted in a high proportion of maternal deaths (Table 2) but the survival of foetuses from the dams not killed by treatment was not significantly decreased. Only foetuses from the dams exposed to the highest dose of viriditoxin on day 10 of pregnancy were significantly smaller ($P < 0·01$) than foetuses from the corresponding propylene glycol-treated dams.

The only gross external defects observed were exencephalies in one foetus from a viriditoxin-treated group, in one from a solvent-treated control group and one from an untreated control. No malformations were observed in either internal soft tissues or in the skeletal structures.

DISCUSSION

Treatment with citrinin during much of the period of major organogenesis had no detectable adverse prenatal effects except at dose levels toxic to the dams. Only the 30 and 40 mg/kg levels of citrinin were associated with adverse effects on foetal growth and survival. The higher dose probably approached the LD_{50} in the strain of mice used, judging from the number of maternal deaths and the published ip LD_{50} of 35 mg/kg for citrinin in mice (Ambrose & DeEds, 1945).

The two exencephalic foetuses found in citrinin-treated litters are of doubtful significance because two similar foetuses were noted in control litters. Furthermore, exencephaly is a common 'spontaneous' congenital defect in mice.

Although nothing is known concerning the foetus, reported effects of citrinin in adults suggest possible

Table 1. *Effects of single ip dose of citrinin on prenatal development in mice*

Treatment		No. of litters†	Total implantations	Survival to day 18 (%)	Externally abnormal foetuses (%)	Foetal weight (g)‡
Day	Dose (mg/kg)					
7	10	5	66	90·3	0	0·97 ± 0·10
	20	8	111	91·9	0	1·00 ± 0·19
	30	6 (1)	77	93·5	1·4	0·89 ± 0·12*
	40	5 (3)	63	76·2**	0	0·89 ± 0·12*
	PG	13	176	94·9	0	0·96 ± 0·12
8	10	5	61	98·4	0	0·95 ± 0·11
	20	6	74	93·2	0	0·91 ± 0·09
	30	6	69	88·4	0	0·91 ± 0·10
	40	7	84	77·4*	0	0·81 ± 0·13**
	PG	24	309	90·0	0	0·93 ± 0·13
9	10	5	70	92·9	0	1·00 ± 0·15
	20	5	60	93·3	0	1·00 ± 0·09
	30	7	84	92·9	1·3	0·98 ± 0·13
	40	5 (2)	57	84·2	0	0·90 ± 0·08**
	PG	21	275	92·7	0·4	0·98 ± 0·09
10	10	6	74	96·0	0	0·98 ± 0·09
	20	6	69	91·3	0	0·89 ± 0·07*
	30	7	86	79·1*	0	0·90 ± 0·21
	40	7	91	80·2*	0	0·87 ± 0·10**
	PG	10	120	90·0	0	0·97 ± 0·11
Untreated control		33	417	92·8	0·3	0·99 ± 0·11

PG = Propylene glycol

†Maternal deaths are indicated in parenthesis.

‡Mean ± S.D.

Values marked with asterisks differ significantly from those for the corresponding PG-treated controls: *$P < 0·05$; **$P < 0·01$.

Table 2. *Effects of a single ip dose of viriditoxin on prenatal development in mice*

Treatment		No. of litters†	Total implant-ations	Survival to day 18 (%)	Externally abnormal foetuses (%)	Foetal weight (g)‡
Day	Dose (mg/kg)					
7	2·0	7	86	94·5	0	0·94 ± 0·07
	3·0	2(4)	25	92·6	0	0·87 ± 0·11
	3·5	0(7)	—	—	—	—
	PG	13	159	94·6	0	0·93 ± 0·08
8	2·0	6	72	97·3	0	1·02 ± 0·08
	3·0	15	156	91·2	0·6	0·98 ± 0·05
	3·5	5(4)	60	90·9	0	0·92 ± 0·02
	PG	11	127	90·1	0	0·96 ± 0·10
9	2·0	7	91	96·8	0	0·96 ± 0·08
	3·0	7(4)	71	89·9	0	1·01 ± 0·12
	3·5	0(6)	—	—	—	—
	PG	11	122	91·0	0·8	0·99 ± 0·10
10	2·0	6	66	95·7	0	1·04 ± 0·09
	3·0	7(5)	63	88·8	0	0·97 ± 0·16
	3·5	5(8)	55	87·3	0	0·86 ± 0·10**
	PG	8	97	91·5	0	0·98 ± 0·09
Untreated control		18	227	92·7	0·4	1·05 ± 0·11

PG = Propylene glycol

†Maternal deaths are indicated in parenthesis.

‡Mean ± S.D.

The value marked with asterisks differs differs significantly from that for the corresponding PG-treated control group: $^{**}P < 0.01$.

mechanisms for its prenatal toxicity. Inhibition of water reabsorption by the kidneys of rats given purified citrinin orally has been reported (Saito, Enomoto & Tutsuno, 1971). In addition, although the presence of additional toxic agents has not been excluded, rats fed rice infected with *P. citrinum* have been shown to suffer kidney damage, while similarly-treated rabbits experienced altered renal glomerular function (Saito *et al.* 1971). Also, mice treated with the culture filtrate of *P. citrinum* sustained kidney damage (Sakai, 1955). In addition to renal toxicity, citrinin has been reported to show parasympathomemetic activity by causing vasodilatation, bronchoconstriction and increased muscular tone in mice (Saito *et al.* 1971), rabbits and dogs (Ambrose & DeEds, 1946).

Although developmental defects were not produced as a result of treating the unborn mouse with citrinin, it must be stressed that other dose regimens might have produced more significant effects. Also, the phenomenon of species difference in reaction to a toxic substance makes it impossible to state that citrinin has little potential as a foetotoxin or teratogen in man or for that matter in other animal species, with only results on mice as a basis for judgement. This is particularly true in view of the fact that citrinin did exhibit some foetotoxicity, albeit at high dose levels. Also, the possibility of cumulative effects has not been eliminated.

Under our experimental conditions, viriditoxin appeared to be more toxic to dams than to developing foetuses. Viriditoxin did not cause an increased incidence of either internal or external abnormalities. Although a decrease in foetal weights in the groups treated on days 7 and 8 with viriditoxin in a dose of 3·0 mg/kg or higher suggested the possibility of a harmful effect on the prenatal growth rate, only litters treated on day 10 exhibited a statistically significant reduction in weight. The possibility again remains, however, that under different conditions viriditoxin might be teratogenic. Use of a variety of animal species and other treatment regimens would be necessary to rule out this possibility completely.

The foregoing results indicate the lack of a differential effect on mother and offspring by either mycotoxin tested. These results are similar to those we have previously obtained with mycotoxins such as moniliformin, penicillic acid and penitrem A (Hayes & Hood, 1975) and with T-2 toxin (Stanford *et al.* 1975). Other mycotoxins, however, have been found to have profound embryotoxic and teratogenic effects at doses well below those toxic to the dam; rubratoxin B (Hood *et al.* 1973) and ochratoxin A (Hayes *et al.* 1974) are examples. Because of the major differences in the chemical nature of various mycotoxins, it is impossible to make generalizations with regard to their effects in particular biological systems. Thus it is necessary to determine the particular hazards inherent in exposure to each mycotoxin that may be present in significant amounts in the environment.

Acknowledgements—This work was supported by US Public Health Service Grants ESO1351 (formerly ESO0464) and ESO1352 (formerly ESO0674) from NIEHS and Grants 636 and 670 from the University of Alabama Research Grants Committee. Dr. Hayes is the recipient of Research Career Development Award 1KO4ES 34113, US Public Health Services, National Institutes of Health. We are grateful to Dr. R. Bickel for assistance with the visceral examinations.

REFERENCES

Ambrose, A. M. & DeEds, F. (1945). Acute and subacute toxicity of pure citrinin. *Proc. Soc. exp. Biol. Med.* **59**, 289.

Ambrose, A. M. & DeEds, F. (1946). Some toxicological and pharmacological properties of citrinin. *J. Pharmac. exp. Ther.* **88**, 173.

Carlton, W. W., Sansing, G., Szczech, G. M. & Tuite, J. (1974). Citrinin mycotoxicosis in beagle dogs. *Fd. Cosmet. Toxicol.* **12**, 479.

Crary, D. D. (1962). Modified benzyl alcohol clearing of alizarin-stained specimens without loss of flexibility. *Stain. Technol.* **37**, 124.

Elis, J. & DiPaolo, J. A. (1967). Aflatoxin B_1. Induction of malformations. *Archs. Path.* **83**, 53.

Hayes A. W. & Hood, R. D. (1974). Mycotoxin induced developmental abnormalities in mice. In *International Union of Pure and Applied Chemistry. Symposium on Mycotoxins in Food, Pulawy, Poland.* p. 11.

Hayes, A. W., Hood, R. D. & Lee, H. L. (1974). Teratogenic effects of ochratoxin A in mice. *Teratology* **9**, 93.

Hood, R. D., Innes, J. E. & Hayes, A. W. (1973). Effects of rubratoxin B on prenatal development in mice. *Bull. env. contam. & Toxicol. (U.S.)* **10**, 200.

Krog, P., Hasselager, E. & Friis, P. (1970). Studies on fungal nephrotoxicity. 2. Isolation of two nephrotoxic compounds from *Penicillium viridicatum* Westling: Citrinin and oxalic acid. *Acta path. microbiol. scand.* **78**, 401.

Langley, R. (1970). *Practical Statistics.* p. 285. Dover Publications, New York.

Lillehoj, E. B. & Ciegler, A. (1972). A toxic substance from *Aspergillus viride-nutans*. *Can. J. Microbiol.* **18**, 193.

Pier, A. C. (1973). An overview of the mycotoxicoses of domestic animals. *J. Am. vet. med. Ass.* **163**, 1259.

Raper, K. B. & Fennell, D. I. (1965). *The Genus Aspergillus.* p. 37. Williams and Wilkins, Baltimore.

Saito, M., Enomoto, M. & Tutsuno, T. (1971). Yellowed rice toxins. Luteoskyrin and related compounds, chlorine-containing compounds, and citrinin. In *Microbiol Toxins. A Comprehensive Treatise. Vol. VI. Fungal Toxins.* Edited by A. Ciegler, S. Kadis and S. J. Ajl. p. 299. Academic Press, New York.

Sakai, F. (1955). The toxic effect, particularly on the kidney, of yellowed rice polluted by *Penicillium citrinum*, as well as of citrinin, a pigment isolated from the mold. *Folia Pharmac., Japan* **51**, 431.

Sokal, R. R. & Rohlf, F. J. (1969). *Biometry. The Principles and Practice of Statistics in Biological Research.* 1st ed., p. 230. W. H. Freeman & Co., San Francisco.

Stanford, G. K., Hood, R. D. & Hayes, A. W. (1975). Effect of prenatal administration of T-2 toxin to mice. *Res. Commun. chem. Path. Pharmac.* **10**, 743.

Staples, R. E. (1974). Detection of visceral alterations in mammalian fetuses. *Teratology* **9**, A37.

Wilson, B. J. & Hayes, A. W. (1973). Microbial toxins. In *Toxicants Occurring Naturally in Foods.* Edited by F. M. Strong, p. 372. National Academy of Sciences, U.S.A., Washington, D.C.

Wilson, J. G. (1965). Embryological consideration in teratology. In *Teratology. Principles and Techniques.* Edited by J. G. Wilson and J. Warkany. Chapter 10. University of Chicago Press, Chicago.

Fd Cosmet. Toxicol. Vol. 14, pp. 179–182. Pergamon Press 1976. Printed in Great Britain

THE DISTRIBUTION AND EXCRETION OF HEXACHLOROPHENE IN RATS OF DIFFERENT AGES

K. BJONDAHL

Research Center Lääke-Medipolar, Box 425, 20101, Turku 10,

and

B. ISOMAA

Institute of Biology, Åbo Akademi, 20500, Turku 50, Finland

(*Received 19 September 1975*)

Abstract—The distribution and excretion of orally administered [^{14}C]hexachlorophene ([^{14}C]HCP) was studied in male rats aged 20, 32 and 85 days. The concentration of radioactivity in the serum, liver, kidneys and brain was significantly higher in the 20-day-old group than in the two other groups during the first 4 days after [^{14}C]HCP administration. Serum radioactivity declined at the same rate in all three groups, but in the liver and kidneys the decline in radioactivity was considerably slower in the 20-day-old group. Most of the administered radioactivity was excreted in the faeces during the first 4 days after [^{14}C]HCP administration. There were no significant differences between the different groups in the proportion of administered radioactivity excreted and no metabolites of [^{14}C]HCP were found by thin-layer chromatography of faecal extracts.

INTRODUCTION

The oral toxicity of hexachlorophene (HCP), a widely used fungicidal and bactericidal agent, has been studied by several workers. Rats fed HCP developed posterior paralysis, and microscopic examination revealed cerebral oedema and degeneration of the white matter of the central nervous system (Kimbrough & Gaines, 1971; Nakaue, Dost & Buhler, 1973; Nieminen, Bjondahl & Möttönen, 1973). The acute oral toxicity data reported for HCP by different workers show great variation. In rats, for example, the acute oral LD_{50} was found by Gump (1969) to be 161 mg/kg, but Gaines & Kimbrough (1971) found LD_{50} values of 66 mg/kg in males and 56 mg/kg in females. Oral toxicity data reported for dogs and mice also show wide variations.

The variability in acute oral LD_{50} values reported for HCP could be due to the different vehicles used by different workers and the variation in the physical state of the HCP administered. However, in a previous study, Nieminen *et al.* (1973) found that the acute oral toxicity of HCP in rats varied with age. In 10-day-old rats the acute oral LD_{50} was 9 ± 2 mg/kg, but subsequently, toxicity decreased almost linearly with age, the LD_{50} reaching a maximum of 111 ± 12 mg/kg in 32-day-old animals. In rats older than 32 days, toxicity increased again and in adult rats the LD_{50} was about half that in 32-day-old rats. The present study was performed in an attempt to determine whether differences in absorption, distribution, metabolism or excretion could explain these age-dependent variations in the toxicity of HCP.

EXPERIMENTAL

Chemicals. [^{14}C-Methylene]hexachlorophene ([^{14}C]HCP) was obtained from the New England Nuclear Corp., Boston, Mass., as a crystalline solid powder. The specific activity was 7·7 mCi/mmol and the chemical purity was 97%, as determined by thin-layer chromatography (tlc).

Experimental procedures

Distribution studies. Male SPF-bred Sprague–Dawley rats, 20, 32 and 85 days old, were used in the experiment. The 20-day-old rats had not been weaned and were kept with their mothers. The other animals received autoclaved standard pelleted food and pasteurized water *ad lib.* HCP was prepared for dosing by dissolving 2·6 mg [^{14}C]HCP (50 μCi) in 2 ml ethanol and adding 400 mg unlabelled HCP; the solution was thoroughly mixed with 48 ml 2% carboxymethylcellulose, and this mixture was administered to the animals by gastric intubation. The amount of HCP administered to each rat was approximately half the previously determined LD_{50} value (Nieminen *et al.* 1973), so that the 20-, 32- and 85-day-old rats received 20, 60 and 35 mg/kg, respectively. Four or five animals from each age group were decapitated 6 hr, 1 day, 4 days and 10 days after the administration of [^{14}C]HCP and blood samples were collected from all of them. The liver, brain and kidneys were weighed and tissue samples weighing 50–200 mg were cut from the central lobe of the liver and from the cerebral cortex, brain stem, cerebellum, medulla and kidneys, for the determination of radioactivity.

Excretion studies. For studying the excretion of ^{14}C-labelled compounds, the animals were kept in cages that permitted the separate collection of urine and faeces, which were removed every day for a period of 10 days and kept refrigerated until assay. Since the 20-day-old rats were still suckling they were kept every second day with their mothers, and urine and faeces were collected only during the 24 hr during which they were separated from the dams. In order

Table 1. *Radioactivity in serum and tissues of 20-, 32- and 85-day-old rats given [^{14}C]HCP in a dose equivalent to approximately half of the LD_{50}*

Time after dosing	Concn of radioactivity (% of administered dose/100 mg tissue) in						
				Brain			
	Plasma	Liver	Kidneys	Cortex	Brain stem	Cerebellum	Medulla
				20-Day-old rats			
6 hr	0·41 ± 0·033*†	0·29 ± 0·030*†	0·14 ± 0·010*†	0·03 ± 0·001	0·03 ± 0·010	0·03	0·03
1 day	0·32 ± 0·017†	0·23 ± 0·042*†	0·13 ± 0·020*†	0·04 ± 0·006	0·04 ± 0·008	0·04 ± 0·009	0·05 ± 0·004
4 days	0·04 ± 0·004†	0·13 ± 0·015*†	0·05 ± 0·004	0·01	0·01	0·01 ± 0·001	0·01 ± 0·003
10 days	<0·01	0·04 ± 0·003	0·04 ± 0·001	0·01	0·01	0·01	0·01
				32-Day-old rats			
6 hr	0·10 ± 0·001	0·08 ± 0·033†	0·04 ± 0·007†	0·01 ± 0·006	<0·01	<0·01	0·01 ± 0·003
1 day	0·04 ± 0·001	0·04 ± 0·004	0·02 ± 0·002	<0·01	<0·01	<0·01	<0·01
4 days	<0·01	0·01 ± 0·001	<0·01	<0·01	<0·01	<0·01	<0·01
10 days	<0·01	0·02 ± 0·009	<0·01	<0·01	<0·01	<0·01	<0·01
				85-Day-old rats			
6 hr	0·04 ± 0·003	0·02 ± 0·008	0·01 ± 0·003	<0·01	<0·01	<0·01	<0·01
1 day	0·02 ± 0·001	0·02 ± 0·006	0·01 ± 0·009	<0·01	<0·01	<0·01	<0·01
4 days	<0·01	<0·01	<0·01	<0·01	<0·01	<0·01	<0·01
10 days	<0·01	<0·01	<0·01	<0·01	<0·01	<0·01	<0·01

Values are means for groups of four or five rats and superscripts indicate those significantly higher ($P < 0·05$ by Student's t test) than the corresponding value for the 32-day-old (*) or 85-day-old (†) group.

to permit collection of samples on each of the 10 days, the 20-day-old group was subdivided into two subgroups and samples were collected from each subgroup on alternate days. The faecal samples from all groups were dried at 105°C for 24 hr and were then thoroughly ground. About 50 mg of each sample was taken for the determination of radioactivity. For determination of radioactivity in the urine, 0·1 ml urine was added to 10 ml Instagel (LKB-Wallac, Turku).

Chromatography. Samples of 1 g dried faeces were shaken for 24 hr with 10 ml 96% ethanol or with acetone. The mixture was filtered and about 80% of the extraction fluid was evaporated. The faecal extracts were subjected to tlc on silica gel-coated plates without fluorescent indicator (Merck AG, Darmstadt) using a heptane–glacial acetic acid (9:1, v/v) solvent system. Autoradiograms were prepared by exposing the chromatograms to X-ray film (CAE Wicor-X RP). [^{14}C]HCP dissolved in ethanol was run as a reference sample.

Radioactivity measurements. The areas of the tlc plates corresponding to the spots on the autoradiograms were scraped off and counted in a toluene-based scintillation fluid (4·2 g 2,5-diphenyloxazole and 0·09 g *p*-bis-(*o*-methylstyryl)benzene in 1 litre toluene). The samples of tissue and faeces were solubilized in 2 ml Protosol (New England Nuclear Corp.) at 55°C for 24 hr and 12 ml scintillation fluid was added. To avoid chemoluminescence, the faeces samples were acidified with HCl prior to addition of the scintillation fluid. Blood serum (200 µl) was counted in Instagel (LKB-Wallac, Turku). All samples were counted in a liquid scintillation spectrometer (LKB-Wallac 81000) and the values obtained were corrected for quenching using [^{14}C]toluene as an internal standard.

RESULTS

Tissue levels of radioactivity were expressed as a percentage of the administered dose of radioactivity and are shown in Table 1. The levels of radioactivity were significantly higher in the serum and tissues of 20-day-old rats than in either the 32- or the 85-day-old rats. The liver and serum showed the highest levels of radioactivity in all groups. In the kidneys, the levels were about half of those in the liver and serum. Only small amounts of radioactivity were found in the brain, and no significant differences were found between the different parts of the brain within one experimental group. The whole liver contained about 3·7% of the given dose of radioactivity 1 day after administration in 20-day-old rats compared with 2·1% in 32- and about 2·9% in 85-day-old rats (Table 2). In the brain the corresponding values were 0·58, 0·08 and 0·04%.

Only minor differences were found in the distribution of radioactivity between the 32- and 85-day-old groups. For example, the amount of radioactivity in the liver and kidneys (Table 2) was smaller in the 85-day-old group than in the 32-day-old group 6 hr after [^{14}C]HCP administration, whereas it was greater on day 1 in the kidneys and on day 4 in the liver in 85-day-old group. Furthermore, the amount of radioactivity found in the brain on day 1 was smaller in the 85-day-old group than in the 32-day-old group.

Calculation of the biological half-life of radioactivity in the serum and tissues on the basis of the data for the first 4 days given in Table 1 reveals a longer half-life in the tissues in the 20-day-old group than in the two others. For the 20-day-old group, the half-life in the liver was 3·7 days and in the kidneys 2·6 days, whereas it was 1·7 days in both the liver and kidneys in the other two groups. In blood serum, the biological half-life was about 1 day in all the groups.

The faecal excretion of radioactivity over a 10-day period following the administration of [^{14}C]HCP is summarized in Table 3. Most of the administered radioactivity was excreted during the first 4 days. After 10 days, 77·5% of the administered amount of radioactivity had been excreted in the 20-day-old group, 76·7% in the 32-day-old and 91·3% in the 85-day-old group. These differences were not statistically significant.

Thin-layer chromatograms of ethanol- and acetone-extracts of the faeces showed that HCP was eliminated unchanged. Autoradiograms of the tlc plates showed, in addition to the HCP spot (R_F 0·22) and additional minor spot (R_F 0·09) accounting for approximately 3% of the total radioactivity. This spot was also present in chromatograms of the drug as supplied and therefore represented a radioactive impurity in the material administered, not a metabolite of HCP.

Only small amounts of radioactivity were found in the urine, equivalent to less than 1% of the administered dose. It is possible that some of this activity could have been due to contamination of urine samples by faeces. No differences in the urinary excretion of radioactivity were observed between the various groups.

DISCUSSION

In a previous study (Nieminen *et al.* 1973) it was found that the toxicity of orally administered HCP was greater in 20-day-old rats than in 32- or 85-day-old rats. In the present study we wished to determine whether this observation could be due to differences in the fate of orally administered [^{14}C]HCP in these three age groups.

The various groups did not differ significantly in the amount of radioactivity excreted in the faeces. Metabolites of HCP have previously been reported in the faeces of rats given HCP orally (Wit & van Genderen, 1962) or ip (Black, Sprott, Howes & Rutherford, 1974), but in the present study, no metabolites of [^{14}C]HCP were detected by tlc of ethanol- or acetone-extracts of the faeces.

In 20-day-old rats, the levels of radioactivity in blood plasma and tissues were higher than in 32- and 85-day-old rats and the decline of radioactivity in the liver and kidneys was found to be slowest in the 20-day-old group. It is tempting, therefore, to assume that the much higher toxicity of HCP to 20-day-old rats is due to a less efficient elimination of HCP. However, the 20-day-old rats were still suckling when [^{14}C]HCP was administered, whereas the other groups were receiving pelleted food. Consequently,

Table 2. *Total amounts of radioactivity in liver, kidneys and brain of 20-, 32- and 85-day-old rats given a dose of [^{14}C]HCP equivalent to approximately half of the LD_{50}*

Time after dosing	Total radioactivity (% of administered dose) in		
	Liver	Kidneys	Brain‡
	20-Day-old rats		
6 hr	4·939 ± 0·37*†	0·336 ± 0·03*†	0·424 ± 0·04*†
1 day	3·707 ± 0·282*†	0·242 ± 0·023*†	0·580 ± 0·071*†
4 days	2·387 ± 0·229*†	0·120 ± 0·007*	0·112 ± 0·014*
10 days	1·48 ± 0·43†	0·163 ± 0·022*†	0·117 ± 0·012*
	32-Day-old rats		
6 hr	2·97 ± 0·125†	0·17 ± 0·026†	0·09 ± 0·045
1 day	2·11 ± 0·18	0·10 ± 0·001	0·08 ± 0·013†
4 days	0·59 ± 0·11	0·04 ± 0·01	0·02 ± 0·003
10 days	0·57 ± 0·16	0·027 ± 0·013	0·02 ± 0·014
	85-Day-old rats		
6 hr	1·48 ± 0·77	0·06 ± 0·041	0·02 ± 0·006
1 day	2·86 ± 0·83	0·16 ± 0·011*	0·037 ± 0·005
4 days	0·88 ± 0·14*	0·053 ± 0·001	<0·01
10 days	0·34 ± 0·06	0·026 ± 0·004	<0·01

‡The amounts for the whole brain are calculated from the means of the concentrations in the different parts of the brain.

Values are means ± SEM for groups of four or five rats and superscripts indicate those significantly higher ($P < 0·05$ by Student's t test) than the corresponding value for the 32-day-old (*) or 85-day-old (†) groups.

Table 3. *Excretion of radioactivity in the faeces from 20-, 32- and 85-day-old rats given a dose of [^{14}C]HCP equivalent to approximately half of the LD_{50}*

Day	Amount of radioactivity (% of administered dose) in faeces from rats aged		
	20 Days	32 Days	85 Days
1	49·40 ± 7·26	53·15 ± 2·16	45·32 ± 9·57
2	25·70 ± 7·56	16·95 ± 3·22	29·10 ± 5·89
3	1·88 ± 0·84	4·81 ± 2·13	12·15 ± 4·09
4	0·55 ± 0·17	1·42 ± 0·67	4·00 ± 0·96
5–10	0·01	0·39 ± 0·01	0·70 ± 0·11
Total...	77·54	76·72	91·27

Values are means ± SEM for groups of four or five animals.

the different diets may have influenced the absorption of [^{14}C]HCP. Furthermore, no clear-cut differences were found in the levels of radioactivity in 32- and 85-day-old rats, although the oral LD_{50} in 85-day-old rats is about half that in 32-day-old rats.

Cerebral oedema and extensive degradation of the white matter of the central nervous system following administration of HCP to rats has been reported by several workers (Kimbrough & Gaines, 1971; Nakaue *et al.* 1973; Nieminen *et al.* 1973), and HCP has been found in *in vitro* experiments to bind rapidly to neural tissues (Pleasure, Towfighi, Silberberg & Parris, 1974; Webster, Ulsamer & O'Connell, 1974). However, in the present study only low concentrations of radioactivity were found in the brain, a finding that suggests a rather slow penetration of the blood–brain barrier by HCP. The amount of [^{14}C]HCP accumulated in the brain was surprisingly small considering the extensive brain damage observed in animals fed HCP, and indicated, perhaps, that the biochemical disturbances underlying changes in the central nervous system are not a direct local effect of HCP. It should be noted that the radioactive compounds found in the brain were not characterized, since insufficient radioactive material was present in this tissue for the radioactive compounds to be identified by tlc. The [^{14}C]HCP administered to the animals had a radioactive purity of 97% and the possibility cannot be excluded that the small amount of radioactivity found in the brain represented radioactive impurities present in the compound administered to the animals.

REFERENCES

Black, J. G., Sprott, W. E., Howes, D. & Rutherford, T. (1974). Percutaneous absorption of hexachlorophene. *Toxicology* **2**, 127.

Gaines, T. B. & Kimbrough, R. D. (1971). The oral and dermal toxicity of hexachlorophene in rats. *Toxic. appl. Pharmac.* **19**, 375.

Gump, W. S. (1969). Toxicological properties of hexachlorophene. *J. Soc. cosmet. Chem.* **20**, 173.

Kimbrough, R. D. & Gaines, T. B. (1971). Hexachlorophene effects on the rat brain. Study of high doses by light and electron microscopy. *Archs envir. Hlth* **23**, 114.

Nakaue, H. S., Dost, F. N. & Buhler, D. R. (1973). Studies on the toxicity of hexachlorophene in the rat. *Toxic. appl. Pharmac.* **24**, 239.

Nieminen, L., Bjondahl, K. & Möttönen, M. (1973). Effect of hexachlorophene on the rat brain during ontogenesis. *Fd Cosmet. Toxicol.* **11**, 635.

Pleasure, D., Towfighi, J., Silberberg, D. & Parris, J. (1974). The pathogenesis of hexachlorophene neuropathy: *In vivo* and *in vitro* studies. *Neurology* **24**, 1068.

Webster, H. deF., Ulsamer, A. G. & O'Connell, M. F. (1974). Hexachlorophene induced myelin lesions in the developing nervous system of *Xenopus* tadpoles: Morphological and biochemical observations. *J. Neuropath. exp. Neurol.* **33**, 144.

Wit, J. G. & van Genderen, H. (1962). Some aspects of the fate of hexachlorophene [2,2'-methylene bis(3,4,6-trichlorophenol)] in rabbits, rats, and dairy cattle. *Acta physiol. pharmac. néerl.* **11**, 123.

Fd Cosmet. Toxicol. Vol. 14, pp. 183–187. Pergamon Press 1976. Printed in Great Britain

THE METABOLISM OF THE ANTIBACTERIAL AGENT BRONOPOL (2-BROMO-2-NITROPROPANE-1,3-DIOL) GIVEN ORALLY TO RATS AND DOGS

D. H. MOORE, L. F. CHASSEAUD and J. D. LEWIS

Department of Metabolism and Pharmacokinetics, Huntingdon Research Centre, Huntingdon, PE18 6ES.

and

P. C. RISDALL and E. L. CRAMPTON

Research Department, The Boots Co. Ltd., Pennyfoot Street, Nottingham NG2 3AA, England

(*Received 7 August 1975*)

Abstract—Oral doses of the antibacterial compound bronopol (2-bromo-2-nitropropane-1,3-diol) were readily absorbed, evenly distributed in tissues and rapidly excreted, mainly in the urine, by rats and dogs. The major urinary metabolite of bronopol was identified by mass and infra-red spectrometric techniques as 2-nitropropane-1,3-diol, formed by reductive dehalogenation, possibly by reaction of bronopol with endogenous thiol-containing compounds. Four other more polar but unidentified metabolites were detected.

INTRODUCTION

Bronopol, 2-bromo-2-nitropropane-1,3-diol ($HO \cdot CH_2 \cdot (NO_2)C(Br) \cdot CH_2 \cdot OH$) is an antibacterial agent with a wide spectrum of activity. Its activity against gram-negative bacteria, particularly Pseudomonads, is high and is greater than that of some commonly used antibacterial and preservative agents (Crowshaw, Groves & Lessel, 1964). This paper describes its metabolic fate, studied in rats and dogs after oral administration of [^{14}C]-labelled material.

EXPERIMENTAL

Materials. [2-^{14}C]Bronopol was synthesized at a specific activity of 21 μCi/mg; radiochemical purity estimated by thin-layer chromatography was not less than 99%. The bronopol used was a commercially available pharmaceutical grade (Bronopol-Boots). 2-Nitropropane-1,3-diol was prepared by the method described by Schmidt & Wilkendorf (1919), and was characterized by nuclear magnetic resonance spectroscopy in deuterated dimethylsulphoxide, using a C-60HL instrument (Japan Electron Optics Ltd., London); peaks at 4·72 ppm (triplet, two protons), 5·0–5·6 (quintuplet, one proton) and 5·9–6·7 (complex pattern, four protons) were assigned respectively to the two O–H exchangeable protons, the 2-C–H proton and the methylene protons.

Animals and experimental design

Rats. Male and female rats of the CFY strain from Carworth Europe, weighing approximately 200 g, were dosed by stomach tube with aqueous solutions of [^{14}C]bronopol buffered to pH 5 to maintain the stability of the compound. A dose of 1 mg/kg (5 μCi) was used throughout.

Dosed rats were placed singly in glass metabolism cages (Jencons Ltd., Hemel Hempstead) for the separate collection of urine and faeces. Urine was collected in receivers cooled in solid CO_2, and all excreta, collected every 24 hr, were stored at −20°C. The expired air of animals was monitored for $^{14}CO_2$ by trapping in 20% (v/v) ethanolamine–2-ethoxyethanol. Animals were given free access to a standard laboratory diet and water. After 5 days the rats were killed and the carcasses were stored at −20°C.

Blood samples were taken into heparinized tubes from the tail veins of three other dosed male rats at intervals after dosing. The samples were centrifuged and the plasma was separated. The bile ducts of one male and one female rat were cannulated with a 00-gauge nylon cannula under halothane/oxygen anaesthesia. Immediately after recovery, the rats were dosed, placed in restraining cages and supplied with a standard laboratory diet and a glucose/saline solution. The animals were kept warm under an infra-red lamp, and bile, urine and faeces were collected for 48 hr.

For the whole-body autoradiographic studies, the dosed animals were asphyxiated with CO_2 at intervals after dosing and were rapidly frozen to −70°C in an acetone–solid CO_2 mixture. The frozen animals were first cut with a jig-saw, then embedded in an aqueous solution of 2% (w/v) carboxymethylcellulose, and finally mounted on to the cold stage of a hydraulically-driven Leitz base-sledge microtome at −16°C in a cryostat (Bright Instrument Co. Ltd., Huntingdon). Sagittal sections 20 μm thick, were cut at several levels to include the spinal cord and the kidneys (Ullberg, 1954). The sections were freeze-dried and placed on Kodak Kodirex X-ray films. Autoradiographs were developed after exposure for 19 days. One animal was studied at each of the intervals 15 min and 1, 4, 8, 24 and 72 hr after dosing.

Dogs. Beagle dogs (8–10 kg bodyweight) were dosed orally with [^{14}C]bronopol (2 mg) mixed with unlabelled bronopol (6–8 mg) as an aqueous solution in gelatin capsules. The animals were housed singly in

stainless-steel metabolism cages for the separate collection of urine and faeces. Urine (collected over solid CO_2) and faeces were collected daily from one male and one female for 5 days. Blood samples were taken from the cephalic vein at intervals and treated as described for the rats. Two other (male) dogs were killed 1·5 and 6 hr after dosing and their dissected organs were stored at −20°C.

Measurement of radioactivity

Faeces and finely minced carcasses or organs were macerated in methanol. After centrifugation, the radioactivity was measured in samples of both supernatant and residue. Samples of urine, plasma, methanolic extracts of faeces, carcasses and organs, contents of expired air traps and cage washings were mixed with a toluene–Triton X-100-based scintillator (Patterson & Greene, 1965). Samples of the residues remaining after solvent extraction were burned in oxygen using a modified plastic-bag technique (Lewis, 1972) and the combustion products were absorbed into a β-phenethylamine-based scintillator (Dobbs, 1963). A Philips Liquid Scintillation Analyser was used for the measurement of radioactivity, using external standard quench correction curves (Kobayashi & Maudsley, 1969).

Nature of metabolites in plasma and urine

Plasma. Freshly prepared plasma samples (1 vol.) were extracted twice with ethyl acetate (3 vols) and the combined ethyl acetate extracts were evaporated under a stream of nitrogen to a small volume and applied to thin-layer plates.

Urine. Urine samples adjusted to pH 5 were freeze-dried, and the residues were extracted with methanol. In later experiments, the residues were extracted first with acetone (to remove urea, which interfered with thin-layer chromatography in polar solvent systems) and then with methanol. The extracts were concentrated and applied to prelayered Kieselgel F_{254} thin-layer plates (E. Merck AG, Darmstadt, Germany) with a layer thickness of 0·25 mm. The solvent systems for chromatography were (a) chloroform–methanol (4:1, v/v), (b) chloroform–methanol–acetic acid (90:16:8, by vol.) or (c) butanol–acetic acid–water (2:1:1, by vol.). The [^{14}C]-labelled metabolites were detected by autoradiography using Kodak Kodirex X-ray film. Areas of silica corresponding to zones of radioactivity were removed and eluted with portions of methanol and water and concentrations of radioactivity in the eluates were measured. Non-radioactive reference compounds were detected by their quenching of the plate fluorescence at 254 nm.

Measurement of spectra

Electron-impact mass spectra were recorded on a Hitachi Perkin-Elmer RMS-4 instrument operating at an electron-beam energy of 70 eV and an ionizing current of approximately 100 μA, and on an AEI MS902S instrument operating at an electron-beam energy of 70 eV and a trap current of 500 μA. Samples were introduced by the direct insertion probe at a chamber temperature of about 130°C. Infra-red spectra were recorded on a Unicam SP200G spectrometer with samples prepared as thin films between NaCl plates.

RESULTS

Excretion of radioactivity

Rats excreted an oral dose of [^{14}C]bronopol rapidly, mainly (80·9% of the dose) in the urine within 24 hr but about 8·4% of the dose was eliminated in the expired air, probably as $^{14}CO_2$, in the course of 3 days. In 2 days, rats with cannulated bile ducts excreted most of the dose (up to 81%) in the urine but small amounts of radioactivity (up to 7%) were excreted in the bile, showing that bronopol was probably completely absorbed. Two dogs also excreted most of an oral dose in the urine, which contained a mean of 79·6% in 24 hr, some in the faeces (Table 1) and presumably some also in the expired air, which was not monitored for $^{14}CO_2$.

Plasma concentrations of radioactivity

After administration of an oral dose of [^{14}C]bronopol to rats and dogs, peak plasma concentrations of

Table 1. *Excretion of radioactivity by animals given oral doses of 1 mg [^{14}C]bronopol/kg*

Species	No. of animals	Sample	Radioactivity found (% of that administered)* 0–24 hr	0–48 hr	0–120 hr
Rats	6	Urine	80·9 ± 2·0	82·7 ± 2·1	83·3 ± 2·1
		Faeces			5·8 ± 0·8
		Expired air	6·3 ± 0·6	8·0 ± 0·9	
		Carcass			1·6 ± 0·2
		Cage washings			0·9 ± 0·3
		Recovery...			99·6
Rats†	2	Urine		80·5, 63·5	
		Bile	1·8, 6·1	1·9, 7·2	
		Recovery...		82·4, 70·7	
Dogs	2	Urine	69·8, 84·3	72·3, 86·9	74·0, 88·3
		Faeces		2·2, 3·0	2·8, 3·4
		Cage washings			0·9, 0·3
		Recovery...			77·7, 92·0

*Findings are expressed as the means ± SEM for the group of six rats and individual values are given for the two cannulated rats and the two dogs.

†With cannulated bile ducts.

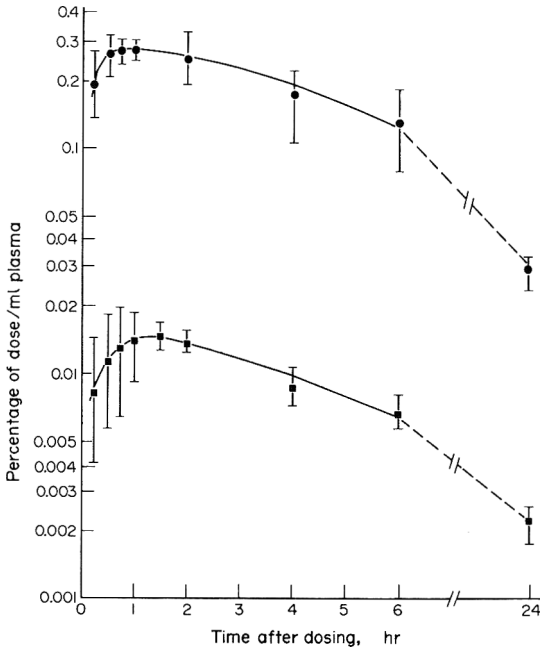

Fig. 1. Plasma concentrations of radioactivity (mean and range of values) after administration of single doses of [^{14}C]bronopol (1 mg/kg) to three rats (●) and two dogs (■).

radioactivity were reached up to 2 hr after dosing in both species (Fig. 1) and corresponded to 2·5–3·1 and 5·9–9·0% of the dose in the total plasma of rats and dogs respectively (assuming plasma volumes of 10 ml for the rats and 450 ml for the dogs). In both species, mean plasma concentrations of radioactivity had declined tenfold 24 hr after dosing, the initial half-life being about 5 hr.

Tissue distribution of radioactivity

Whole-body autoradiography of rats (Fig. 2) showed that the radioactivity was rapidly absorbed and distributed, although systemic levels of radioactivity were never high in relation to the levels in the gastro-intestinal tract. The distribution of [^{14}C]-bronopol and/or its metabolites was remarkably even, and there was no evidence of accumulation in any organ. No radioactivity was detected in sections from rats killed 24 hr or later after dosing. Radioactivity was fairly evenly distributed in the tissues of two dogs, killed 1·5 and 6 hr after administration of oral doses of [^{14}C]bronopol, exceptions being higher concentrations in the kidneys and lower concentrations in fatty tissues (Table 2). Except in the kidneys, concentrations of radioactivity were greater in the plasma than in the tissues, a finding similar to the results obtained in rats by the use of the more qualitative autoradiographic technique.

Table 2. *Tissue distribution of radioactivity in two dogs dosed orally with [^{14}C]bronopol*

	Percentage of dose after			
	1·5 hr		6 hr	
Tissue	Total	%/g*	Total	%/g*
Brain	0·44	0·0053	0·32	0·0042
Eyes	0·057	0·0059	0·049	0·0051
Fatty tissue†	4·28	0·0034	1·03	0·0008
Heart	0·39	0·0052	0·25	0·0033
Kidneys	0·93	0·0168	0·55	0·0091
Liver	3·10	0·0072	2·00	0·0043
Lungs	0·47	0·0059	0·31	0·0032
Muscle†	27·06	0·0077	12·18	0·0035
Testes	0·034	0·0075	0·019	0·0041
Plasma†	4·46	0·0099	2·52	0·0056
Urine	22·04	—	31·57	—
Faeces	0	—	0·62	—

*These may be converted to ppm equivalent to bronopol by multiplication by 90.
†Assuming these represent, respectively, 14, 39 and 5% of the body weight (9 kg).

Metabolites of [^{14}C]bronopol in urine and plasma

Five metabolites were detected by thin-layer chromatography and autoradiography in the urine of rats and dogs (Table 3).

The major metabolite (metabolite 1) was shown to be 2-nitropropane-1,3-diol, by comparison of its chromatographic properties and of its infra-red (Fig. 3) and mass spectra (Fig. 4) with those (Figs 3 & 5) of the authentic compound. No molecular ion (*m/e* 121) was observed in either mass spectrum, but fragment

Table 3. *Metabolites of [^{14}C]bronopol in the urine of rats and dogs given oral doses*

		R_F values in solvent systems*				Percentage of dose in 0–24 hr urine†	
Compound		(a)	(b)	(c)	PC‡	Rats	Dogs
Metabolite	1	0·47	0·41	0·61	0·35	42·6 ± 2·2	43·7 (38·4, 49·0)
	2	0·03	0·13	0·55	0·1	11·7 ± 0·8	9·7 (9·4, 10·0)
	3	0·00	0·04	0·43	0	4·2	11·3 (10·6, 12·0)
	4	0·00	0·00	0·34	0	2·7	
	5	0·00	0·00	0·22	0	3·0	
Bronopol		0·64	0·62	0·72	3·6	ND	ND

ND = none detected
*(a) Chloroform–methanol (4:1, v/v); (b) chloroform–methanol–acetic acid (90:16:8, by vol.); (c) butanol–acetic acid–water (2:1:1, by vol.).
†Values are means ± SEM for groups of six rats and means with individual figures in parentheses for the two dogs.
‡Approximate partition coefficient in ethyl acetate–water, pH 6.

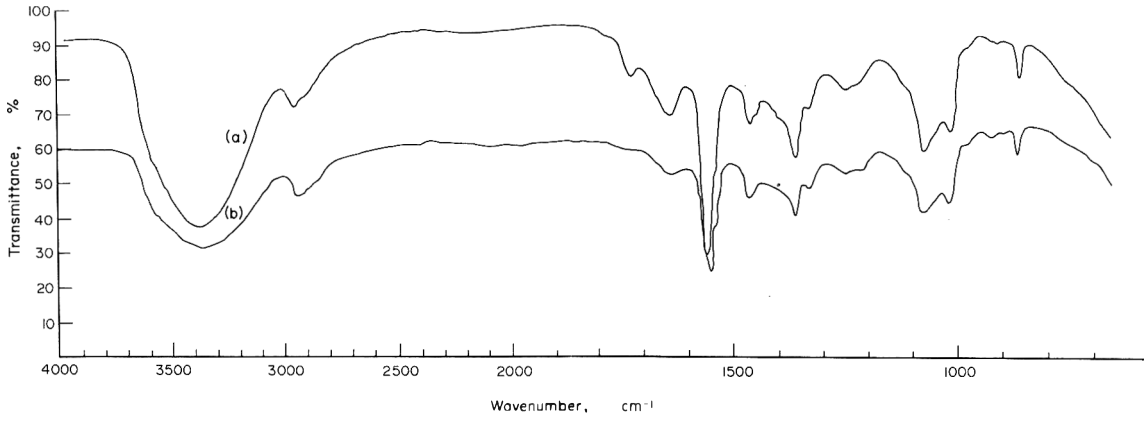

Fig. 3. Infra-red spectra of (a) major metabolite 1 and (b) authentic 2-nitropropane-1,3-diol.

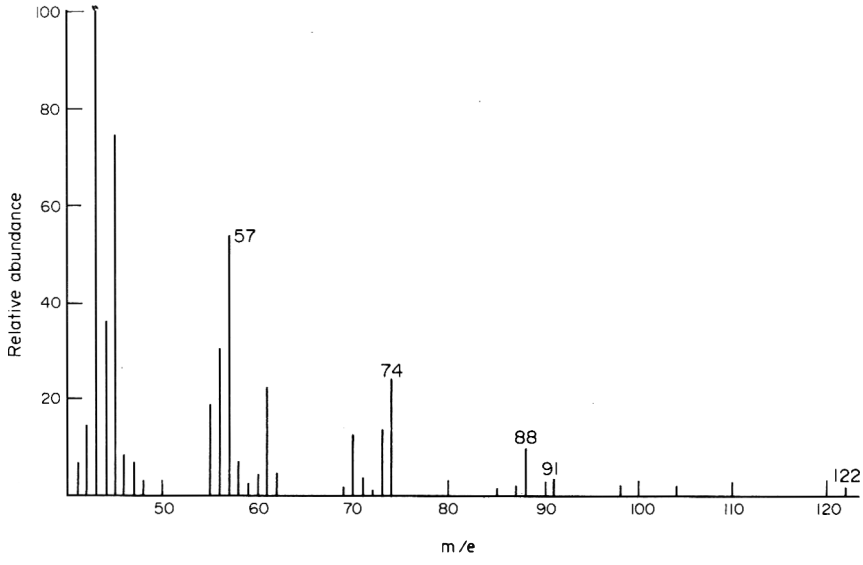

Fig. 4. Mass spectrum of major metabolite 1.

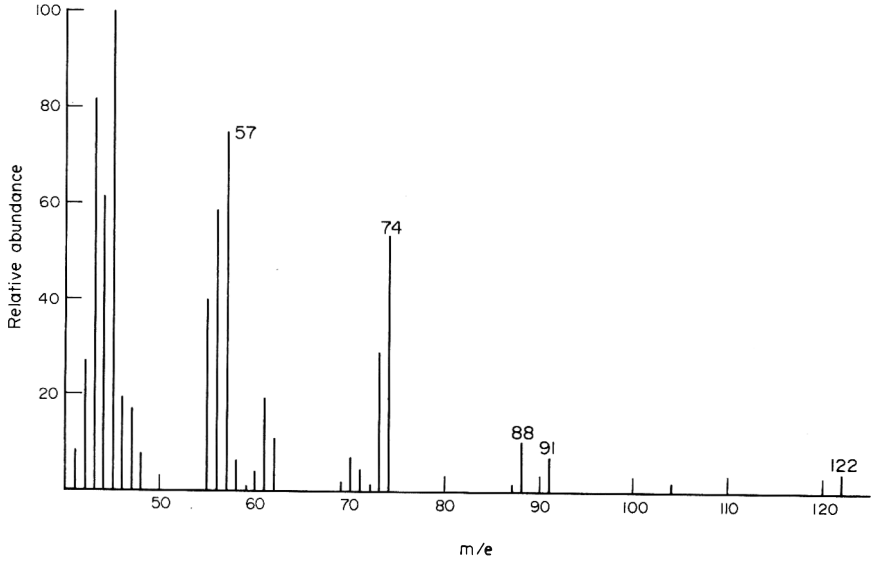

Fig. 5. Mass spectrum of authentic 2-nitropropane-1,3-diol.

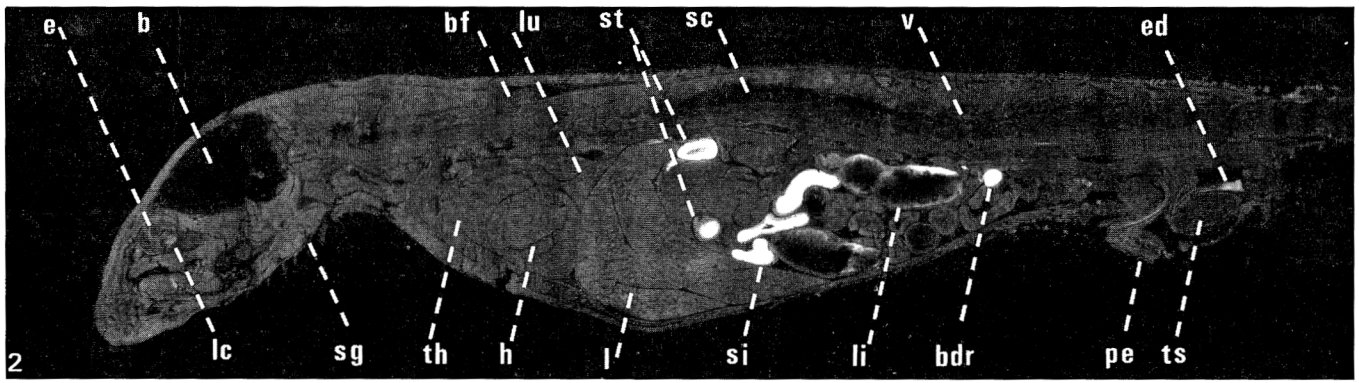

Fig. 2. Distribution of radioactivity in the rat 15 min after oral administration of [^{14}C]bronopol (1 mg/kg), showing brain (b), bladder (bdr), brown fat (bf), epididymis (ed), eye (e), heart (h), lachrymal gland (lc), liver (l), large intestine (li), lung (lu), penis (pe), spinal cord (sc), salivary gland (sg), small intestine (si), stomach (st), thymus (th), testis (ts) and vertebra (v).

Facing p. 186

ions of *m/e* 91, 74, 73 and 57 were of similar intensity in both spectra, and corresponded respectively to loss from the molecule of CH_2O, HNO_2, $CH_2O + H_2O$ and $NO_2 + H_2O$, as established by the high-resolution mass spectroscopy of the synthetic 2-nitropropane-1,3-diol (*m/e* ratios 91·0276, calculated 91·0269 ($C_2H_5NO_3$): 74·0366, calculated 74·0368 ($C_3H_6O_3$): 73·0161, calculated 73·0164 ($C_2H_3NO_2$): 57·0336, calculated 57·0340 (C_3H_5O)). Other peaks at *m/e* 122 and 88 were of variable intensity and sometimes absent from different mass spectra of the substances and may have been due to an impurity resulting from a decomposition process. Peaks of *m/e* below 50 are not shown. The mass spectrum of bronopol (principal peaks at *m/e* 169/171 (58%), 151/153 (18%), 135/137 (90%), 123/125 (60%), 109 (40%), 107 (100%) and 105 (58%) had similar features; no molecular ion (*m/e* 199/201) was observed and fragments corresponded to loss of CH_2O, $CH_2O + H_2O$ and $NO_2 + H_2O$ (respectively, *m/e* 168·9369/170·9339, calculated 168·9374/170·9355 ($C_2H_4NO_3Br$), 150·9258/152·9233, calculated 150·9269/152·9249 ($C_2H_2NO_2Br$) and 134·9421/136·9393, calculated 134·9446/136·9426 (C_3H_4OBr)).

2-Nitropropane-1,3-diol (metabolite 1) was formed rapidly *in vitro* by reaction of bronopol in aqueous solution at pH 7 with various thiol compounds (cysteine, 2-mercaptoethanol, 2-mercaptoethylamine, thioglycollic acid and glutathione). [^{14}C]Bronopol added to samples of rat or dog plasma at 37°C decomposed completely within 5 min, yielding 2-nitro-[^{14}C]propane-1,3-diol, which was extracted into ethyl acetate prior to confirmation of its identity by thin-layer chromatography. This compound was the only metabolite of bronopol detected in the plasma of rats or dogs; it accounted for about 60% of the plasma radioactivity 15 min after dosing, a figure which decreased steadily to 40% after 4 hr, 30% after 5 hr and about 12% after 24 hr in both species. When [^{14}C]bronopol was added to dog plasma *in vitro* at 37°C, 80% of the radioactivity was recovered as 2-nitro-[^{14}C]propane-1,3-diol after incubation for 1 hr compared with only 30% after incubation for 24 hr, so the latter compound was also unstable on prolonged incubation in plasma.

Attempts to characterize the other metabolites of [^{14}C]bronopol in urine failed. Rats dosed orally with [^{35}S]cysteine daily for 7 days, to radiolabel the body sulphur pool and facilitate detection of possible cysteine conjugates, and with non-radioactive bronopol on day 6 (0·2 mg) and day 7 (8 mg) excreted chromatographically similar [^{35}S]-labelled substances before and after the bronopol doses. Treatment of extracts of the rat urine with β-glucuronidase and sulphatase in aqueous solution at pH 5 did not alter the chromatographic properties of the other urinary metabolites of [^{14}C]bronopol.

DISCUSSION

After administration of oral doses of [^{14}C]bronopol, the radioactivity was readily absorbed, evenly distributed in tissues and rapidly excreted by rats and dogs. The novel reductive dehalogenation of bronopol to 2-nitropropane-1,3-diol took place rapidly *in vitro* in plasma or in the presence of thiol compounds including glutathione, and thus did not require enzymic activity for the conversion, in contrast to the *in vivo* reduction of 2-bromo-2-ethylbutyrylurea (carbromal) to 2-ethylbutyrylurea, reported to require the activity of tissue enzymes (Butler, 1964). In this context it is of interest to note that thiols antagonize the antibacterial activity of bronopol (Crowshaw *et al.* 1964) and that it has been suggested that the mode of antibacterial action is by oxidation of thiol groups (Bowman & Stretton, 1972; Stretton & Manson, 1973). Other minor radioactive metabolites were probably the products of further metabolism of 2-nitropropane-1,3-diol, and the presence of significant amounts of $^{14}CO_2$ in the expired air indicated complete metabolism of some of the compound, possibly via [^{14}C]glycerol (or serine); [^{14}C]glycerol would have been converted mainly to $^{14}CO_2$ but some would have been incorporated into body lipids and carbohydrates (Doerschuk, 1951; Gidez & Karnovsky, 1954).

REFERENCES

Bowman, W. R. & Stretton, R. J. (1972) Antimicrobiol activity of series of halo-nitro compounds. *Antimicrob. Agents & Chemother.* **2**, 504.

Butler, T. C. (1964). The metabolic fate of carbromal (2-bromo-2-ethylbutyrylurea). *J. Pharmac. exp. Ther.* **143**, 23.

Crowshaw, Betty, Groves, M. J. & Lessel, B. (1964). Some properties of bronopol, a new antimicrobial agent active against *Pseudomonas aeruginosa*. *J. Pharm. Pharmac.* **16**, (Suppl.) 127*T*.

Dobbs, H. E. (1963). Oxygen flask method for the assay of tritium-, carbon-14-, and sulphur-35-labelled compounds. *Analyt. Chem.* **35**, 783.

Doerschuk, A. P. (1951). Some studies on the metabolism of glycerol-1-C^{14}. *J. biol. Chem.* **193**, 39.

Gidez, L. I. & Karnovsky, M. L. (1954). The metabolism of C^{14}-glycerol in the intact rat. *J. biol. Chem.* **206**, 229.

Kobayashi, Y. & Maudsley, D. V. (1969). Practical aspects of liquid scintillation counting. In *Methods of Biochemical Analysis*. Vol. 17. Edited by D. Glick. p. 55. Interscience Publishers, New York.

Lewis, J. D. (1972). A modified plastic bag combustion technique for the radioassay of ^{14}C and ^{3}H in biological tissues. *Int. J. appl. Radiat. Isotopes* **23**, 39.

Patterson, M. S. & Greene, R. C. (1965). Measurement of low energy beta emitters in aqueous solution by liquid scintillation counting of emulsions. *Analyt. Chem.* **37**, 854.

Schmidt, E. u. Wilkendorf, R. (1919). Über einige Derivate des Trimethylenglykols. *Ber. dt. chem Ges.* **52**, 389.

Stretton, R. J. & Manson, T. W. (1973). Some aspects of the mode of action of the antibacterial compound Bronopol (2-bromo-2-nitropropane-1,3-diol). *J. appl. Bact.* **36**, 61.

Ullberg, S. (1954). Studies on the distribution and fate of ^{35}S-labelled benzyl-penicillin in the body. *Acta radiol.* Suppl. 118, p. 22.

Fd Cosmet. Toxicol. Vol. 14, pp. 189–192. Pergamon Press 1976. Printed in Great Britain

THE PERCUTANEOUS ABSORPTION AND DISPOSITION OF THE ANTIBACTERIAL AGENT BRONOPOL IN RATS AND RABBITS

D. H. MOORE and L. F. CHASSEAUD
Department of Metabolism and Pharmacokinetics,
and
D. BUCKE
Department of Cell Biology, Huntingdon Research Centre, Huntingdon PE18 6ES,
and
P. C. RISDALL
Research Department, The Boots Co. Ltd., Pennyfoot St., Nottingham NG2 3AA, England

(*Received 7 August 1975*)

Abstract—The antibacterial agent bronopol, 2-bromo-2-nitropropane-1,3-diol (labelled with carbon-14) was only slowly absorbed through the skin of rats and rabbits (11% in 24 hr), although greater absorption was found when solutions in acetone rather than in water were applied. Excretion of absorbed radioactivity occurred mainly in the urine and expired air. Relatively even tissue distribution of radioactivity was found in rabbits, but autoradiographs of the treated areas of skin showed high concentrations of radioactivity around the hair follicles. Binding of [^{14}C]bronopol to hair *in vitro* was small, however, suggesting that percutaneous absorption probably occurred through the hair follicles.

INTRODUCTION

Bronopol, 2-bromo-2-nitropropane-1,3-diol, is widely used in cosmetic, toiletry and pharmaceutical preparations as a preservative agent. It may also be used as an antibacterial agent in topical formulations. This paper describes the percutaneous absorption and disposition of [^{14}C]bronopol applied to the skin of rats and rabbits. Its metabolic fate after oral administration to rats and dogs is described elsewhere (Moore, Chasseaud, Lewis, Risdall & Crampton, 1976).

EXPERIMENTAL

Material. [2-^{14}C]Bronopol was synthesized at a specific activity of 21 μCi/mg. Its radiochemical purity, estimated by thin-layer chromatography, was not less than 99%.

Cutaneous application. Solutions of [^{14}C]bronopol (4 mg/ml) were applied to areas of skin clipped free of hair (and, in the case of one rabbit, treated with depilating cream) on the backs of rats (CFY strain from Carworth Farm Europe, body weight 200 g) and rabbits (New Zealand Whites, weighing 3 kg) in a dose of 1 mg/kg body weight. The treated areas were covered with occlusive dressings consisting of polythene kept in place with adhesive tape. The animals were placed in glass or stainless-steel metabolism cages, and urine (collected over solid CO_2) and faeces were collected at intervals. Expired air from some animals was monitored for $^{14}CO_2$ by trapping in ethanolamine–2-ethoxyethanol (1:4, v/v). The animals were killed after application of bronopol for intervals ranging from 6 to 48 hr (rabbits) or to 72 hr (rats) for radioactivity determinations. The tissues of the rabbits were dissected out and stored at −20°C for subsequent measurement of radioactivity. [^{14}C]Bronopol was left in contact with the skin throughout each experiment to simulate chronic exposure to the compound and to enhance percutaneous absorption as much as possible.

Measurement of radioactivity. The radioactivity in the samples was measured by liquid scintillation counting (Moore *et al.* 1976).

Microhistoautoradiography. Areas of skin treated with the radioactive compound, as well as untreated areas, were taken from rabbits killed at intervals after application of [^{14}C]bronopol and were subjected to microhistoautoradiography by the method of Appleton (1964).

RESULTS

Influence of the solvent on the percutaneous absorption of [^{14}C]bronopol in rats

The amounts of radioactivity excreted in the urine and faeces and trapped from the air drawn through the cages were greater when rats were treated with [^{14}C]bronopol dissolved in acetone alone than when a solution in an acetone–water mixture (9:1 v/v) or water alone was used. The values obtained in these three cases indicated percutaneous absorption to the extent of about 20, 7 and 10% of the dose, respectively, during the 5 days after application (Fig. 1). Because of this difference in the absorption of radioactivity, acetone was chosen as the solvent for further studies.

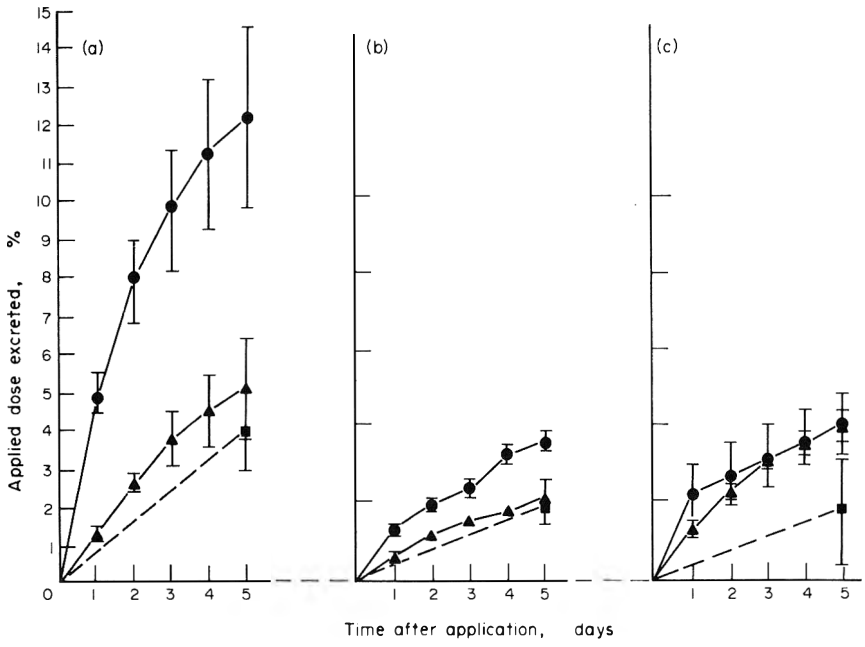

Fig. 1. Excretion of radioactivity in the urine (●), expired air (▲) and faeces (■) after application of [^{14}C]bronopol dissolved in 50 µl of (a) acetone, (b) acetone–water (9:1, v/v) or (c) water to the shaved backs of rats.

Disposition of radioactivity in rats and rabbits after cutaneous application of [^{14}C]bronopol dissolved in acetone

Most of the applied radioactivity remained associated with the treated area of the skin in both rats (about 80–90% up to 3 days after treatment) and rabbits (Table 1). Less than 3% of the applied radioactivity was found in the carcasses of individual rats killed 6, 12, 24 or 48 hr after application or at 72 hr, when about 10% of the applied dose had been excreted, mainly in the urine and expired air.

Table 1. *Tissue distribution of radioactivity after cutaneous application of [^{14}C]bronopol to the shaved backs of rabbits*

Tissue or other material	Percentage of applied radioactivity* detected after application of [^{14}C]bronopol for							
Hours...	6		12		24		48†	
	Total	%/g	Total	%/g	Total	%/g	Total	%/g
Brain	0·0066	0·00080	0·0146	0·00173	0·0062	0·00079	0·0078	0·00097
Eyes	0·0042	0·00094	0·0087	0·00185	0·0038	0·00081	0·0028	0·00066
Fatty tissue‡	0·143	0·00034	0·315	0·00075	0·080	0·00019	1·092	0·0026
Gastro-intestinal tract	0·51		1·40		0·84		2·89	
Heart	0·0049	0·00089	0·0150	0·00183	0·0099	0·00097	0·0160	0·0024
Kidneys	0·049	0·0024	0·079	0·0031	0·032	0·00163	0·098	0·0059
Liver	0·156	0·00138	0·563	0·00344	0·286	0·00153	0·459	0·0046
Lungs	0·0140	0·0010	0·046	0·0021	0·020	0·00118	0·072	0·0040
Muscle‡	1·111	0·00095	2·118	0·00181	0·737	0·00063	1·474	0·00126
Ovaries	—	—	0·00025	0·00138	—	—	0·0027	0·0044
Plasma‡	0·176	0·00117	0·474	0·00316	0·197	0·00131	0·413	0·00275
Skin	—	0·00066	—	0·00118	—	0·00133	—	0·0042
Testes	0·0092	0·00096	—	—	0·0050	0·00068	—	—
Urine	0·27	—	3·92	—	6·62	—	25·37	—
Faeces	0·0057	—	0·244	—	0·256	—	0·819	—
Skin at site of application§	64·24	—	36·91	—	48·50	—	59·60	—
Occlusive dressing	11·54	—	6·22	—	9·16	—	2·62	—
Rest of carcass	2·01	0·00115	4·06	0·0023	1·94	0·0010	2·86	0·00217

* Figures are values derived in each case from a single rabbit.
† Since considerable amounts of fur were left on this animal's back by the hair clippers, the appropriate area of skin was treated with depilating cream, which was removed before application of the [^{14}C]bronopol solution.
‡ Assuming these tissues represent 14, 39 and 5%, respectively, of the body weight (3 kg).
§ Not including skin taken for autoradiography.

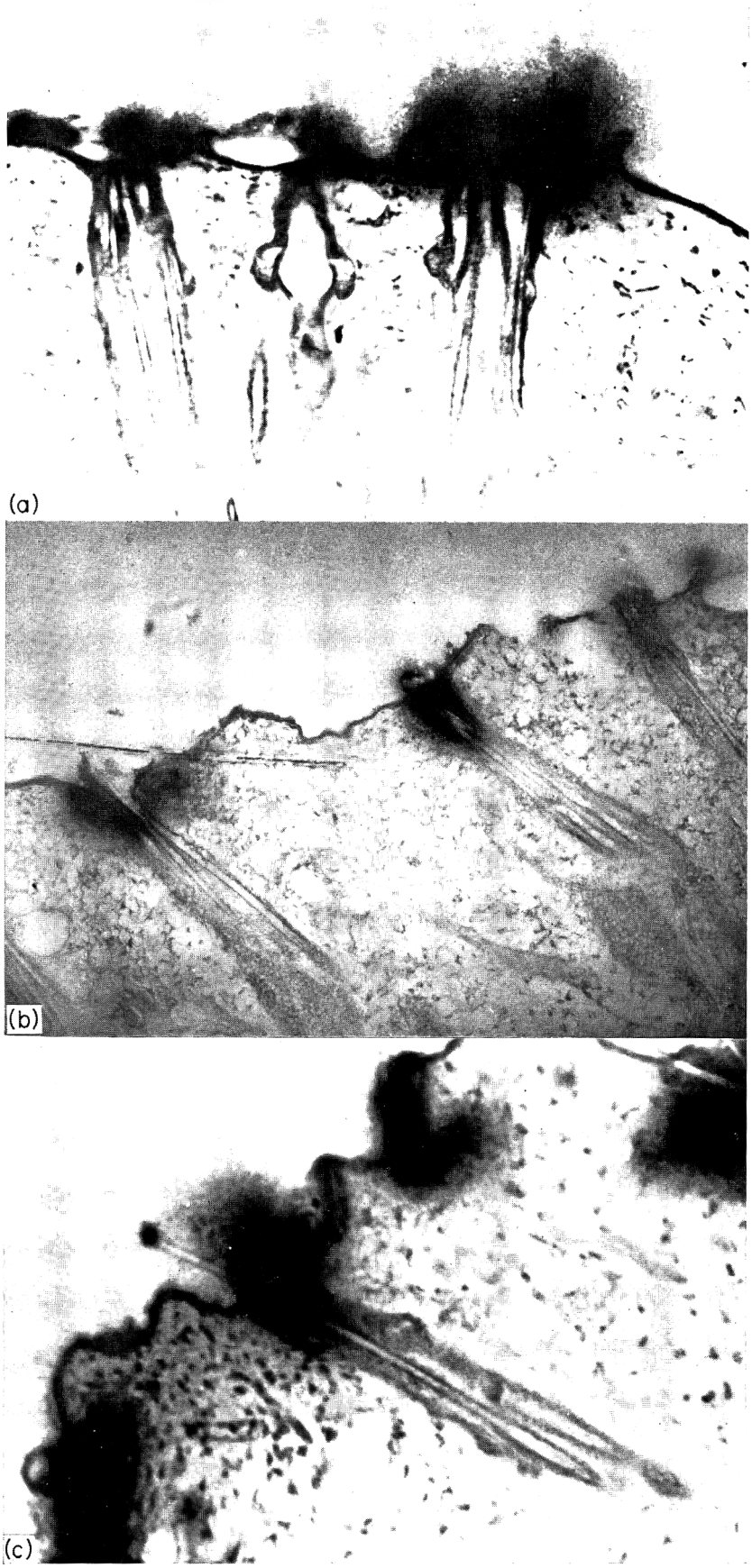

Fig. 2. Microhistoautoradiographs of sections of [^{14}C]bronopol-treated areas of skin from rabbits killed (a) 6, (b) 12 and (c) 24 hr after application. Radioactivity was localized mainly on the epidermis around the hair follicles. (a) × 200, (b) × 125 and (c) × 300.

Facing p. 190

Table 2. *Binding of [^{14}C]bronopol to rat skin and fur*

Site of application	Solvent vehicle	Duration of contact (hr)	Percentage of applied radioactivity found in successive washes				
			Water	Methanol	Acetone	In the residue	In the occlusive dressing
Shaved skin	Water	0·5	37·0	10·7	3·4	9·2	8·9
		1	52·4	7·4	4·3	10·0	3·7
		1·5	41·0	12·8	6·2	19·2	3·2
	Acetone	0·5	58·4	22·3	7·7	13·5	8·0
		1	32·0	42·9	13·6	26·4	8·2
		1·5	16·9	56·3	11·1	18·0	8·3
		24	30·5	11·2	9·7	20·3	2·6
		24	41·5	10·7	4·9	18·1	2·4
Fur	Acetone	0·5	89·7	6·9	1·4	1·3	
		0·5	85·9	6·9	1·2	1·7	

During 6, 12 and 24 hr about 5, 17 and 11%, respectively, of a dose applied to rabbits was absorbed. Pretreatment of one rabbit with a depilating cream had a marked effect on absorption, the urinary excretion of radioactivity indicating that about 30% was absorbed. Only small variations in the tissue distribution of radioactivity were found in the rabbits and only the kidneys and liver, the organs associated with the elimination of bronopol, had greater concentrations than the plasma (Table 1). The extent of absorption was probably underestimated since the air expired by the rabbits was not monitored for $^{14}CO_2$.

Microhistoautoradiographs of the treated areas of skin from the four rabbits (as exemplified in Fig. 2a–c) showed that radioactivity was confined mainly to the epidermis, with superficial penetration restricted to the areas surrounding the hair follicles. No radioactivity could be detected in the dermis or muscle layers, or in sections of untreated areas of skin.

Binding of bronopol to rat skin and fur

Since autoradiographs of rabbit skin treated with [^{14}C]bronopol showed pronounced localization of radioactivity near the hair follicles (Fig. 2a–c), [^{14}C]bronopol dissolved in acetone or water was applied to the shaved backs of rats and to quantities of rat fur *in vitro*. After various time intervals, the rats were killed and the treated areas of skin were removed and washed first with water and then with methanol and acetone. Samples of the residue were measured for radioactivity not removed by the solvents. The fur was similarly extracted. Although much of the radioactivity could be extracted by these methods, a significant proportion remained on or in the skin, particularly after longer periods of application. No significant binding of radioactivity to fur alone occurred (Table 2).

Metabolites of [^{14}C]bronopol in the urine of rats and rabbits

Thin-layer chromatography of methanol extracts of freeze-dried urine, followed by autoradiography, showed a pattern of [^{14}C]-labelled metabolites similar to that found in the urine from rats dosed orally with [^{14}C]bronopol (Moore *et al.* 1976). The major metabolite was chromatographically indistinguishable from 2-nitropropane-1,3-diol, and other more polar unidentified metabolites were also present.

DISCUSSION

Only small amounts were absorbed by rats and rabbits after cutaneous application of [^{14}C]bronopol, despite the use of occlusive dressings to enhance percutaneous absorption. Most of the radioactivity remained on the epidermis at the site of application in association with the hair follicles. The greater absorption demonstrated in the rabbit treated with depilating cream may have been due to removal of other skin components as well as of all remaining fur from the treated area.

Some of the radioactivity bound to the skin proved difficult to remove by washing with water or organic solvents, particularly if the period of contact had been prolonged, but most of the radioactivity appeared to be superficial. Binding of radioactivity to fur itself was minimal. Thus it may be concluded that skin was a more effective barrier to the absorption of bronopol than was the gastro-intestinal epithelium, especially when aqueous rather than acetone solutions were applied.

The percutaneous absorption of many compounds seems to be greater in rabbits than in rats, as demonstrated, for example, by Bartek, LaBudde & Maibach (1972), but with respect to bronopol absorption, both species were similar.

As after oral administration of the compound (Moore *et al.* 1976), metabolism of [^{14}C]bronopol appeared to involve reductive dehalogenation to 2-nitropropane-1,3-diol and the formation of the other unidentified metabolites, with $^{14}CO_2$ as an end-product.

Most foreign compounds are thought to traverse the skin through the stratum corneum by a process of passive diffusion, but bronopol seems to be an exception since it is probably absorbed mainly through the hair follicles. The reason for this is not known, but it may be connected with the requirement

for thiol groups in the initial biotransformation of bronopol.

REFERENCES

Appleton, T. C. (1964). Autoradiography of soluble labelled compounds. *J. R. microsc. Soc.* **83,** 277.

Bartek, M. J., LaBudde, J. A. & Maibach, H. I. (1972). Skin permeability *in vivo*: Comparison in rat, rabbit, pig and man. *J. invest. Derm.* **58,** 114.

Moore, D. H., Chasseaud, L. F., Lewis, J. D., Risdall, P. C. & Crampton, E. L. (1976). The metabolism of the antibacterial agent bronopol (2-bromo-2-nitropropane-1,3-diol) given orally to rats and dogs. *Fd Cosmet. Toxicol.* **14,** 183.

Fd Cosmet. Toxicol. Vol. 14, pp. 193–195. Pergamon Press 1976. Printed in Great Britain

SHORT PAPERS

FORMATION OF DIMETHYLNITROSAMINE FROM PESTICIDES CARRYING METHYLATED TERTIARY AMINOGROUPS IN THE PRESENCE OF NITRITE AT pH 3*

G. EGERT
Abteilung für Toxikologie, Universität Tübingen, D-74 Tübingen,

and

H. GREIM†
Abteilung für Toxikologie, Gesellschaft für Strahlen- und Umweltforschung, D-8042 Neuherberg, Bundesrepublik Deutschland

(*Received* 15 *November* 1975)

Summary—Pesticides and nitrite present in human food may be a hazard to man, since many pesticides are secondary or tertiary amines, which are known to form nitroso compounds in the presence of nitrite under conditions resembling those in the human stomach. Four tertiary amine pesticides—two dimethylurea derivatives, one thiuram disulphide and one phosphine compound—were incubated for 4 hr at 37°C in the presence of excess nitrite at pH 3. The reaction products were extracted with dichloromethane and were analysed qualitatively and quantitatively by infra-red spectrophotometry, nuclear-magnetic-resonance spectrometry, gas chromatography–mass spectrometry and light spectrophotometry. All four pesticides formed carcinogenic dimethylnitrosamine, the yields obtained being cycluron 4%, chloroxuron 3%, thiram 9% and dimefox 22%.

Introduction

The formation of carcinogenic *N*-nitroso derivatives by interaction of nitrite with secondary and tertiary amines and quaternary ammonium compounds at a pH between 3 and 1 has evoked much concern recently since such reactions may occur in the human stomach (Druckrey, Preussmann, Ivankovic & Schmähl, 1967; Lijinsky, Conrad & Van de Bogart, 1972; Lijinsky, Taylor, Snyder & Nettesheim, 1973; Magee & Barnes, 1956 & 1967; Sander & Schweinsberg, 1971). This offers a potential health hazard to man because nitrite as well as amines are present in human food. Nitrite is found in many leafy vegetables (Ashton, 1970; Phillips, 1968) and is used as a preservative in certain foods in various countries, including the UK, Canada and the USA.

Amines with a potential for forming nitroso compounds may be ingested in the form of drugs (Lijinsky, 1974; Lijinsky *et al.* 1973) and as pesticide residues present in human food (Ceaborn, Radeleff & Bushland, 1960; Maier-Bode, 1968). Although much work has been done on the nitrosation of pesticides such as methylcarbamates, methylthiocarbamates or triazine derivatives (Eisenbrand, Ungerer & Preussmann, 1974 & 1975; Elespuru & Lijinsky, 1973; Sen, Donaldson & Charbonneau, 1975), little information is available on the interaction of nitrite with pesticides carrying tertiary amino groups. We have investigated, therefore, the interaction of nitrite with four pesticides, namely the dimethylureas, chloroxuron and cycluron, the phosphine oxide, dimefox, and the thiuram disulphide, thiram. The quantitative and qualitative estimation of the reaction product, dimethylnitrosamine (DMN), is reported in this communication.

Experimental

Materials. Chloroxuron (3-(4-(4-chlorophenoxy) phenyl)-1,1-dimethylurea), cycluron (3-cyclooctyl-1,1-dimethylurea), dimefox (bis(dimethylamido)phosphoryl fluoride) and thiram (tetramethylthiuram disulphide) were purchased from Riedel de Haën AG, Seelze-Hannover, bromoacetic acid, dichloromethane, KOH, sodium nitrite, HCl, sulphanilic acid and sodium thiocyanate from E. Merck AG, Darmstadt, and *N*-α-naphthylenediamine dihydrochloride from Serva, Heidelberg.

Nitrosation. A reaction mixture containing 20 ml 10^{-3} M aqueous solution or suspension of the pesticide, 1 ml 10^{-3} M-thiocyanate and 1 ml acetic acid was adjusted to pH 3 with 6 N-HCl. Subsequently 20 ml 10^{-1} M sodium nitrite was added under adequate stirring and kept at 37°C. After incubation for 4 hr, during which the pH was kept constant, the nitroso derivatives formed were extracted three times by vigorous shaking with 20 ml dichloromethane. The organic extract was filtered through magnesium sulphate and the filtrate was then reduced in volume by exposure to a stream of nitrogen.

Identification and estimation of DMN. DMN was identified qualitatively by infra-red spectroscopy (Perkin Elmer 577) by demonstration of the typical curve

*This work was supported by the Deutsche Forschungsgemeinschaft.

†To whom reprint requests should be sent.

Table 1. *Yields of DMN from the reaction of pesticides and nitrite at 37°C and pH 3 for 4 hr*

Pesticide*	Concn of reactants (mg/ml)		Yield of DMN (% of theoretical)
	Pesticide	Sodium nitrite	
Cycluron	0.19	6.9	4
Chloroxuron	0.29	6.9	3
Thiram	0.24	6.9	9
Dimefox	0.15	6.9	22

DMN = Dimethylnitrosamine

*Cycluron: 3-cyclo-octyl-1,1-dimethylurea; chloroxuron: 3-(4-(4-chlorophenoxy)-phenyl)-1,1-dimethylurea; thiram: tetramethylthiuram disulphide; dimefox: bis(dimethylamido)phosphoryl fluoride.

at the wave number 1500–1430 cm^{-1} and by gas chromatography–mass spectroscopy (using the LKB 9000 S instrument) demonstrating the molepeak of 74 *m/e* at a relative intensity of 2%, and of the NO-fragment of 30 *m/e* at a relative intensity of 100%.

Quantitative analysis of DMN was performed by colorimetry (Eisenbrand & Preussmann, 1970) and by nuclear-magnetic-resonance spectroscopy using the Perkin Elmer instrument R 32, 90 MHz. The unknown samples and an authentic sample of DMN were solubilized in $CDCl_3$, tetramethylsilane being used as an internal standard.

Results and Discussion

It was demonstrated by infra-red and mass spectroscopy that all the four pesticides investigated formed DMN in the presence of nitrite at pH 3. The yields of the reactions are given in Table 1.

The formation of DMN from thiram and from ziram (zinc dimethyldithiocarbamate) has already been shown by Sen *et al.* (1975) and by Eisenbrand (1974) and Elespuru & Lijinsky (1973). Similarly ferbam (ferric dimethyldithiocarbamate) as well as dimethyldithiocarbamate itself, a component of the fungicide products tecoram and zireb, should also form DMN. Elespuru & Lijinsky (1973) have demonstrated the formation of DMN from 1,1-dimethylurea, and we have now shown that the dimethylurea derivatives, chloroxuron and cycluron, which are used as herbicides, also form DMN. It is probable, therefore, that other 1,1-dimethylurea herbicides, such as chlortoluron, diuron, fluometuron, isonuron, metoxuron and monoxuron, form DMN.

The formation of DMN from the dimethylaminophosphine oxide derivative, dimefox, has not been demonstrated before, but by analogy, the fungicide triamphos (5-amino-3-phenyl-1,2,4-triazolyl-bis(dimethylamino)phosphine oxide) may also be expected to form DMN.

Since all these reactions were studied at body temperature at pH 3, it is likely that they also occur in the human stomach. Feeding tests in which these pesticides are given with nitrite to laboratory animals will help to evaluate the potential health hazard for man. The yield of DMN from these thiocarbamates, dimethylureas or phosphines in the human stomach may be low, because excess nitrite has to be present for maximal DMN formation. However, DMN is such a highly carcinogenic and mutagenic agent (Czygan, Greim, Garro, Hutterer, Schaffner, Popper, Rosenthal & Cooper, 1973; Druckrey *et al.* 1967; Magee & Barnes, 1956) that any possible formation should be prevented. Unless it is possible to eliminate nitrite from human food, exposure of man to potentially nitrosatable drugs and pesticides must be avoided.

REFERENCES

Ashton, M. R. (1970). The Occurrence of Nitrates and Nitrites in Foods. The British Food Manufacturing Industries Research Association, Leatherhead (Literature Survey No. 7).

Ceaborn, H. V., Radeleff, R. D. & Bushland, R. C. (1960). Pesticide residues in meat and milk. USDA Agric. Res. Serv. December, p. 33.

Czygan, P., Greim, H., Garro, A. J., Hutterer, F., Schaffner, F., Popper, H., Rosenthal, O. & Cooper, D. Y. (1973). Microsomal metabolism of dimethylnitrosamine and the cytochrome P-450 dependency of its activation to a mutagen. *Cancer Res.* **33**, 2983.

Druckrey, H., Preussmann, R., Ivankovic, S. u. Schmähl, D. (1967). Organotrope carcinogene Wirkungen bei 65 verschiedenen N-Nitroso-Verbindungen an BD-Ratten. *Z. Krebsforsch.* **69**, 103.

Eisenbrand, G. u. Preussmann, R. (1970). Eine neue Methode zur kolorimetrischen Bestimmung von Nitrosaminen nach Spaltung der *N*-Nitrosogruppe mit Bromwasserstoff in Eisessig. *Arzneimittel-Forsch.* **20**, 1513.

Eisenbrand, G., Ungerer, O. & Preussmann, R. (1974). Rapid formation of carcinogenic *N*-nitrosamines by interaction of nitrite with fungicides derived from dithiocarbamic acid *in vitro* under simulated gastric conditions and *in vivo* in the rat stomach. *Fd Cosmet. Toxicol.* **12**, 229.

Eisenbrand, G., Ungerer, O. & Preussmann, R. (1975). Formation of *N*-nitroso compounds from agricultural chemicals and nitrite. In *N-Nitroso Compounds in the Environment*. Edited by P. Bogovski and E. A. Walker. IARC Scient. Publ. no. 9, p. 71. International Agency for Research on Cancer, Lyon.

Elespuru, R. K. & Lijinsky, W. (1973). The formation of carcinogenic nitroso compounds from nitrite and some types of agricultural chemicals. *Fd Cosmet. Toxicol.* **11**, 807.

Lijinsky, W. (1974). Reaction of drugs with nitrous acid as a source of carcinogenic nitrosamines. *Cancer Res.* **34**, 255.

Lijinsky, W., Conrad, E. & Van de Bogart, R. (1972). Carcinogenic nitrosamines formed by drug/nitrite interactions. *Nature, Lond.* **239**, 165.

Lijinksy, W., Keefer, L., Conrad, E. & Van de Bogart, R. (1972). Nitrosation of tertiary amines and some biologic implications. *J. natn. Cancer Inst.* **49.** 1239.

Lijinsky, W., Taylor, H. W., Snyder, C. & Nettesheim, P. (1973). Malignant tumours of liver and lung in rats fed aminopyrine or heptamethyleneimine together with nitrite. *Nature, Lond.* **244.** 176.

Magee, P. N. & Barnes, J. M. (1956). The production of malignant primary hepatic tumours in the rat by feeding dimethylnitrosamine. *Br. J. Cancer* **10.** 114.

Magee, P. N. & Barnes, J. M. (1967). Carcinogenic nitroso compounds. *Adv. Cancer Res.* **10.** 163.

Maier-Bode, H. (1968). Zur Verbreitung von Schädlingsbekämpfungsmitteln. *Naturwissenschaften* **55.** 470.

Phillips, W. E. J. (1968). Changes in the nitrate and nitrite contents of fresh and processed spinach during storage. *Agric. Fd Chem.* **16.** 88.

Sander, J. & Schweinsberg, F. (1971). *In vivo* and *in vitro* experiments on the formation of *N*-nitroso compounds from amines or amides and nitrate or nitrite. In *N-Nitroso compounds Analysis and Formation*. Edited by P. Bogovski, R. Preussmann and E. A. Walker. IARC Scient. Publ. no. 3. p. 97. International Agency for Research on Cancer, Lyon.

Sen, N. P., Donaldson, B. A. & Charbonneau, C. (1975). Formation of nitrosodimethylamine from the interaction of certain pesticides and nitrite. In *N-Nitroso Compounds in the Environment*. Edited by P. Bogovski and E. A. Walker. IARC Scient. Publ. no. 9. p. 75. International Agency for Research on Cancer, Lyon.

Fd Cosmet. Toxicol. Vol. 14, pp. 197–198. Pergamon Press 1976. Printed in Great Britain

INHIBITION OF SENSITIZATION REACTIONS INDUCED BY CERTAIN ALDEHYDES

D. L. J. OPDYKE

Research Institute for Fragrance Materials, Inc., P.O. Box 1152, Englewood Cliffs, New Jersey 07632, USA

(*Received 4 December* 1975)

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

Introduction

Fragrance materials have been a part of the skin's environment since before recorded history. In modern times, their use in cosmetics, tobacco, household products (such as floor waxes and room deodorants), garden sprays and a host of other applications makes for broad distribution and lifelong human exposure to fragrance materials. Hence, one is all the more impressed by the fact that in very few instances is injury to man attributable to these components.

Nonetheless, the Research Institute for Fragrance Materials, Inc., has embarked on an extensive testing programme to ensure the continued safety of the materials involved and to date has published monographs on 348 of these materials (Opdyke, 1973, 1974 & 1975). Where some fragrance components have been found to be skin sensitizers, the fragrance industry has been alerted so that their use may be discontinued.

Observations on an apparent "quenching" of the induction of sensitization in man are reported in this paper, with the object of drawing attention to possible differences between the sensitizing potential of simple compounds and that of mixtures containing them.

Experimental and Results

The maximization test (Kligman, 1966; Kligman & Epstein, 1975) has been used as the test procedure for screening 604 fragrance components over a 6-yr period. The test materials, dissolved in warmed petrolatum, have been tested at levels representing ten times the highest concentration known to be present in any product that comes in contact with human skin.

In the course of this testing programme, three instances arose in which an aldehyde, occurring widely in nature but tested alone in its pure form, induced sensitization reactions in human subjects. In each case, the natural essential oils in which the aldehyde occurred did not elicit sensitization under the same test conditions; something present in the natural product mix seemed to interfere with the induction of sensitization. This observation led to the initiation of a study involving the experimental addition to the pure aldehyde of some of the alcohols and terpenes with which the aldehyde occurs in nature; the effect of this was to block the induction of sensitization or its manifestation under the conditions of the test.

Three examples have been discovered so far:

(a) Phenylacetaldehyde, an aldehyde identified among the constituents of several essential oils, notably of neroli, *Citrus sinensis* leaves, other citrus species (flowers and leaves), narcissus, magnolia, lily, rose, tea and others (*Fenaroli's Handbook of Flavor Ingredients*, 1971).

(b) Citral, mixture of two geometric isomers—α-citral and β-citral—each existing as *cis*- and *trans*-isomers, originally reported to be found in lemongrass oil (*Cymbopogon flexuosus* (Nees.) Stapf.) in amounts up to 75% and also reported to be found in citrus peel and leaf oils, *Litsea citrata*, *L. cubeba* Blume., *Lindera citriodora*, *Backhousia citriodora*, *Calypranthes parriculata* and many others (*Fenaroli's Handbook of Flavor Ingredients*, 1971).

(c) Cinnamic aldehyde, identified in the essential oils of cinnamon leaves and cinnamon bark and also of hyacinth, myrrh, Bulgarian rose, patchouli and others (*Fenaroli's Handbook of Flavor Ingredients*, 1971).

The results of tests on these materials are summarized in Table 1.

Discussion

There is no suggestion at this point that these results represent anything more than observations that require more intensive study, which is now in the second year. The implications are that some materials may interfere with the induction or expression of sensitization in man, and that some individual compounds that are sensitizers may be innocuous when present in oils derived from natural sources or in various fragrance mixtures.

Table 1. *Results of sensitization tests on phenylacetaldehyde, citral and cinnamic aldehyde*

Test	Materials tested* First	Second	Relative proportions (v/v)	Overall concentration(s) applied* (%)	Result of sensitization test
a	Phenylacetaldehyde	—	—	2	+
	Rose oil	—	—	2	−
	Neroli oil	—	—	4	−
	Phenylacetaldehyde	Phenylethyl alcohol	1:1	4	−
	Phenylacetaldehyde	Dipropylene glycol	1:1	4	−
b	Citral	—	—	8,4.2,1,0·1	+
	Lemongrass oil				
	East Indian†	—	—	4	−
	West Indian	—	—	4	−
	Citral	*d*-Limonene	4:1	5	−
	Citral	α-Pinene	4:1	5	−
	Citral	Mixed citrus terpenes	4:1	5	−
c	Cinnamic aldehyde	—	—	2	+
	Cinnamon bark oil	—	—	8	+
	Cinnamon leaf oil	—	—	10	−
	Cinnamic aldehyde	Eugenol	1:1	6	−
	Cinnamic aldehyde	Eugenol	2·5:1	6	+
	Cinnamic aldehyde	*d*-Limonene	1:1	6	−

*The test agents, dissolved in petrolatum in the concentrations indicated, were subjected to the maximization test (Kligman, 1966; Kligman & Epstein, 1975).
†85% citral.

At the present time, with these observations currently under investigation so that they may be more completely understood, it is worth drawing attention to the fact that there appear to be safe conditions for the use of these three materials.

REFERENCES

Fenaroli's Handbook of Flavor Ingredients (1971). Edited by T. E. Furia and N. Bellanca. pp. 329, 337 & 573. Chemical Rubber Co., Cleveland, Ohio.

Kligman, A. M. (1966). The identification of contact allergens by human assay. III. The maximization test. A procedure for screening and rating contact sensitizers. *J. invest. Derm.* **47**, 393.

Kligman, A. M. & Epstein, W. (1975). Updating the maximization test for identifying contact allergens. *Contact Dermatitis* **1**, 231.

Opdyke, D. L. J. (1973, 1974 & 1975). Monographs on Fragrance Raw Materials. *Fd Cosmet. Toxicol.* **11**, 95–115, 477–495, 855–876, 1011–1081; **12**, 385–405, 517–537, 703–736, 807–1016; **13**, 91–112, 449–457, 545–553.

Fd Cosmet. Toxicol. Vol. 14, p. 199. Pergamon Press 1976. Printed in Great Britain

Review Section

REVIEWS OF RECENT PUBLICATIONS

Chemicals in Food and Environment. Edited by M. Webb. British Medical Bulletin, Vol. 31, no. 3. Medical Department, The British Council, 65 Davies Street, London W1Y 2AA, 1975. pp. 86. £3.

As is their custom, the publishers of the British Medical Bulletin have assembled an august body of writers to review various topics on the general theme of chemicals in food and the environment. The resulting scatter of interests seems at first sight somewhat discordant. However, on closer examination the relationships between the various aspects emerge.

Of particular interest is the opening paper by E. E. Pochin on the acceptance of risk. It is timely to be reminded that virtually every human activity involves some risk, and that some substantial risks, such as those involved in smoking or riding motor cycles, are taken quite voluntarily. The somewhat mathematical view is taken that, in the consideration of toxic chemicals, the levels of risk that can be regarded as acceptable by the public have to be quantitated. However, it is M. Webb who, in concluding a workmanlike review on cadmium toxicity and after speculating about some of the hazards of cadmium to small groups of people, notes that "in the end, it is the individual, not statistics, that matters". These views polarize the extremes between which toxicologists vacillate. In the volume under review, contributions on epidemiological studies and on the assessment of hazards from low doses of toxic substances highlight points between these two extremes.

Other papers in this publication illustrate some of the complexities that have to be faced not only in connexion with heavy metals in the environment and the increasing pressures on water supplies, but also in the fields of food additives and contaminants and novel food sources. This series of reviews should be of interest to a wide range of readers concerned with questions of health, nutrition and environmental and occupational medicine, if only to provide some insight into the wide-ranging problems involved.

Fd Cosmet. Toxicol. Vol. 14, pp. 201–204. Pergamon Press 1976. Printed in Great Britain

BOOK REVIEWS

Toxicologie du Styrène Monomère. Recherches expérimentales et cliniques chez l'Homme. By M. Oltramare, E. Desbaumes, C. Imhoff and W. Michiels. Editions Médecine et Hygiène, Geneva, 1974. pp. 100. Sw. fr. 12.00.

In view of the widespread industrial use of styrene monomer in the production of plastics, synthetic rubber and fibreglass, it is particularly important that the effects in man of exposure to this compound are well understood. This small paperback looks into this problem in some detail.

The first section takes the form of a monograph in which the authors present a review of studies published up to and including 1971 and relating particularly to the toxicity and metabolism of styrene monomer in experimental animals and in man. Styrene absorbed via the lungs, and more slowly through intact skin, is excreted mainly in the urine. As the authors point out, however, there are conflicting views as to whether haematological and hepatic changes are induced by styrene. There is a similar lack of agreement about the minimum atmospheric concentrations at which the generally recognized toxic manifestations of styrene—such as those associated with its narcotic and irritant actions—occur, and consequently about the maximum admissible concentrations (MACs) that should be permitted in factory atmospheres. The accepted MACs vary, for example, from 1·2 ppm (5 mg/m^3) in the Soviet Union and Bulgaria to 100 ppm (426 mg/m^3) in various other countries including the UK and the German Federal Republic. In between are various East European countries in favour of an MAC of 11·7 or 47 ppm.

To clarify this situation, the authors exposed six volunteers (three of whom were industrially exposed to styrene) to concentrations of 50–300 ppm styrene, and for comparative purposes to 200 ppm toluene and 300 or 600 ppm Freon 113, for 1–3 hours. Parameters studied included subjective symptoms, such as irritation of the mucous membranes and digestive disturbances, reactions to various psychomotor tests, and estimations of urinary mandelic acid and of solvent concentrations in expired and alveolar air. Their findings are presented in detail in the second section of the book. In parallel with these studies, the authors report in the third section the results of investigations, including haematology and liver-function tests, carried out among 15 workers who had been exposed to concentrations of 10–560 ppm styrene for periods ranging from 6 months to 8 years.

On the basis of all their findings, the authors conclude that the MAC for styrene should be set below 50 ppm. The main effects of exposure to 50 ppm were eye irritation, headache, drowsiness and fatigue. Except in workers predisposed to digestive disturbances, the signs characteristic of 'styrene sickness' were generally associated with exposures in the region of 100 ppm or more. Most workers exposed for several years to high concentrations of styrene showed signs of conjunctivitis, but no evidence of styrene hepatotoxicity was apparent from the liver-function tests. Four of nine workers examined had a relative lymphocytosis without leucopenia, but no other haematological lesion was found. The concentration of mandelic acid in the urine varied among individuals, but increased significantly after exposure to styrene.

Although the book is published in French, a fairly detailed English summary of the authors' findings in man is appended. The data set out in this publication provide a useful addition to previously available information on the effects of industrial exposure to styrene.

Peptide Transport in Protein Nutrition. Frontiers of Biology Vol. 37. Edited by D. M. Matthews and J. W. Payne. North-Holland Publishing Company, Amsterdam, 1975. pp. xxii + 503. $54.25.

This book is the direct result of a somewhat unusual Ciba Foundation Symposium held in 1971. The meeting was unusual in that contributions came partly from a group working on the intestinal absorption of peptides in the mammal and partly from microbiologists interested in the mechanism by which micro-organisms take up peptides from growth media and in the subsequent metabolic fate of such peptides. The editors of the volume now under consideration represent the mammalian and bacterial interests, respectively.

The book is important for a number of reasons. Firstly it brings together much scattered information indicating clearly that peptides are not merely the products of interim stages of protein digestion preceding the formation of absorbable amino acids, a concept widely held for some time and based on the demonstration of active absorption mechanisms for amino acids in the small intestine. Secondly, it demonstrates the merits of communication between widely separated groups of experimental biologists. Thirdly, it contains exhaustive lists of references, which have been abstracted and used in the text in a scholarly manner.

The final chapter on the occurrence and biological activities of peptides is a brief (70-page) review of a very wide field of scientific endeavour. Contributed by the book's two editors, it surveys the very large number of peptides found in nature, considering briefly their nutritional, enzymic, hormonal, toxic and pharmacological functions or properties. In addition there are notes on antibiotic, antiviral and cryoprotective peptides, and finally on such groups as the phospho-, lipo- and glycopeptides.

The book will be of practical value to many, and of scientific interest to all biologists. It is appropriately introduced by Professor D. H. Smyth, whose

original observation on peptide transport was made 15 years ago.

Petroleum and the Continental Shelf of North-West Europe. Vol. 2. Environmental Protection. Edited by H. A. Cole. Applied Science Publishers Ltd., Barking, Essex, 1975. pp. 126. £8.

The problems of oil pollution are certainly not new, but with the arrival of oil and a radical new technology in the North Sea, there has developed an increasing awareness of the toxicological problems arising from accidental oil leaks and their dispersion.

This volume provides an overview of the technology involved in the prevention of oil leakage, and a description of some of the toxic effects this type of off-shore industry may have on the marine fauna and flora. While the concise descriptions of oil-spill prevention schemes are not of direct interest to the toxicologist, it is reassuring to know that both government and industry are giving thought to this matter. A chapter on the effects of long-term low-level exposure to oil describes some of the strategy behind analyses of the environmental impact of oil. The range of flora and fauna remaining after contamination is a better indication of pollution than the overall abundance. Depletion of food supplies will modify the populations of organisms further up the food chain. At the top of the food chain, man may become aware of pollution because of taint, particularly in delicacies such as oysters. Although part of the initial accumulation of hydrocarbons in shellfish exposed to an oil spill will clear after a short while, it has been shown that some substances may be retained over a long period of time. It has also been demonstrated that some of these materials may be transformed by the shellfish, although in general the evidence seems to suggest that most invertebrates cannot metabolize foreign hydrocarbons. In contrast, fish can apparently metabolize polycyclic hydrocarbons quite readily.

The assessment of the effects of oil contamination on our marine environment is still far from complete. However, it would seem that prevention is better than cure and this volume demonstrates to those not directly involved the precautions that are being taken, while suggesting that the cure is not yet known.

Dangerous Properties of Industrial Materials. By N. I. Sax. 4th ed. Van Nostrand Reinhold Co., New York, 1975. pp. vii + 1258. £21.25.

For manufacturers, users and other handlers of industrial chemicals, there exists no more compact summary, in a single volume, of the potential hazards and toxicological status of these materials than is found in this compilation. Although 7 years have elapsed since the publication of the 3rd edition, the latter is still widely consulted.

The intervening years have, of course, witnessed a dramatic upsurge in the toxicological testing of industrial chemicals, and many of these new data have been incorporated into the modified 'toxic hazard ratings' of the 4th edition. Unfortunately, however, some of the shortcomings of previous editions have also been retained in this section and the incorporation of additional mis-statements detracts much from the value of the publication. In the former category, it is dismaying to see that carrageenan is still listed as of "unknown" toxicity, despite the extensive feeding tests that have been conducted on this food additive. Even the systemic toxicity of butylated hydroxytoluene apparently remains unknown to the author, although in this case he does concede that "limited" animal experiments suggest it to be of a low order. Other errors are of commission rather than omission, and include the unjustified branding of a number of compounds as carcinogens. This incriminating label has persisted from the previous edition for compounds such as pyrene, and has now been added to some more, including formaldehyde, petroleum waxes, anthracene and the chloronitroanilines. Such misleading statements are only too likely to have the effect of crying "wolf", and to lead to the ignoring of more accurate warnings of hazard.

As usual, the first 300 pages of the book offer background information on the control of industrial pollution and noise, on radiation hazards and on food additives. Novel aspects of this part include a review of procedures involved in the development of legislation for the control of industrial materials, the reproduction in full of the threshold limit values recommended in the USA for 1974 and a discussion on the hazards of exposure to microwave, laser and ultraviolet radiation.

Of special interest is a short chapter on industrial cancer risks, written by an expert in the field and free from the type of mistake found in the hazard-rating section. This introductory chapter includes discussions of fairly recently discovered carcinogens, such as vinyl chloride and chloromethyl methyl ether, although surprisingly bis-chloromethyl ether receives no specific mention here. Attention is drawn to the factors affecting the design of toxicity tests and consideration is given to the possible implications for man of demonstrations of the carcinogenicity of various industrial chemicals in experimental animals. While recognizing the need for caution in such cases, the author puts into perspective the likely hazard arising, for example, from infrequent exposure to a chemical which has been alleged to produce cancer when administered relentlessly to a species known to be susceptible to tumour growth.

Progress in Medicinal Chemistry. Vol. 11. Edited by G. P. Ellis and G. B. West. North-Holland Publishing Co., Amsterdam. 1975. pp. x + 277. Dfl. 85.00.

This is the eleventh volume in a series that aims to publish critical reviews on highly specialized topics.

The first of the five reviews now presented is concerned with the stereochemical aspects of parasympathomimetics and their antagonists. In this physicochemical approach to the problem, the author deals with the subjects of X-ray crystallography, nuclear magnetic resonance studies of conformation and mol-

ecular orbital calculation of conformation. This is followed by a long discussion on conformationally restrained analogues, subdivided into derivatives of acetylcholine and of muscarine. In each division, isomer type and relevant pharmacology are detailed. This is followed by a summary of the synthetic and stereochemical methodology involved. Finally there is a short discussion on the possible relationship between receptors for compounds that block acetylcholine and those that antagonize histamine. In all, this is an impressive review, but it demands some sound chemical knowledge from readers who wish to appreciate the more complex details of the work described.

The second article, entitled "Quantum chemistry in drug research", deals with quantum chemical methods, the calculation of geometries and of conformation, charge density, orbital energies and frontal electron densities. This is followed by a discussion on theories of drug activity and binding to receptors. The application of quantum mechanics to drug research and the value of molecular orbital calculations make fascinating reading even for a 'non-chemical biologist', such is the clarity of this review.

The third review is concerned with the psychomimetics of various members of the Convolvulaceae family. The first part of the work deals with some of the "magic drugs" of the Aztecs, in particular ololiuqui and tlitliltzin. Their phytochemical, physiological, pharmacological and psychopharmacological properties are described in some detail. The second part deals in a similar way with certain varieties of Ipomoea (morning glory). The possible non-horticultural use of these seeds—to provide an experience of the LSD type—is discussed, and LD_{50} values for certain varieties of Ipomoea seed are given. The author suggests that there is concern that "a new type of potentially dangerous psychoactive drug abuse" may be arising.

The fourth review is a very long account of seven types of antihyperlipidaemic agents. Following a brief introduction describing the different types of hyperlipoproteinaemia and the possible connexion between raised blood-lipid levels and atherosclerosis, the author gives a very detailed group-by-group account of the many substances that affect these blood levels. The paper has a very strong chemical bias and, with over 600 references, presents a very thorough—if somewhat daunting—review. By contrast, the nine-line conclusion is somewhat less adequate.

The final article is a long and detailed account of the medicinal chemistry of lithium salts and their clinical value in psychiatry. Apart from the clinical studies, the main aspects reviewed are the effects of various lithium salts on thyroid and adrenocortical activity, antidiuretic-hormone action, ion metabolism, electrolyte distribution, electro-encephalograms, biogenic amines, GABA and acetylcholine, intermediary metabolism and animal behaviour. It is of interest that this review was compiled (by a group of 12 authors) from the results of a Medlars Search for the period of 1968–1973. A welcome general summary concludes this long but lucid account of the manifold actions of lithium salts.

Thus, yet again, the high standards set by these editors have been maintained. Each review is a mine of information.

Principles and Practice of Electron Microscope Operation. By A. W. Agar, R. H. Alderson and D. Chescoe. Practical Methods in Electron Microscopy. Vol. 2. Edited by Audrey M. Glauert. North-Holland Publishing Co., Amsterdam, 1974. pp. xiii + 345. Dfl. 44.00 (paperback).

The important role that the transmission electron microscope has played in contemporary biology is in no doubt, but the technical complexity of the instrument has often deterred the biologist from making full use of its potential. The aim of this book is to introduce the prospective user to the electron microscope and to provide instruction in sufficient depth to enable him to obtain maximum benefit from the machine. In accomplishing this task, the authors have produced a book that is both clear and concise, using mathematical formulae only where they help to clarify important points.

Theoretical design and construction aspects are dealt with in the first two chapters, an introduction that proceeds naturally into the consideration of image formation dealt with in the third. The following three chapters discuss the adjustment, performance and operation of the electron microscope, while the next deals with image recording and display. The latter is concerned with the important role of photography in electron microscopy, a subject that is often neglected in introductory texts. Chapter 8 re-emphasizes the practicalities of image interpretation in the narrow sense of the ways in which the interpretation is influenced by the design, construction and performance of the instrument. The final chapter reviews modern trends in transmission electron microscopy, and although the high-energy machine described (operating at 3 MV and above) will probably find limited use in biology, analytical machines are proving to be powerful tools in biological research.

The book is available in a clothbound edition as well as in paperback. The photographs are well chosen and reproduced and the volume as a whole maintains the high standard achieved by others in this series. This book will help all prospective and many established electron microscopists to obtain better results from their instruments.

Writing Scientific Papers in English. An ELSE-Ciba Foundation Guide for Authors. By Maeve O'Connor and F. P. Woodford. Associated Scientific Publishers, Amsterdam, 1975. pp. vii + 108. Dfl. 21.00.

All aspiring authors in the scientific field should be encouraged to read this book, for although it is concerned primarily with the writing of papers reporting original research, most of the advice given is equally applicable to any form of scientific writing. The book's approach is simple and direct. The author of a paper is advised to write his conclusions first, so that he can prepare his methods and results accordingly. In most cases this preliminary exercise will also clarify his thoughts on the kind of publication that will be most appropriate and on the audience at which it should be aimed. Having guided the aspirant through the critical planning stages, the authors of this slim volume outline a series of well

defined steps to the submission of the final manuscript.

This blow-by-blow account does not end here, but also deals with editorial queries and the correction of proofs. Perhaps the most valuable (and entertaining) part of the book is Appendix 5, which provides a list of "expressions to avoid". Most of these can be recognized readily as old friends encountered in many papers. The remaining appendices are useful lists of standard abbreviations, units and symbols, with the exception of Appendix 1, which gives a brief outline of each step involved in the writing of a paper, and identifies those parts of the main text dealing in more detail with each aspect. Elsewhere in the book, useful advice is given on the design and layout of tables, illustrations and graphs to encourage clarity in presentation.

An important aspect of this "Guide for Authors" is the declared aim of ELSE (the European Association of Editors of Biological Periodicals) that the text should form the nucleus of a system of specific guidelines concerned with the problems of specialized groups. To this core manual will be added booklets for particular scientific disciplines that may require special treatment and for authors of various nationalities who encounter particular problems when writing in English. The latter difficulties are touched upon in general in the main volume, and a brief but thorough section on 'style' is of relevance both to the English-speaking writer and to his foreign counterpart.

As one would expect from authors who are editors themselves, this book is clearly written, helpfully arranged and well referenced and indexed. Perhaps it will encourage all of us to look more closely at the way we write.

BOOKS RECEIVED FOR REVIEW

Intestinal Absorption and Malabsorption. Edited by T. Z. Csáky. Raven Press, New York, 1975. pp. x + 308. $18.95.

Behavioral Toxicology. Vol. 5 in the Environmental Science Research Series. Edited by B. Weiss and V. G. Laties. Plenum Press. New York, 1975. pp. xxi + 469. $47.50.

Toxicology of Insecticides. By F. Matsumura. Plenum Press, New York, 1975. pp. xvii + 503. $33.10.

Pharmacology and Pharmacokinetics. Edited by T. Teorell, R. L. Dedrick and P. G. Condliffe. Plenum Press, New York, 1974. pp. xii + 388. $29.50.

Intestinal Absorption in Man. Edited by I. McColl and G. E. Sladen. Academic Press Inc. (London) Ltd., London, 1975. pp. ix + 363. £11.00.

Organochlorine Insecticides: Persistent Organic Pollutants. Edited by F. Moriarty. Academic Press Inc. (London) Ltd., London, 1975. pp. xii + 302. £7.90.

Heavy Metal Toxicity, Safety and Hormology. By T. D. Luckey, B. Venugopal and D. Hutcheson. **Environmental Quality and Safety.** Suppl. Vol. 1. Edited by F. Coulston and F. Korte. Academic Press Inc. (London) Ltd., London, 1975. pp. 120. £6.95.

Toxicology. The Basic Science of Poisons. Edited by L. J. Casarett and J. Doull. Baillière Tindall, London, 1975. pp. xiii + 768. £14.50.

Fd Cosmet. Toxicol. Vol. 14, pp. 205–212. Pergamon Press 1976. Printed in Great Britain

Information Section

ARTICLES OF GENERAL INTEREST

DIETARY NITROSAMINES

The known carcinogenicity of *N*-nitrosamines (NOAs) has prompted many investigations into their occurrence in foodstuffs and their synthesis in the human gastro-intestinal tract as a result of the action of nitrosating reagents upon normal food and body constituents. A review of the interrelationships between nitrate, nitrite and nitrosatable amino compounds present in food and drugs has been published by Sander & Schweinsberg (*Zentbl. Bakt. Hyg.* 1972, **156,** 299 & 321). Major factors in this field include the stability of the compounds, leading to their environmental accumulation, their ability to be formed in the stomach under physiological conditions, and the distribution of their precursors.

The recent development of sensitive analytical procedures for the detection and estimation of volatile NOAs by gas chromatography (GLC) and mass spectrometry (MS) now permits the screening of foodstuffs for NOAs as well as nitrite. Möhler & Hallermayer (*Z. Lebensmittelunters. u. -Forsch.* 1974, **156,** 206) have pointed out that photometric methods of detection are inadequate for investigating the formation or degradation of carcinogenic NOAs, GLC being the most suitable method in most instances where volatile compounds are involved. However, Fine *et al.* (*Nature, Lond.* 1974, **247,** 309) have described a group-specific NOA analyser capable of detecting both volatile and non-volatile NOAs at the μg/kg (ppb) level and have reported recoveries in the 75–100% range for analysis of dimethylnitrosamine (DMNA) added at levels of 20–240 μg/kg to fresh beef or fresh herring or of the non-volatile diphenylnitrosamine added at 60 or 120 μg/kg to fresh herring.

Animal products

The addition of nitrite to spiced meat preparations has been justified by the argument that such treatment is necessary to minimize the risk of botulism from material that has not been refrigerated during storage. The addition of nitrite to canned meat has been shown to inhibit significantly the production of toxin by *Clostridium botulinum*, the level of added nitrite at the time of formulation being more important than the residual level during storage (Christiansen *et al. Appl. Microbiol.* 1973, **25,** 357). These authors also reported that in an analysis of samples of cured meat containing varying concentrations of nitrite and nitrate, a screening procedure based on the FDA's GLC–MS multidetection system was negative for all 14 of the volatile nitrosamines sought. Further investigations into the same problem by Hustad *et al.* (*ibid* 1973, **26,** 22) involved the keeping of experimental batches of wieners, containing nitrite and nitrate at levels of 0–300 and 0–450 mg/kg respectively and contaminated with *Cl. botulinum* spores (620/g), under conditions simulating commercial distribution and possible temperature abuse during storage by consumers. In the absence of nitrite, botulinum toxin could be detected after 14 days at 27°C, while with a low nitrite concentration (50 mg/kg), the toxin took 56 days to appear under the same conditions of storage. With higher nitrite concentrations, toxin production was inhibited completely, but the effect of nitrate on toxin production was insignificant. Flavour was considered to be improved by the addition of nitrite. In tests for the 14 volatile NOAs by the FDA's GLC–MS procedure, none was detected in the treated products at the limiting concentration of 10 μg/kg.

The apparently random occurrence of up to 80 μg DMNA/kg in samples of frankfurters has not been convincingly attributed to any one of a number of possible factors (Fiddler *et al. J. Fd Sci.* 1972, **37,** 668). When up to some five times the amount of nitrite (156 mg $NaNO_2$/kg) permitted in the US for comminuted meat products was added to an experimental frankfurter formulation, no significant level of DMNA (limit of confirmable detection 10 μg/kg) appeared after the normal 2-hour processing procedure, but apparent quantities (about 8 μg/kg) appeared after the product had been heated for an additional 2 hours. Addition of at least 1500 mg $NaNO_2$/kg was necessary before DMNA in excess of 10 μg/kg was produced in the frankfurters.

Sen *et al.* (*Nature, Lond,* 1973, **245,** 104) detected fairly high levels of *N*-nitrosopyrrolidine (NPyr) in some mettwurst sausages made in Canada, while other samples of the same commodity from different manufacturers contained none. When sausages were made with and without nitrite and with and without the curing mixture of spices, salt, nitrite and nitrate, it was found that NPyr occurred only in association with the curing mixture, which contained both NPyr and *N*-nitrosopiperidine (NPi). Both the black pepper (*Piper nigrum*) and the paprika (*Capsicum annuum*) present in the curing mixture were shown to react with nitrite to produce NOAs. Black pepper, which contains piperidine in the form of piperine and chavicine, formed mainly NPi, while paprika was largely responsible for the NPyr produced.

Nitrosamines have been reported to occur in fried bacon and it appears that nitrosation of other bacon constituents may also occur during the production, frying or digestion of this food. A report by Knowles *et al.* (*ibid* 1974, **249,** 672) that spray-smoked or traditionally smoked bacon samples may contain nitro-

or nitroso-derivatives of cresols, guaiacols and 4-alkylguaiacols, particularly after frying, is of interest in the present context in view of the possibility that phenol nitrosation may compete with *N*-nitrosation for the nitrite available in bacon. However, the quantitative significance of this observation is still in question and at present relatively little is known about the possible biological effects of the nitrosophenols.

Although their earlier work had indicated that cooking had little effect on the DMNA content of bacon, Fazio *et al.* (*J. Ass. off. analyt. Chem.* 1973, **56,** 919) have reported that while they detected no NPyr in the raw samples, fried bacon was found to contain 10–108 μg NPyr/kg and the fat remaining in the pan after frying contained 45–207 μg/kg. Much lower levels of NPyr were found in fried bacon by Sen *et al.* (*Nature, Lond.* 1973, 241, 473) and by Panalaks *et al.* (*J. Ass. off. analyt. Chem.* 1974, **57,** 806), who also reported similarly low levels of DMNA and diethylnitrosamine. The latter authors mentioned, however, that the level of NPyr in cooked bacon was directly related to the amount of nitrite used in the manufacturing process, a point discussed in more detail by Sen *et al.* (*J. agric. Fd Chem.* 1974, **22,** 540). The latter showed that bacon prepared with a curing mixture containing no nitrite produced no NPyr or DMNA when fried, while in bacon cured with mixtures containing 50–200 mg $NaNO_2$/kg. NPyr was found in concentrations ranging from 2 to 20 μg/kg.

The NPyr concentrations in the fried bacon studied by Sen *et al.* (1974, *loc. cit.*) correlated well with the $NaNO_2$ concentration added initially to the curing mixture but not with the nitrite level in the cured bacon just before frying. This suggests that the NPyr may be produced during the frying process from an intermediate nitroso compound probably produced during the early stages of the curing process. It has been suggested (Lijinsky *et al. Tetrahedron* 1970, **26,** 5137) that this intermediate may be nitrosoproline, which may undergo decarboxylation to NPyr when the bacon is fried. Alternatively, the NPyr in bacon may arise directly from the interaction of nitrite with pyrrolidine released from proline or putrescine (*Cited in F.C.T.* 1970, **8,** 458). Further information on possible precursors has been provided by Bills *et al.* (*J. agric. Fd Chem.* 1973, **21,** 876) who found that while no NPyr was formed from glutamine, glutamic acid or hydroxyproline in a simulated frying system containing $NaNO_2$ and running at 170°C, the same system produced NPyr in easily detectable and identifiable amounts from *N*-nitrosoproline, pyrrolidine, spermidine, proline or putrescine. Spermidine, a common constituent of mammalian tissues yielded as much NPyr as did pyrrolidine itself.

Fish meal is another animal product that is a potential source of NOAs. After the death of a large number of farm mink which had been fed herring meal, Sen *et al.* (*ibid* 1972, **20,** 1280) investigated seven samples of the feed material and found that six contained 0·12–0·45 mg DMNA/kg but none contained detectable nitrite. However, nitrate in concentrations of 13–22 ppm was detected in four of the samples. The source of the nitrite necessary for NOA formation is obscure; its absence from the final products corroborated the manufacturer's claim that no nitrite was used in their preparation, but a small amount may have been present in the raw fish or in the drying gases used in preparation of the fish meal. The authors conclude from these findings that the routine analysis of fish meals for DMNA is desirable. The possible influence of nitrate-containing feeds on the NOA content of edible meat has also been studied. Cantoni *et al.* (*Arch. vet. ital.* 1974, **25,** 21) found that the addition of nitrate to calf feeds at 400 or 937 mg/kg increased significantly the concentration of nitrite, nitrate and NOA in the carcass and viscera of the animals, and recommended that in order to minimize NOA residues in edible meats the addition of more than 400 mg/kg nitrate to feeds should be avoided.

Wheat and tobacco

By cultivating wheat in a divided field and dressing the soil with a high dose of nitrate fertilizer plus either dimethylamine citrate, methylbenzylamine or *N*-methylaniline, Sander *et al.* (*Z. Krebsforsch.* 1973, **80,** 11) provided very favourable conditions for the uptake of NOAs by the wheat. Nevertheless, no NOAs could be detected in any of the wheat kernels examined by a method with a sensitivity of 0·3–2·0 μg/kg. Moreover, rats fed the treated wheat developed no tumours in a 2-year study. The nitrate content of the wheat samples was apparently not determined.

It has been reported that the level of NOAs in cigarette-smoke condensate depends on the concentration of total volatile bases in the tobacco, and on its nitrate content (McCormick *et al. Nature, Lond.* 1973, **244,** 237). Kadar & Devik (*Beitr. Tabakforsch.* 1972, **6,** 117) have shown that NOAs occur in cigarette smoke in quantities no greater than 50 ng/cigarette, and their studies suggest that as a rule the amounts are even smaller. Slightly higher values have been reported, however, by Morie & Sloan (*ibid* 1973, 7, 61), who found that smoke condensate from cigarettes made from tobacco with a high nitrate content and from those made of blended tobacco containing 8% $NaNO_3$ contained 50 ng and 95 ng DMNA/cigarette, respectively. A method capable of detecting 10 ng/cigarette, revealed no DMNA in the smoke from two popular domestic cigarettes and another of mixed blend.

[P. Cooper—BIBRA]

PARAQUAT REVISITED

Paraquat is notorious for the severe and frequently lethal pulmonary changes it can induce in people who have ingested small quantities (*Cited in F.C.T.* 1972, **10,** 700). Delay in the excretion of paraquat, which

may continue to appear in the urine to a detectable degree for up to 1 month in man, is presumed to be due to tissue binding and the slow release of the compound (*ibid* 1972, **10,** 701), and it has been noted that in rats and mice the uptake of paraquat by lung tissue, and to a lesser extent by cardiac muscle, differentiates its activity from that of diquat (*ibid* 1974, **12,** 571). Some further studies of paraquat intoxication throw a little more light on the processes involved.

Human poisoning

Fatal pulmonary intra-alveolar fibrosis has been reported by Copland *et al.* (*New Engl. J. Med.* 1974, **291,** 290) in the case of a 39-year-old man who drank a mouthful of paraquat concentrate. After 3 days, renal damage was indicated by a rise in blood urea nitrogen and serum creatinine, and this was followed by dyspnoea and progressive respiratory impairment leading to death on day 26. Necropsy showed heavy, dark-purple lungs in which the major change was extensive obiliterative fibrosis. It is noteworthy that the immediate cause of this man's death was sinus arrest following ventricular arrhythmia. Myocardial oedema characterized paraquat poisoning in two patients, a man of 67 and a woman of 44, described by Borchard *et al.* (*Klin. Wschr.* 1974, **52,** 657). These died 11 and 10 days, respectively, after ingesting the herbicide. Autopsy indicated damage to the pneumocytes and basal membrane followed by pulmonary oedema and fibrosis. Liver lesions were characterized by giant mitochondria and intracellular paracrystalline inclusion bodies, myofibril fragmentation and myocardial cell oedema were observed and necrosis was apparent in the kidney tubules.

Another phenomenon, described by Lautenschläger *et al.* (*Dt. med. Wschr.* 1974, **99,** 2348) in five patients who had drunk paraquat in quantities of 10–50 ml, was a rapidly progressive normocytic anaemia appearing within 2–3 days and showing little improvement within the 30 days during which the two survivors were treated in hospital. Erythrocyte production in the bone marrow was arrested without evident interference with the production of other types of blood cells. Inhibition of erythropoiesis was not dose-dependent, and reached its peak after 5–14 days. In one of the survivors who was examined 6 months after the incident, the blood and bone marrow appeared to be normal.

Tissue distribution studies

Murray & Gibson (*Toxic. appl. Pharmac.* 1974, **27,** 283) determined the tissue distribution of radioactivity after administration of ^{14}C-labelled paraquat to rats, guinea-pigs and monkeys in an oral dose equivalent to the LD_{50}. Maximal concentrations of paraquat appeared in the serum after 0·5–1 hour, and were followed by an initially rapid and subsequently slower and prolonged decline in all species. Radioactivity in the lung reached its peak after 32 hours, indicating slow selective accumulation. Nevertheless, gastro-intestinal absorption was poor; in rats 52% of the dose was still within the gastro-intestinal tract after 32 hours, at which time faecal and urinary excretion had accounted for 17 and 14%, respectively, of the administered dose and no radioactivity had been detected in the expired air. No paraquat metabolites were identified in rat urine. In monkeys, detectable excretion of paraquat in the urine continued for more than 21 days, but 21·5% of the dose was excreted during the first day.

A study by Rose *et al.* (*Nature, Lond.* 1974, **252,** 314) on the energy-dependent accumulation of bipyridilium herbicides in the rat lung has shown that slices of rat lung accumulate paraquat but not diquat. After incubation of lung slices with [^{14}C-methyl]paraquat or [^{14}C-ethylene]diquat, the diquat concentration in the tissue remained constant over a period of 30–120 minutes, but lung-tissue concentrations of paraquat rose throughout and beyond this period. Accumulation of paraquat could be inhibited by cyanide or iodoacetate or by rotenone, which inhibits mitochondrial respiration. Since the concentration of paraquat in the lungs of rats given an oral dose of 126 mg/kg rose from about 4 μg/g wet weight at 4 hours to 14 μg/g at 32 hours, while the plasma concentration remained constant at about 1 μg/ml (Murray & Gibson, *loc. cit.*), it appears that paraquat is released into the circulation from other organs, and probably to a major extent from the gastro-intestinal tract, and is thus transferred to accumulating tissues. If this happens in man, it may be important after paraquat poisoning to remove the compound not only from the stomach but also from the remaining gastro-intestinal tract and the blood.

Treatment on this principle has been advocated by Smith *et al.* (*Br. med. J.* 1974, **4,** 569) on the strength of findings in rats given a lethal oral dose of [^{14}C-methyl]paraquat (680 μmol/kg). In these animals, plasma concentrations of paraquat remained above 5 nmol/ml for 30 hours or more, while lung concentrations exceeded the toxic threshold of 15 nmol/ml. Concentrations of paraquat in the contents of the stomach and small intestine 1–16 hours after dosing showed a linear relationship between the plasma and intestinal concentrations of the herbicide, but not between concentrations in the plasma and stomach contents. After 10–16 hours, 10–40% of the dose still remained in the stomach after 16 hours. When the same dose of paraquat was followed after 4 or 10 hours with a saline stomach wash and dosing with 0·5 ml caster oil, 250 mg magnesium sulphate/kg and 7–10 ml of 7% (w/v) bentonite suspension (the oil and bentonite administration being repeated a further three times at 2–3 hour intervals), the plasma concentration of paraquat was reduced within 30 hours to 6% of that in animals not given this antidotal treatment. Lung concentrations of paraquat also failed to reach the levels recorded in rats given the paraquat treatment only. Of the rats given the antidotal treatment, 80% survived even when the treatment was delayed for 10 hours. Slices of human lung were shown *in vitro* to accumulate paraquat from relatively low plasma concentrations in the same manner as did the rat lung, so that similar treatment designed to prevent absorption from the gut into the plasma might well prove beneficial in preventing a fatal accumulation of paraquat in the lung in cases of human poisoning.

Biochemical studies

Ilett *et al.* (*Toxic. appl. Pharmac.* 1974, **28,** 216)

have reported biochemical differences in the responses of rats and rabbits given paraquat iv in a dose of 20 and 10–80 mg/kg respectively. The anticipated lung damage in the rat was accompanied by decreases in microsomal cytochrome *P*-450 concentrations in the lung and in the metabolism of [^{14}C]bromobenzene by lung microsomes at 24 and 72 hours after paraquat treatment. Although mortality was high in rabbits given 40 or 80 mg paraquat/kg, the lung damage seen in the rat did not occur at any of the dosage levels given to rabbits and there were no parallel reductions in cytochrome *P*-450 concentration and bromobenzene metabolism. Moreover there was no evidence of the NADPH oxidation blockade that occurred in rats after 48 hours. In experiments with [^{14}C-methyl]paraquat, the highest tissue concentrations of radioactivity were found in the lungs in each animal species, although rabbits showed no histopathological or biochemical evidence of lung damage. The fall in paraquat concentration from its peak value was more rapid in rabbits than in rats, and this applied also to all subcellular lung fractions. No evidence emerged of covalent binding of paraquat to lung macromolecules, and since the rate of H_2O_2 formation was higher in the rabbit than in the rat lung, the liberation of H_2O_2 was not a satisfactory explanation for the lung lesions in the rat. Witschi & Kacew (*Med. Biol.* 1974, **52,** 104) have remarked on the relative mildness of the biochemical changes in the rat lung compared with the tissue lesions produced by paraquat. Injection of 40–60 mg paraquat/kg produced a dose-related reduction of amyl hydrocarbon hydroxylase (AHH) activity, but the difference was insufficient to serve as an indicator of toxic lung damage. Decreased glucose utilization by paraquat-poisoned lung tissue was accompanied by lowered pyruvate kinase (PK) and lactic dehydrogenase (LDH) activities. However, calculations of total enzyme activity indicated that paraquat had increased the total activity of pulmonary PK, glucose-6-phosphate dehydrogenase and isocitric dehydrogenase and had not increased the true LDH activity. A significant fall in monoamine-oxidase activity after doses of 50–60 mg paraquat/kg correlated well with toxic lung damage. However, the enzyme changes that have been observed are considered less useful for the detection of lung toxicity than of liver toxicity.

Rose *et al.* (*Biochem. J.* 1974, **138,** 437) have found that disturbances of corticosteroid synthesis in the rat adrenal, and the subsequent alteration of liver glycogen utilization are common to both paraquat and diquat and therefore have no direct relevance to the lung lesions characteristic of paraquat poisoning. Adrenal stimulation may, however, explain the appearance of adrenal necrosis as a result of paraquat poisoning in man, the atrophy of lymphoid tissue in the paraquat-treated rabbit, and the changes in thymus, spleen and adrenals induced in the rat by both diquat and paraquat. In rats given 20 mg paraquat/kg ip, the depletion of liver glycogen induced by starvation for 24 hours was less than in controls. Blood glucose levels rose rapidly to a peak at 1 hour, falling to control levels after about 7 hours; plasma corticosteroid levels reached their peak after 30 minutes and remained elevated for at least 24 hours, while plasma adrenocorticotrophin remained high for only 4 hours.

The changes induced by paraquat on the surfactants of animal lungs have been the subject of previous comment (*Cited in F.C.T.* 1974, **12,** 781). Observations by Malmqvist *et al.* (*Scand. J. resp. Dis.* 1973, **54,** 206) on the phospholipid composition of lung homogenates and alveolar washings from rats killed 24 hours after a single sc injection of 35 mg paraquat/kg included a significant decrease in the lecithin fraction of the homogenates, and a more marked decrease in this fraction of alveolar washings, the lecithin concentration here being reduced by more than 50%. The protein content of the wash was increased fourfold, while the cell content increased only slightly. A discrepant narrowing of the hysteresis loop was observed in Wilhelmy balance recordings, despite a slightly reduced minimal surface tension. It seems possible from these observations that the alveolar collapse that is part of the picture of paraquat poisoning is attributable to the interference of paraquat with the phospholipid metabolism of the alveolar epithelium.

Cytological effects

A parallel degree of cytotoxicity was observed when either paraquat or diquat was added to a culture of rat alveolar or peritoneal macrophages (Styles, *Br. J. exp. Path.* 1974, **55,** 71). Exposure of the cultures to either herbicide in a concentration of 10^{-6} mol/litre for 30 minutes produced irreversible cellular damage, as indicated by the uptake of vital stain. The viability of fibroblasts was not reduced, although their cloning efficiency was impaired, by exposure to 10^{-5} mol/litre or less. The toxicity of diquat to fibroblasts was some ten times that of paraquat, a finding which might suggest that if concentrations above this level were reached in the lung following herbicide ingestion, repair would be less inhibited by paraquat than by diquat. However, as the opposite is the case, it appears that the lung lesion characteristic only of paraquat is probably due to the prolonged retention of this compound in the lung, rather than to its absolute concentration at any one time.

The mutagenic properties of paraquat have been compared with that of diquat in the dominant lethal test in mice (Pasi *et al. Mutation Res.* 1974, **26,** 171). Males were given a single injection of 76 mmol diquat/kg or 66 mmol paraquat/kg and then mated over one complete spermatogenic cycle. The females were examined for total implantations after 9–15 days of gestation. Toleration of the given doses was good, and neither compound had a mutagenic effect on any maturation stage of spermatogenesis, as would have been indicated by a significant increase in the incidence of early foetal deaths and preimplantation losses. However, both compounds caused a significant reduction in the pregnancy rates, for weeks 1–5 in the diquat group and for week 3 in the paraquat group. This antifertility effect thus involved only the postmeiotic late spermatids in the case of paraquat, whereas diquat apparently affected all the postmeiotic maturation stages and the premeiotic early and late spermatocytes.

[P. Cooper—BIBRA]

DIET AND CANCER OF THE COLON

In north-west Europe and the USA, cancer of the large bowel has the dubious distinction of being responsible for more deaths than any other type of cancer except that affecting the lungs, and at least in the USA, the colon and rectum together account for more new cancer cases than any other organ site (Berg *et al. Hlth Servs Rep.* 1973, **88,** 915).

Correlation with other forms of cancer

With one or two exceptions, countries with high rates of colon cancer also have high rates of rectal cancer (Wynder *et al. Cancer, N.Y.* 1967, **20,** 113). In Japan, however, the low incidence of colon cancer is not accompanied by a similarly low rate of rectal cancer, and for this and other reasons (Wynder & Shigematsu, *ibid* 1967, **20,** 1520) it seems that the two cancers may have a different aetiology. The incidences of colon and breast cancer, on the other hand, are very closely correlated ($r = +0{\cdot}81$), so a common aetiology seems likely for these two (Drasar & Irving, *Br. J. Cancer* 1973, **27,** 167). A positive correlation has also been found between cancer of the colon and cancer of the pancreas, kidney and prostate (Wynder & Reddy, *Am. J. dig. Dis.* 1974, **19,** 937). It is interesting to note that, in contrast, the incidence and mortality rates for cancer of the colon show a strongly negative correlation with those for stomach cancer (Drasar & Irving, *loc. cit.*; Wynder & Shigematsu, *loc. cit.*).

Correlation with other diseases

Patients with ulcerative colitis carry a greatly increased risk of developing cancer of the large bowel, the risk being dependent on the location and spread of the colitis (MacDougall, *Lancet* 1964, **ii,** 655). Similarly a link between familial polyposis, a disease of genetic origin, and colon cancer is well established (Wynder & Shigematsu, *loc. cit.*). Apart from this association with a genetically determined disease, there is little evidence to suggest that heredity plays a major role in the aetiology of colon cancer. A positive correlation also exists between colon cancer and myocardial infarction, although factors related to the latter, such as hypertension, obesity, smoking and elevated levels of blood cholesterol, do not appear to be associated with colon cancer (Wynder & Shigematsu, *loc. cit.*).

Epidemiological investigations

Many epidemiological studies of large-bowel cancer have been carried out and all provide strong indications of the involvement of some environmental factor or factors (Burkitt, *Cancer, N.Y.* 1971, **28,** 3; Draser & Irving, *loc. cit.*; Haenszel & Correa, *Cancer, N.Y.* 1971, **28,** 14; Wynder & Reddy, *loc. cit.*; Wynder & Shigematsu, *loc. cit.*). These studies have shown that the incidence of colon cancer is highest in industrialized areas of the world, such as the UK, USA and northern Europe, whereas the disease is comparatively rare in countries and areas of lower socio-economic status, such as Uganda, Portugal, Israel, Puerto Rico, Asia and South America (Wynder & Shigematsu, *loc. cit.*). It must be pointed out that the differences in incidence are so great that the findings cannot be attributed to differences in diagnostic procedure or the efficiency of medical care. In the USA, large-bowel cancer is less common in southern areas than in the north and the incidence is also lower in rural areas than in urban regions.

The most decisive evidence, however, for the involvement of environmental factors in the aetiology of colon cancer comes from studies of populations that have migrated from areas of low incidence to areas of high incidence. Notable among these are studies of groups that have settled in the USA from Japan, Norway or Poland. Mortality from colon cancer among these groups has risen to the higher level characteristic of US residents (Haenszel, *J. natn. Cancer Inst.* 1961, **26,** 37). Furthermore, the children of such migrants experience risks from colon cancer typical of the USA (Haenszel & Correa, *loc. cit.*). Among Polish migrants to Australia, the incidence of cancer of the colon has again been found to show an increase from the low Polish level to the higher Australian rate (Staszewski *et al. Br. J. Cancer* 1971, **25,** 599). Migration within the USA also alters the risks of bowel cancer, mortality again being typical of the place of residence rather than that of birth (Haenszel & Dawson, *Cancer, N.Y.* 1965, **18,** 265). In this context, it is interesting that similar investigations of the incidence of stomach cancer in migrant populations have revealed exactly the opposite pattern, the risks being characteristic of the country of origin (Haenszel, *loc. cit.*).

Environmental factors

Wynder & Shigematsu (*loc. cit.*) carried out an exhaustive investigation of a large number of factors that could play some role in the aetiology of colon cancer. Factors found not to be related to, or only inconsistently associated with, the incidence of the disease included alcohol, obesity, blood group, constipation, the use of purgatives and gastro-intestinal surgery, including appendectomy. There was an apparent association between smoking and cancer of the large bowel and especially between cigar- or pipe-smoking and the incidence of sigmoid cancer, but the complex association of smoking with other factors such as obesity, alcohol, socio-economic status and nutritional habits makes interpretation of the results hazardous.

Diet is considered by the majority of investigators as the environmental factor most likely to be involved in the aetiology of large-bowel cancer, but there are considerable differences of opinion as to the actual component of the diet responsible. It seems unlikely that food additives are a source of large-bowel carcinogens, since Denmark, which has very strict controls on the use of additives, still has a high mortality rate from colon cancer. Moreover, in countries such as India, where exposure to food additives is minimal, there are considerable regional differences in the incidence of the disease (Berg *et al. loc. cit.*).

Comparisons of the diets of patients with cancer of the colon and those of healthy individuals have revealed no consistent differences (Higginson, *J. natn. Cancer Inst.* 1966, **37,** 527; Pernu, *Annls Med. intern.*

Fenn. 1960, **49,** 33; Stocks, *Rep. Br. Emp. Cancer. Campn* 1957, no. 35 (suppl. II), p. 51; Wynder & Shigematsu, *loc. cit.*), although Wynder *et al.* (*Cancer, N.Y.* 1969, **23,** 1210) found that the diet of Japanese patients with colon cancer contained less rice and more fruit and milk than that of control patients, a finding indicative of the higher socio-economic status and 'westernized' diet of the cancer patients.

More interesting results have been obtained from studies of diets of population groups rather than of individuals. These investigations have led to three hypotheses, postulating respectively that colon cancer is related to a low intake of fibre in the diet, to the consumption of large quantities of fat or to the intake of large amounts of animal protein, since these three factors are said to characterize the diets of western societies having a high incidence of cancer of the large bowel.

Dietary fibre

Cleave (*J. R. nav. med. Serv.* 1956, **42,** 55) suggested that many of the diseases common in affluent western societies, such as atherosclerosis, obesity, diverticular disease and appendicitis, were due to the adoption of diets containing highly refined carbohydrates and in particular to the elimination of fibre from sugar and flour. The term 'fibre' covers a wide variety of unavailable carbohydrates, notably water-soluble polysaccharides, hemicellulose, cellulose and lignin (Southgate, *J. Sci. Fd Agric.* 1969, **20,** 331). Burkitt (*loc. cit.*; *idem, J. natn. Cancer Inst.* 1971, **47,** 913; *idem, Pathologia Microbiol.* 1973, **39,** 177) has developed the Cleave theory and has attributed the high incidence of colon cancer in certain countries to a lack of fibre in the diet, basing his hypothesis on the effects of unabsorbable fibre on the physiology of the large bowel.

It has been shown that people consuming diets high in fibre have faster intestinal transit times and much greater faecal bulk than people on low-fibre diets (Burkitt, *Cancer, N.Y.* 1971, **28,** 3; Burkitt *et al. Lancet* 1972, **ii,** 1408). For example, the transit time in rural Africans is often only 40 hours, whereas in the UK population, transit times of 80 hours are common; in addition, the weights of faeces passed by Africans are 2–4 times as great as those passed by the UK population. It is suggested, therefore, that in people on an unrefined high-fibre diet, the large bulk of faeces passing through the colon effectively dilutes any carcinogen ingested or formed in the gut, and thus reduces the amount of carcinogen in contact with the gut wall. In addition, the faster transit times associated with high-fibre diets are assumed to reduce the time available for the production of carcinogens by bacteria in the gut (discussed in more detail below) and, more importantly, to shorten the time of contact of carcinogens with the bowel wall.

Assessment of the evidence for and against this hypothesis is complicated by the fact that chemically ill-defined fibre has been used in many of the experimental studies. Since the various types of fibre appear to have quite different physiological effects on the gut, apparently conflicting results may be due to the use of totally different types of dietary fibre in the experiments. It is interesting in this context that Robertson (*Nature, Lond.* 1972, **238,** 290) has shown that there has, in fact, been very little change in the total dietary intake of fibre in the past 100 years, although the sources of the fibre have altered, with a reduction in the consumption of fibre from cereal products and an increase in that from vegetables and fruit. Drasar & Irving (*loc. cit.*), using data from 37 countries, have shown that the incidence of colon cancer does not correlate at all with dietary levels of fibre, but in view of the highly complex nature of the material in question this evidence should perhaps not be considered too damning to the cause of dietary fibre.

The dilution of possible colon carcinogens by high-fibre diets appears to be well substantiated. Of these possible carcinogens, faecal steroids (bile acids, neutral steroids, such as cholesterol, and their bacterial metabolites) have been the most intensively studied. They have been found to be present in much lower concentrations in the stools of people in areas with a low incidence of colon cancer than in people in the UK and USA and, additionally, the steroids are more extensively degraded in the latter groups (Aries *et al. Gut* 1969, **10,** 334). It must be remembered, however, that differences in the diets of the two groups are not only confined to the amounts of fibre, so these differences in steroid concentration may equally well be attributable to differences in the fat or meat content of the diets. Eastwood *et al.* (*Br. med. J.* 1973, **4,** 392) found that the addition of wheat bran or cellulose to the normal English diet increased the weight of faeces passed and reduced the faecal concentrations of bile acids, but intestinal transit time was unaffected. Similar results have been reported by Walters *et al.* (*ibid* 1975, **2,** 536), who studied the effects of dietary supplements of wheat bran (mainly hemicellulose) or bagasse (mostly cellulose and lignin). The two types of fibre had no effect on the total loss of neutral steroids, but because of an increase in faecal bulk the steroids were effectively diluted. The faecal concentration of acid steroids was reduced by wheat bran, but not by the bagasse supplement, which increased the excretion of bile acids. No changes in the extent of degradation of acid or neutral steroids by bacteria were detected. This contrasted with the work of Pomare & Heaton (*ibid* 1973, **4,** 262), who found that the addition of wheat bran to the diet reduced the metabolism of bile salts by intestinal bacteria and, in addition, increased the excretion of bile acids.

The relatively unimportant role of transit time in the aetiology of colon cancer is evident from the work of Glober *et al.* (*Lancet* 1974, **2,** 80), who found that the increase in colon-cancer incidence in Japanese migrants to Hawaii was not accompanied by any change in transit time. The findings of Wynder & Shigematsu (*loc. cit.*) are also apposite in this context, since these authors showed that there was no correlation between bowel habits, especially constipation and the use of laxatives, and colon cancer. Thus it appears that if dietary fibre does have any effect on the interaction of faecal carcinogens with the bowel mucosa, it does so by increasing faecal bulk rather than by altering the transit time.

Another possible mechanism by which dietary fibre protects the gut against the action of potential carcinogens is the adsorption of such compounds on

to the fibre or their sequestration within a colloid matrix of fibre. There is evidence that several types of fibre can bind bile salts *in vitro* (Kritchevsky & Story, *J. Nutr.* 1974, **104,** 458), although Heaton *et al.* (*Scand. J. Gastroent.* 1971, **6,** 281) could not demonstrate adsorption *in vivo*.

There are distinct differences in the composition of the gut flora of people in areas of high and low incidence of colon cancer (Aries *et al. loc. cit.*), and although diet appears to be the controlling factor, it is difficult to determine which component of the diet is responsible. Moreover, it seems unlikely that the differences in gut flora can be attributed to changes in the fibre content of the diet, since an alteration in diet from one containing fibre to one completely absorbable, or conversely from a low-fibre to a high-fibre content, has little or no effect on the bacterial flora (Attebery *et al. Am. J. clin. Nutr.* 1972, **25,** 1391; Walters *et al. loc. cit.*).

Animal protein and fat

Gregor *et al.* (*Gut* 1969, **10,** 1031) using data from 28 countries, found that mortality rates for intestinal cancer were closely correlated with the intake of animal protein. Furthermore, there appeared to be an association between the rate of increase in the incidence of colon cancer and an increase in the consumption of animal protein. In a more extensive study, Drasar & Irving (*loc. cit.*) confirmed the close association of colon cancer with the intake of meat and also with that of 'combined fat', consisting mainly of fat derived from meat. The correlation between combined fat and animal protein was too close for them to be distinguished and it is possible that the correlation of meat consumption with colon cancer is due to its contribution to the overall fat intake of a population.

Wynder & Shigematsu (*loc. cit.*) had previously suggested from an epidemiological investigation of migrant populations that fat consumption was implicated in colon cancer. They showed that the incidence of large-bowel cancer among Japanese migrants to Hawaii changed from that characteristic of Japan to that typical of the USA, and that the diet of the migrants changed from a Japanese diet in which only 12% of the calorie intake was in the form of fat, mostly unsaturated, to the American type of diet in which 40–44% of the calorie content was fat, approximately half of which was saturated. Furthermore, they reported that the Jewish population of New York, which generally consumes a diet with a content of animal fat somewhat higher than that consumed by other groups, had a higher rate of colon cancer than other New York residents, while Seventh Day Adventists, who eat little meat and have a lower intake of saturated fats than most Americans, had a somewhat lower rate.

Given that dietary constituents such as fat are associated with cancer of the colon, one must question what potential carcinogens or co-carcinogens are present in the intestinal lumen and whether the concentration of such compounds changes when the diet is altered. The compounds suspected by most workers are acid and neutral steroids and their bacterial metabolites, notably the bile acids, such as cholic and deoxycholic acids, and cholesterol. One of the reasons for implicating faecal steroids in the aetiology of colon cancer is that they bear a steric similarity to some potent carcinogens, including 3-methylcholanthrene.

Hill (*J. Path.* 1970, **104,** 239) has shown that the faecal concentration of bile acids and neutral steroids in adults depends on the intake of fat. When healthy volunteers changed from a normal high-fat diet to one low in fats (less than 30 g fat/day), the concentration of faecal acid steroids fell immediately and had reached a new equilibrium level of 25–30% of the control value within a few days. The faecal concentration of cholesterol-derived neutral steroids declined more slowly but after 4 weeks had reached an equilibrium of 40% of the control value.

Steroids such as deoxycholic acid and apocholic acid have been shown to produce sarcomas at the site of injection in animals (Cook *et al. Nature, Lond.* 1940, **145,** 627; Lacassagne *et al. ibid* 1961, **190,** 1007; *idem, ibid* 1966, **209,** 1026), and bacterial metabolites of bile acids have been reported to possess carcinogenic activity (Druckrey *et al. Klin. Wschr.* 1941, **20,** 781). In addition, Nigro *et al.* (*Dis. Colon Rectum* 1973, **16,** 438) have shown that increased secretion of bile acids by the liver enhances the formation of intestinal tumours by azoxymethane and other carcinogens.

In a similar study, Wynder & Reddy (*loc. cit.*) have shown that, in rats, taurodeoxycholic acid or lithocholic acid increases the yield of colorectal tumours induced by 1,2-dimethylhydrazine, a finding that suggests that these bile acids can act as tumour promoters although they may not be carcinogens *per se*. Interestingly, it has also been shown that rats fed a diet high in beef fat are more susceptible to the induction of tumours by azoxymethane than are rats fed a normal diet (Nigro *et al. J. natn. Cancer Inst.* 1975, **54,** 439).

The microbial flora of the gut appears to play an important role in the metabolism of bile acids and neutral steroids. In a series of papers, Hill and his colleagues have shown that intestinal bacteria can deconjugate bile salts (Draser *et al. Lancet* 1966, **i,** 1237) and can effect dehydrogenation, aromatization and dehydroxylation of the steroid nucleus (Aries *et al. Biochim. biophys. Acta* 1971, **248,** 482; Goddard & Hill, *ibid* 1972, **280,** 336; Hill & Drasar, *Gut* 1968, **9,** 22). In view of the metabolic versatility of intestinal micro-organisms, it is possible that they can also convert acid and neutral steroids into carcinogens. Evidence to support this hypothesis has been sought by comparing the bacterial flora, faecal steroid composition and products of bile degradation in people from areas of high and of low colon-cancer incidence. People in areas of high incidence (England, Scotland and the USA) had a faecal flora containing larger numbers of gram-negative non-sporing anaerobes (Bacteroides) and fewer enterococci than the flora of people in areas of low incidence (Uganda, India and Japan) and faecal steroids were present in much lower concentration in the latter (Aries *et al.* 1969, *loc. cit.*; Hill & Aries, *J. Path.* 1971, **104,** 129; Hill *et al. Lancet* 1971, **i,** 95). Moreover, the faecal steroids present in people from the high-risk areas were far more extensively metabolized than those from Ugandans, Indians and Japanese, a finding

which was to be expected since it is the anaerobic bacteria of the gut flora (i.e. Bacteroides. Clostridia and Bifidobacteria) that are most active in steroid metabolism. In addition, it was found that a far greater proportion of strains of these anaerobes isolated from the British and Scottish people could metabolize steroids (by dehydroxylation at positions 7 and 12 of the steroid nucleus) than of those isolated from Ugandans or Indians (Hill & Aries, *loc. cit.*). Wynder & Reddy (*loc. cit.*) obtained similar results by comparing American with Japanese and Chinese populations, finding that the former population group degraded and excreted bile acids and cholesterol to a greater degree than the two latter groups.

More recently, the faecal bile acids and the levels of Clostridia capable of dehydrogenating the steroid nucleus have been compared in patients with colon cancer and patients with other diseases (Hill *et al. Lancet* 1975, **I**, 535). High faecal bile-acid concentrations were found in 82% of the cancer patients but in only 17% of the controls and most of the former group had faecal Clostridia capable of bile-salt dehydrogenation. It is possible that the estimation of faecal levels of bile acids and nuclear-dehydrogenating Clostridia might provide a useful method for the detection of large-bowel cancer, especially in people with familial polyposis or ulcerative colitis, who are particularly at risk from this type of cancer.

To summarize, therefore, epidemiological investigations strongly suggest a link between diet and cancer of the colon, the incidence of which appears to reflect the degree of 'westernization' of the diet. The causative agent in the diet is still a hotly debated issue, but there is good evidence to implicate high dietary concentrations of fat and animal proteins. Lack of dietary fibre also appears to be involved, probably because it decreases faecal bulk and thus increases the concentration of carcinogens in the gut. If cancer of the colon does prove to be due to dietary factors, this cancer, and possibly cancers of the breast and rectum, should ultimately be preventable by the adoption of suitable diets.

[I. R. Rowland—BIBRA]

Fd Cosmet. Toxicol. Vol. 14, pp. 213–225. Pergamon Press 1976. Printed in Great Britain

TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS

FLAVOURINGS, SOLVENTS AND SWEETENERS

2995. Canadian carcinogenicity study on saccharin

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.

Saccharin has been used as an artificial sweetener for over 70 years. Although no adverse effects have been associated with its use in man, there has been continual controversy regarding its safety, particularly its potential capacity to produce bladder cancer following oral administration to laboratory animals (*Cited in F.C.T.* 1973, **11,** 1126). As part of an exhaustive scientific effort to determine unequivocally its carcinogenic potential, the study cited above was undertaken to test the carcinogenicity of saccharin sold in Canada. The sample tested was manufactured in Japan and conformed to the USP, BP and Food Chemicals Codex specifications.

Groups of 60 male and 60 female Charles River rats, free from the bladder parasite *Trichosomoides crassicauda*, were fed diets containing sodium saccharin at levels to provide a daily intake of 0, 90, 270, 810 or 2430 mg/kg body weight for 26 months. A dose-related increase in mortality was found in male rats, and rats of both sexes on the highest dose level showed a reduction in body weight, probably caused in part by the saccharin-induced diarrhoea that occurred in these animals. Throughout the study, no effect was observed on haematological parameters or the results of urine analyses. Pituitary tumours, lymphomas and mammary tumours occurred frequently, but the incidence was unrelated to saccharin administration. One bladder tumour was found in a male control and four occurred in treated animals, one each in a male and female rat in the group on the lowest dose level and two in male rats on the 810-mg/kg dose. All four tumours in the test groups were observed macroscopically and diagnosed histologically as transitional-cell papillomas, none of which were invasive. The tumour in the control rat was considered to be an angiosarcoma. Bladder calculi were observed in three rats, and in 67 others small calculi were seen microscopically in filtered urine samples. No relationship was found between the dose level of saccharin and the calculus incidence, or between the incidence of calculi and of bladder tumours.

These results demonstrate that under the conditions of this test, saccharin was not found to be carcinogenic in rats fed maximally tolerated doses for 26 months. The findings complement the results of Schmähl (*Arzneimittel-Forsch.* 1973, **23,** 1466) but contrast with experiments carried out by the FDA (Taylor & Friedman, *Toxic. appl. Pharmac.* 1974, **29,** 154) and by the Wisconsin Alumni Research Foundation (*Food Chemical News* 1973, **15** (4), 18) showing the development of bladder tumours in rats exposed both *in utero* and throughout their lifespan to high doses of saccharin.

[A report of the National Academy of Sciences subcommittee on saccharin (*Food Chemical News* 1975, **16** (43), 48) has called for the study of various additional aspects, including transplacental carcinogenesis of saccharin, the toxicological significance of impurities in commercial saccharin, and changes in urine composition after high doses of saccharin and their relationship to the induction of bladder calculi.]

ANTIOXIDANTS

2996. A butylhydroquinone assessment

Astill, B. D., Terhaar, C. J., Krasavage, W. J., Wolf, G. L., Roudabush, R. L., Fassett, D. W. & Morgareidge, K. (1975). Safety evaluation and biochemical behavior of monotertiarybutylhydroquinone. *J. Am. Oil Chem. Soc.* **52,** 53.

Mono-*tert*-butylhydroquinone (2-*tert*-butylhydroquinone; TBHQ) is approved in the USA for use as an antioxidant in edible oils and fats, alone or with other antioxidants at a maximum total antioxidant level of 200 ppm (21 CFR 121.1244). Data submitted to the FDA in support of the petition for clearance of this material have now been published. The studies reported include the feeding of TBHQ to rats at dietary levels of 0·016–0·5% for 20 months, a three-generation reproduction study in rats fed 0·5% TBHQ and the feeding of rats for 6 months on 0·02–0·5% TBHQ that had been heated at 190°C (375°F) for 3 hr. In addition, beagles were fed 0·05–0·5% TBHQ for 2 yr, and metabolism and excretion studies were performed in rats, dogs and man.

The wide range of parameters studied in each of the feeding and reproduction tests revealed no evidence of toxicity on the part of TBHQ. The only change was a slight but statistically significant increase in serum glutamic–oxalacetic transaminase activity in rats fed the highest level (0·5%) of heated TBHQ for 3 or 6 months, but this was not considered to indicate cellular damage.

Studies in the dog have shown that TBHQ is itself a major metabolite of butylated hydroxyanisole (BHA). In rats, most of a single dose of TBHQ was found to be excreted in the urine as the *O*-sulphate conjugate, with a small amount as the *O*-glucuronide and even less as uncombined phenolic compounds. Single doses of 100 mg TBHQ/kg were almost completely excreted within 48 hr, and after 400 mg/kg excretion accounted for 65% of the dose within 4 days. In dogs, a major part of the dose appeared in

the urine as unchanged TBHQ, together with some *O*-glucuronide and *O*-sulphate conjugates, the latter being a less important end-product than in the rat. In the human studies, volunteers given 0·5–4 mg TBHQ/kg in 30% corn oil excreted almost the entire dose in the urine within 40 hr, whereas when taken in 10% corn oil less than half the dose appeared in the urine. The serum levels of TBHQ reflected the urinary concentrations and the fall in urinary output of total TBHQ, and it was concluded, therefore, that absorption was much lower with a low-fat than with a high-fat vehicle. Again the *O*-sulphate accounted for a major part of the dose and a small amount of *O*-glucuronide was excreted, but no free TBHQ was detected. In rats given ^{14}C-labelled TBHQ in single doses or at a low level in the diet for 17 days, no significant body burden was detected.

In the USA, the maximum daily intake of phenolic antioxidants by man from foods containing the maximum permitted level of 200 ppm has been estimated to be about 0·3 mg/kg, of which up to about 0·07 mg/kg would probably be TBHQ. It is calculated, therefore, that the results now reported suggest safety margins of 1000–10,000 for this compound.

AGRICULTURAL CHEMICALS

2997. Reassurance on carbaryl absorption

Comer, S. W., Staiff, D. S., Armstrong, J. F. & Wolfe, H. R. (1975). Exposure of workers to carbaryl. *Bull. env. contam. & Toxicol. (U.S.)* **13,** 385.

Carbaryl has been shown to be one of the pesticides most readily absorbed through the skin (*Cited in F.C.T.* 1975, **13,** 477). The exposure of workers engaged in mixing and bagging it, as well as of those applying it in the field, is therefore of importance.

Workers engaged in mixing and bagging dusts containing 4 or 5% carbaryl were subjected to higher levels of dermal and respiratory exposure than field-workers handling 0·045–0·06% carbaryl sprays in orchards. The mean potential exposure of formulators was 73·9 mg/hr through the skin and 1·1 mg/hr via the lungs, compared with 59 and 0·09 mg/hr, respectively, for spray operators. In plant workers, the regions most exposed to carbaryl were the hands, forearms and the front of the body, whereas in spray operators, droplet contamination affected mainly the shoulders and back of the neck. Urinary 1-naphthol levels after exposure to carbaryl were in the range 0·2–65 ppm (mean 8·9 ppm), 26 of the 102 findings being in the "high" concentration range above 10 ppm. The mean excretion rate of 1-naphthol was 0·5 mg/hr with a range of 0·004–3·4 mg/hr. Urinary excretion increased from the start of the working day to reach a maximum in the late afternoon and evening, and fell again by the next morning.

The excretion of 0·5 mg 1-naphthol/hr represents excretion of about 0·7 mg carbaryl/hr, indicating an 8-hr absorption figure of 5·6 mg carbaryl. As noted above, however, the potential exposure of packers was some 75 mg/hr, or 600 mg in an 8-hr day, so that dermal absorption of carbaryl in dust form is probably far from complete. In view of the relatively low toxicity of carbaryl, working exposure to large quantities is considered reasonably safe. Nevertheless, in the interests of safety, the use of protective clothing and respirators is considered advisable during the formulation, packing and spraying of this pesticide.

2998. Toxicity studies on MCPA and MCPP

Verschuuren, H. G., Kroes, R. & den Tonkelaar, Engelina M. (1975). Short-term oral and dermal toxicity of MCPA and MCPP. *Toxicology* **3,** 349.

A farmer whose clothing became saturated with 2-methyl-4-chlorophenoxyacetic acid (MCPA) developed aplastic anaemia, accompanied by haemorrhagic gastritis and by possible indications of slight liver damage (*Cited in F.C.T.* 1976, **14,** 69). When rats were fed MCPA or 2-(2-methyl-4-chlorophenoxy)propionic acid (MCPP) for 7 months at dietary levels of 100–2500 ppm, kidney weights were increased at all levels, and 400 ppm and above produced anaemia and an increase in female liver weights, with liver-cell enlargement at the highest level of MCPP (Gurd, Harmer & Lessel, *Fd Cosmet. Toxicol.* 1965, **3,** 883). LD_{50} values ranging from 300 to 600 mg/kg for the ip route and from 550 to 1060 mg/kg given orally were established in rats and mice for the sodium and diethanolamine salts of MCPA and MCPP, but the compounds were only poorly absorbed through mouse skin, even when it was damaged. MCPA has been shown to be dehalogenated by soil bacteria (*Cited in F.C.T.* 1968, **6,** 805).

The study cited above established LD_{50} values for MCPP, as the acid, of 402 and 1210 mg/kg in rats treated ip and orally, respectively. MCPA given orally or dermally to rabbits appeared unchanged in the urine in the first 24 hr. Single application of MCPA or MCPP as an aqueous paste to the abraded or shaved skin of rabbits produced slight erythema, and MCPP-treated skin became sclerotic after a few days but had healed in 7–12 days. Either compound applied five times weekly for 3 wk to shaved rabbit skin at dose levels of 0·5, 1·0 or 2·0 g/kg produced a dose-related erythema, and there was a reversible decrease in skin elasticity following MCPA treatment. Significant growth retardation occurred during treatment with the lowest dose level of either compound, although recovery was observed after treatment ceased. At higher levels there was severe weight loss and some deaths occurred, particularly in MCPA-treated rabbits. Haematological changes involved only a reversible decrease in lymphocytes with 0·5 g MCPA/kg, evaluation at higher MCPA levels being precluded by the small number of animals. Organ weights were normal in the MCPP groups, but were not determined in the MCPA rabbits. At the two highest levels of MCPA, there was evidence of focal liver necrosis, renal tubular degeneration and marked lymphocyte depletion in the spleen and thymus, all of which could, however, have been due to a concurrent dysbacteria infection.

When rats were fed MCPA or MCPP for 90 days at dietary levels of 50, 400 or 3200 ppm, the highest level of either compound depressed food consumption and body-weight gain. Rats fed 3200 ppm MCPA had enlarged erythrocytes with an increased haemoglobin content, while rats fed 3200 ppm MCPP had decreased haemoglobin and erythrocyte counts, accompanied in the males by a decreased haematocrit and leucocyte count and in the females by a decrease in neutrophils. This level of MCPP also increased alkaline-phosphatase activity, indicating possible liver damage, and decreased relative ovary and prostate weights. Erythrocyte levels were depressed in males fed MCPP even at 400 ppm, and each compound produced a significant increase in relative kidney weight at 400 and 3200 ppm, but no histological changes were detected in any organ. At 50 ppm there was only an insignificant increase in kidney weight, unaccompanied by other signs of adverse effect.

FEED ADDITIVES

2999. The persistence of diethylstilboestrol residues

Aschbacher, P. W., Thacker, E. J. & Rumsey, T. S. (1975). Metabolic fate of diethylstilbestrol implanted in the ear of steers. *J. Anim. Sci.* **40**, 530.

The use of diethylstilboestrol (DES) implants for fattening cattle and sheep was banned by the FDA in 1973, after residues of this compound and/or its conjugates had been detected in the livers of steers slaughtered up to 60 days after implantation. The residues were detected by radioactive-tracer studies and confirmed by isotope-dilution and recrystallization techniques and by thin-layer chromatography, rather than by the less sensitive official method involving mouse uterine assay. The FDA subsequently proposed to revoke the official method but substitution of an alternative method awaits agreement on the necessary level of sensitivity. Meanwhile, a court order for a public hearing had the effect of voiding the FDA ban and there has followed a sharp increase in the number of illegal DES residues detected. The regulation governing ear implants for beef cattle (Sec. 135b.6) specifies only a 21-day withdrawal period before slaughter, but the study now under review shows that low residues can persist far beyond this time.

Four steers were implanted in the ear with pellets containing about 28 mg [^{14}C]DES and were slaughtered after 30, 60, 90 and 120 days. Levels of radioactivity in tissues and excreta were determined by liquid scintillation, allowance being made for background levels as determined in control animals, and the labelled compounds were characterized by thin-layer chromatography and isotope-dilution procedures after extraction and hydrolysis (to free any conjugated DES present). In addition, some faecal extracts were subjected to gas-liquid chromatography (GLC).

Radioactivity persisted in the plasma throughout the 120 days, the levels at the end of this period being about one-third of the early values. Urinary and faecal excretion rates conformed to a similar pattern. The total excreted had accounted for 24·7, 56·8, 52·5 and 84·9% of the implanted radioactivity after 30, 60, 90 and 120 days, respectively, activity in the faeces being some 2–3 times greater than that in the urine. In four animals, total recovery of radioactivity from excreta and tissues ranged from 82·7 to 90·9% of that implanted. The proportion remaining in the ears fell from 65·9% after 30 days to 4·2% after 120, but that detected in the carcass and internal organs remained relatively constant at 0·03–0·05% of that implanted. Highest tissue concentrations were found in the bile and gall bladder (homogenized together), salivary gland, lungs, liver and kidney, but there was considerable variation between animals and no evidence of a decline with time. In the liver, 9–34% of the label was identified by isotope dilution as DES or a conjugate, equivalent to 0·07–0·13 ppb ($b = 10^9$) DES, and omission of the hydrolysis step from one sample indicated that about 7% (0·04 ppb DES) was in unconjugated form. The major part of the urinary radioactivity appeared to be present as either DES or a conjugate, but in the faeces less than half was in these forms, and GLC data suggested the presence of a much smaller molecule than DES.

THE CHEMICAL ENVIRONMENT

3000. Chromates as lung carcinogens

Langård, S. & Norseth, T. (1975). A cohort study of bronchial carcinomas in workers producing chromate pigments. *Br. J. ind. Med.* **32**, 62.

Chromates have come under fire chiefly for their sensitizing properties, but there is also some evidence that calcium chromate, formed during the processing of crude chrome ores, has some carcinogenic potential (*Cited in F.C.T.* 1969, **7**, 690).

The study by Langård & Norseth (cited above) involved 133 workers employed in making chromate pigments between 1948 and 1972. Of the 24 workers employed for more than 3 yr, there were three with bronchial carcinoma. Compared with an incidence rate of 0·079 for a corresponding group of the general population of Norway, the risk ratio for chrome workers was about 38. The average age of these workers when they were found to have bronchial carcinoma was 50 yr. The workers in this company were exposed mainly to zinc chromate, and those develop-

ing carcinomas had probably been exposed to 0·5–1·5 mg chromium/m^3 for 6–9 yr. One may also have been exposed to lead chromate, and two were tobacco smokers.

While most cases of respiratory cancer previously associated with chromate exposure have occurred in the chromate-producing industry, it seems that some cancer hazard exists also in the chromate-pigment industry. Although the intervention of the raw material, sodium dichromate, cannot be ruled out entirely and the possible influence of tobacco smoking cannot be assessed in this small group, the data presented suggest that exposure to zinc chromate may increase the risk of bronchial carcinoma.

3001. The lead line and its significance

Eisenstein, R. & Kawanoue, S. (1975). The lead line in bone—a lesion apparently due to chondroclastic indigestion. *Am. J. Path.* **80,** 309.

Chronic ingestion of lead by children leads to a lesion in the long bones characterized by a line of increased density on X-ray films. This so-called 'lead line' is situated anatomically within the growing, metaphyseal, region of the bone, and its development has been noted in association with lead poisoning in several animal species.

The histology of this lesion in man was described some 40 yr ago by Park *et al.* (*J. Pediat.* 1933, **3,** 265), whose findings of impaired resorption of calcified metaphyseal cartilage, depressed bone deposition on cartilaginous surfaces and the accumulation of numerous multinucleate giant cells, some containing lead inclusions, have been confirmed by the authors cited above. The latter point out that closely similar lesions are found in the bones of young monkeys with lead encephalopathy and they developed also in guinea-pigs given 4% lead subacetate in the diet for 6 wk.

The bone lesions in guinea-pigs were examined ultrastructurally, particular attention being paid to the appearance of the giant cells. These conformed morphologically to osteoclasts (cells that resorb bone) and chondroclasts (cells that resorb cartilage) and contained large amounts of calcified cartilage. It seems that lead impairs the normal ability of these cells to degrade cartilage, so that the lead line is more a manifestation of a persistence of calcified cartilage than of a primary change in bone metabolism.

3002. More lead in the mouse kidney

Choie, D. D., Richter, G. W. & Young, L. B. (1975). Biogenesis of intranuclear lead-protein inclusions in mouse kidney. *Beitr. Pathol.* **155,** 197.

In a recent issue (*Cited in F.C.T.* 1976, **14,** 156) we referred to a demonstration that injection of lead into the mouse promotes a rapid increase in protein synthesis in the kidneys. Earlier, this group had shown that after a single injection of lead, characteristic intranuclear inclusion bodies could be demonstrated in the proximal tubule of the rat within as little as 24 hr (*ibid* 1973, **11,** 919). These inclusion bodies are composed of a lead–protein complex and it has been postulated that the protein component arises either from preformed cellular protein or from plasma proteins.

The authors cited above continued the work of this same group by investigating the appearance of both intranuclear and intracytoplasmic inclusion bodies in the renal cells of mice given a single intracardiac injection of lead acetate in a dose of 5–30 mg lead/kg body weight either as the sole treatment or after inhibition of protein synthesis by injections of 20 mg cycloheximide/kg body weight. In addition, cultures of kidney cells were prepared and incubated in a medium containing 20 μg lead/ml.

The appearance of inclusion bodies *in vivo* was found to be both time- and dose-related. Following treatment with 30 mg lead/kg, intranuclear inclusions were demonstrated in two of five animals at 6 hr, while at 24 hr they were present in all mice following all levels of treatment. No intranuclear inclusions were found in mice treated also with cycloheximide. Inclusion bodies were demonstrated after 15 hr in a few cells in lead-treated tissue culture.

The authors postulate that the protein does not arise from preformed or plasma protein but is synthesized as a direct result of lead stimulation, since the inclusion did not occur after protein synthesis had been inhibited. They suggest that this synthesis could occur in both the nucleus and cytoplasm or in the cytoplasm alone. If the latter is true, the lead and protein must form a soluble complex, which can be partly transported to the nucleus and polymerized there as well as in the cytoplasm. It is also proposed that cytoplasmic inclusions may be removed by autophagy, a concept supported by the presence of intranuclear and the absence of intracytoplasmic bodies after chronic lead poisoning.

3003. Coping with nickel

Samitz, M. H. & Katz, S. A. (1975). Nickel dermatitis hazards from prostheses: *in vivo* and *in vitro* solubilization studies. *Br. J. Derm.* **92,** 287.

Katz, S. A. & Samitz, M. H. (1975). Leaching of nickel from stainless steel consumer commodities. *Acta derm.-vener., Stockh.* **55,** 113.

The occurrence of dermatitis attributable to stainless-steel prostheses has been disputed (*Cited in F.C.T.* 1974, **12,** 279). These two papers provide evidence that leaching of nickel (Ni) from stainless steels (which usually contain 10–14% Ni for surgical purposes and more variable proportions for domestic appliances) is quite possible.

In the first study, stainless-steel suture wires, screws and prostheses were cut and immersed for 1 wk at room temperature (20–22°C) in sweat, blood, plasma or physiological saline. The liquid phase was then assayed for Ni spectrophotometrically. Saline was found to be capable of leaching out 0·1–9·8 ppm Ni, sweat 0·3–99 ppm and whole blood 2·6–17·4 ppm under these conditions. When stainless-steel spheres were implanted into the leg muscles of rabbits, there was a local distribution of up to 46·2 ppm Ni after 3 wk and 53·3 ppm after 6 wk in the tissue immediately adjacent to the implant, but no appreciable

quantity was detected at a distance of 1 cm or more from the sphere.

In the second study, various Ni alloys and plated metals were immersed for 1 wk at room temperature in saline, synthetic sweat or a solution of household detergent. From many specimens (2–10 sq cm surface area) far more than 0·5 μg Ni could be leached into the liquid phase (5 ml), although an extraction level of this order has been suggested as an "eliciting safety limit". The conditions of the experiment did not reflect actual domestic exposure, but since the use of Ni in kitchen furniture and equipment is increasing, and exposure to coins and jewellery also occurs, sensitized persons are likely to be subjected to significant Ni exposure. Some samples of stainless steel from which Ni could be leached experimentally in significant quantities failed to give positive reactions when spot-tested with dimethylglyoxime. It seems, therefore, that this screening test, though widely used by dermatologists, cannot be regarded any longer as a reliable index of safety for Ni-sensitized persons.

3004. The trace metal saga continues

Schroeder, H. A. & Mitchener, Marian (1975). Life-term effects of mercury, methyl mercury, and nine other trace metals on mice. *J. Nutr.* **105,** 452.

The extensive trace-metal studies conducted by H. A. Schroeder and his colleagues have been reported for journal readers over the last decade or more. The most recent contribution considered was concerned with nickel (*Cited in F.C.T.* 1975, **13,** 399), but the latest instalment of this work to appear in print covers no less than ten trace metals and is said to complete the group's studies of the effects of 27 elements in mice.

Groups of 36–54 mice of each sex were given a diet of known trace-element content together with drinking-water containing aluminium (Al), barium (Ba), beryllium (Be), boron (B), lead (Pb), inorganic mercury (Hg), nickel (Ni), titanium (Ti), tungsten (W) or vanadium (V), each at a level of 5 ppm, or methylmercury (MeHg) at a level of 5 ppm Hg for 70 days and thereafter at 1 ppm or at 1 ppm from weaning until death. Treatment was continued for the animals' entire lifespan and records of body weight, survival and tumour incidence were kept.

In the group given V, the males were slightly larger than the controls and both sexes had a longer lifespan. Mice given Ni also lived somewhat longer than the controls. Be, although known to be toxic when inhaled or when ingested in large doses, had no substantial effect at 5 ppm. Ba slightly reduced survival times but did not affect body weight significantly. Neither B nor inorganic Hg had any notable effect. The body weights of both males and females were increased by ingestion of 1 ppm MeHg but were decreased by the 5 ppm level, which caused a high rate of mortality during the first 2 months of treatment. Mice given 1 ppm MeHg after an initial 70 days on 5 ppm lived longer than those given 1 ppm MeHg from weaning and the males gained weight faster. These somewhat unexpected effects of MeHg require further investigation. Tumours were infrequent in MeHg-treated mice of both sexes. None of the trace elements included in this study caused any significant increase in tumour incidence.

3005. Liver tumours from dichlorodiaminodiphenylmethane

Russfield, Agnes B., Homburger, F., Boger, E., Van Dongen, C. G., Weisburger, Elizabeth K. & Weisburger, J. H. (1975). The carcinogenic effect of 4,4′-methylene-bis-(2-chloroaniline) in mice and rats. *Toxic. appl. Pharmac.* **31,** 47.

4,4′-Methylenebis-(2-chloroaniline), also known as 3,3′-dichloro-4,4′-diaminodiphenylmethane (I) has been used in the dye, plastics and rubber industries. Toxic hepatitis has been described in workers exposed to the related compound, diaminodiphenylmethane (*Cited in F.C.T.* 1975, **13,** 589), and there is evidence that this compound may be a weak carcinogen in rats (*ibid* 1968, **6,** 809).

Male rats were fed I at dietary levels of 1000 or 500 ppm while male and female mice received levels of 2000 or 1000 ppm for 18 months. All the animals were then fed a control diet for a further 6 months. The higher level given in each case was the maximum dose tolerated in preliminary studies. Survival rates were similar in test and control groups of rats. In mice, the lower dose level made no difference to survival, but the higher one increased early mortality in females. There was a significant incidence of hepatomas in female but not in male mice at both dose levels of I. In females, hepatomas occurred in 50% of those on the high dose and in 43% on the low dose but in none of the controls. In mice there was also an increase in the incidence of haemangiosarcomas and haemangiomas in both sexes. Treated rats showed an increased incidence of hepatomas and lung adenomatosis, although the latter increase was not statistically significant. The incidence of malignant lymphomas and amyloidosis in treated mice was reduced from the control level, but no explanation of this phenomenon is offered.

3006. Allergy to ethylenediamine

Eriksen, K. E. (1975). Allergy to ethylenediamine. *Archs Derm.* **111,** 791.

Ethylenediamine (ED) was established as the fifth most common contact allergen when tested on patients attending a New York clinic during 1968–70 (*Cited in F.C.T.* 1974, **12,** 582). In these patients it provoked a 13·2% incidence of positive reactions, ranking after mercuric chloride, mercaptobenzothiazole, *p*-phenylenediamine and potassium dichromate, but before nickel sulphate and turpentine oil. Cross-reactions were detected in ED-sensitive patients to the chelating agent ethylenediaminetetraacetic acid (EDTA), which is a weak sensitizer in its own right (*ibid* 1973, **11,** 706), and to structurally-related antihistamines such as promethazine hydrochloride.

The author cited above has now provided information on allergy to ED and to other common sensitizers in Denmark. Of 1111 eczematous patients who were patch-tested in the dermatology department of

the Finsen Institute in Copenhagen during the 10 months ending in July 1974, 23 (2·07%) gave positive reactions to ED. The sensitizing agent in 17 cases was Mycolog cream, in which ED is used as a stabilizer. In three cases, cross-reaction was found to EDTA, which because of its widespread use was suspected of being the primary sensitizer. Other cross-reactions detected were to promethazine hydrochloride and to diethylenetriamine, but not to triethylenetetramine. Over the same period, the 1111 patients gave positive reactions to neomycin in 86 cases, nickel in 75, dichromate in 46, balsams in 38, *p*-phenylenediamine in 36, rubber in 27 and parabens in five. ED thus took seventh place as a contact sensitizer in Denmark, a position not markedly different from that in the United States. However, the incidence of diagnosed reactions to this and other allergens was much lower in Danish patients than in the New York study, and the ranking of other leading sensitizers was also different.

3007. Polyneuropathy from MBK

Allen, N., Mendell, J. R., Billmaier, D. J., Fontaine, R. E. & O'Neill, J. (1975). Toxic polyneuropathy due to methyl n-butyl ketone. An industrial outbreak. *Archs Neurol.* **32,** 209.

An epidemic of polyneuropathy which occurred in 1973 at an Ohio plant producing plastics-coated and colour-printed fabrics was linked to methyl *n*-butyl ketone (MBK) exposure (*Cited in F.C.T.* 1975, **13,** 403) and animal studies subsequently confirmed the aetiological role of MBK (*ibid* 1976, **14,** 157). Further details of the outbreak have now been presented.

Of 1157 employees at the factory who were screened by electrodiagnostic examinations, 86 were identified as having a toxic polyneuropathy. Eleven of these cases were classified as moderate to severe, 38 as mild and 37 as minimal in intensity. Sensory signs predominated in the mildly affected men, and the sensory effects were accompanied by motor involvement in the more severe cases. In those classified as minimally involved, there were no objective neurological findings but abnormalities of muscle and nerve conduction were apparent. Distribution of such abnormalities and of muscle weakness was predominantly distal; the weakness most commonly affected the intrinsic muscles of the hands and feet and the long extensors or flexors of the digits. Sensory loss was usually limited to the feet or fingers, and involved approximately equal loss of light touch, fast or pricking pain and temperature discrimination, and occasionally also of vibration sense. Reflex loss was minimal, being generally confined to the disappearance of ankle and finger jerks.

Initial symptoms were frequently an intermittent tingling paraesthesia of hands or feet, or a slowly developing weakness of these extremities leading to a slapping gait or to difficulty in coping with pincer movements or in grasping heavy objects. Weight loss was apparent in severe cases. Haematological examination revealed a reduction in mean erythrocyte acetylcholinesterase activity and an increase in mean plasma butyrocholinesterase activity, but these changes were unrelated to the severity of neuropathy. In experimental studies, chickens exposed to MBK were found to show an increase in butyrocholinesterase activity.

The epidemic afflicted printing workers, particularly machine operators, their helpers and pan washers, and was correlated with the number of hours worked each week. Workers in a base-coating section were also affected. The first case appeared in December 1972, 4 months after MBK had been substituted for methyl isobutyl ketone in a solvent mixture with methyl ethyl ketone (MEK). One man was affected after only 5 wk of exposure. Average levels of 36 ppm MBK and 516 ppm MEK were detected behind the printing machines. Workers in another plant exposed only to MEK showed no toxic neuropathy, but two cases were identified among workers elsewhere who were exposed to MBK alone. However, although MEK alone was not found to induce neuropathy in animals, combinations of MEK and MBK had a markedly synergistic effect. MEK was also found to produce haematological changes in animals similar to those in the affected men.

Discontinuation of the use of MBK in the Ohio plant prevented the occurrence of further cases, and improvement followed in all severely affected men and in the majority of those only mildly or minimally affected. Animal studies have since suggested that the threshold limit value for MBK should be set at 5 ppm, rather than at 100 ppm, as is now the case.

3008. Health hazards of toluene diisocyanate in industry

Adams, W. G. F. (1975). Long-term effects on the health of men engaged in the manufacture of tolylene di-isocyanate. *Br. J. ind. Med.* **32,** 72.

Sensitization resulting in difficulty in breathing and pain in the chest is an established phenomenon in workers exposed to toluene diisocyanate (tolylene diisocyanate; TDI) during the manufacture of polyurethane plastics (*Cited in F.C.T.* 1971, **9,** 280).

A 9-yr prospective study of workers manufacturing TDI involved an assessment of symptoms and lung function in men who reported no respiratory symptoms, and an assessment of the long-term effects of TDI on men whose development of respiratory disturbance led to their removal from the manufacturing plant. Among the men removed from further contact with TDI because of their respiratory symptoms, the proportion of the work force becoming symptomatic during their first year was 14·8%. The incidence of removals during the second and subsequent years ranged from 0·7 to 3·5% of the force working in any given year. Of the healthy men on the plant, none showed any symptoms significantly different from those in a control group apart from a tendency to wheeze, which was more frequent among the controls. In men who continued to work on the plant, TDI exposure had no significant effect on the values for forced expiratory volume (FEV_1) and forced ventilatory capacity (FVC) in lung-function tests, compared with the measurement in an unexposed control group. However, among the symptomatic men removed from further exposure to TDI, breathlessness and wheezing were persistent and were commoner than in controls;

the FEV_1 was on average 267 ml lower and the FVC 269 ml lower than the predicted value in these affected men.

It appears, therefore, that sensitization to TDI can lead to long-term persistence of respiratory symptoms, with slightly impaired ventilatory function. On the other hand, some 79% of the total number of men who have worked for 1–11 yr in the two plants with which this study was concerned have shown no signs of sensitization.

3009. No chloracne from trichlorobenzene

Powers, Marcelina B., Coate, W. B. & Lewis, T. R. (1975). Repeated topical applications of 1,2,4-trichlorobenzene: Effects on rabbit ears. *Archs envir. Hlth* **30**, 165.

1,2,4-Trichlorobenzene (TCB) is used as an industrial intermediate. Since chlorinated benzenes have been incriminated as cutaneous porphyria inducers (*Cited in F.C.T.* 1965, **3**, 528), further investigation of the industrial hazard of handling TCB has been indicated.

The chloracnegenic potential of TCB was assessed by application of the compound three times weekly to the inner surface of the rabbit ear. TCB was applied undiluted or as a 5 or 25% solution in petroleum ether, in a volume of 0·2 ml for each exposure. Controls were treated with an equal volume of the solvent three times weekly or, in the case of the positive controls, with a 10% hexachlorodiphenyl oxide emulsion in olive oil applied weekly. The latter treatment was continued for 4 wk only, while the solvent and test solutions were applied for 13 wk.

During wk 1–4 neither TCB nor the control treatments produced signs of overt systemic toxicity, but during month 2 positive-control animals were emaciated and one died. Dermal responses on the exposed surface of the ear were seen in the positive controls from wk 3, and by wk 4 typical signs of chloracne had appeared. With 5% TCB in petroleum ether, slight redness, scaling and desquamation occurred but the severity of these effects did not increase after 39–40 exposures and they were comparable with the effects induced by the solvent alone. In concentrations of 25 or 100%, TCB produced moderate to severe local irritation accompanied by erythema, scaling, desquamation and encrustation, mild follicular enlargement, some hair loss and scarring. Thickening of the ear was not evident. No gross evidence of acneiform dermatitis appeared, no systemic toxic effects were apparent and at autopsy no significant visceral changes were observed. Marked hepatic damage was seen in all the positive-control animals. It appears, therefore, that the local effects of TCB are akanthosis and hyperkeratosis without the primary follicular involvement typical of chloracne. The compound seems likely to induce dermal irritation rather than chloracne in those handling it in industry.

3010. Triethyltin and water on the brain

Lijinsky, W. & Aldridge, W. N. (1975). Increase in cerebral fluid in rats after treatment with triethyltin. *Biochem. Pharmac.* **24**, 481.

Triethyltin (TET) is recognized as a producer of brain oedema. This development is associated with interference in the utilization of adenosine triphosphate and inhibition of oxidative phosphorylation (*Cited in F.C.T.* 1971, **9**, 892).

Rats were given an iv injection of TET sulphate in a dose of 10 mg/kg, which was estimated to produce a brain concentration of about 20 μM TET. When the animals were kept at an environmental temperature of 5 or 19°C, there was a delay of 12 hr after the injection before the water content of the brain tissue started to increase. When the environment was kept at 34°C, the brain-water content started to increase immediately. Incubation of the microsomal fraction of rat brain with 20 μM TET in the absence of sodium and potassium ions reduced by 54% the activity of adenosine triphosphatase (ATPase), the enzyme thought to be involved in the transfer of sodium across membranes, while in the presence of sodium the reduction was 35%. A brain-microsomal fraction derived from rats given a dose of TET and maintained at 34°C failed to show any reduction in ATPase activity. It is unlikely, therefore, that the brain oedema observed in TET-poisoned animals was directly attributable to the inhibition of the sodium-transport enzyme system.

3011. No systemic effects from vinyl chloride copolymer implants

Brand, K. G., Buoen, L. C. & Brand, I. (1975). Foreign-body tumorigenesis by vinyl chloride vinyl acetate copolymer: No evidence for chemical cocarcinogenesis. *J. natn. Cancer Inst.* **54**, 1259.

The association of vinyl chloride monomer with tumour induction in rats and man suggested the possibility that residual monomer might contribute to the tumorigenic activity of vinyl chloride copolymer implanted sc in rats. In some 10,000 mice of various inbred strains and hybrids given sc implants of vinyl chloride–vinyl acetate copolymer (VCA), the local sarcomas recorded showed a predictable incidence and latencies and there was no increase in the expected natural incidence of tumours remote from the implantation site. In a further search for evidence of a chemical influence as well as a foreign-body reaction in the tumorigenic effect of VCA implants, studies were carried out on two strains of CBA mice.

VCA powder (particle size 50–100 μm), in amounts corresponding by weight to two film implants of standard size, was implanted sc in 30 male and 46 female mice, which were then observed until death. Only one fibrosarcoma developed at the site of implantation and there was no increase over the expected natural incidence of tumours distant from the implantation site. In another experiment, standard-sized VCA films were roughened to increase the area of contact between implant surface and tissue and facilitate the release of chemicals from the film. Tumours occurred in eight of the 26 mice implanted with this film, whereas 52 of 53 mice given a smooth implant developed tumours. The first of these tumours developed 9 months after implantation and the majority had appeared by month 21. Smooth films of a standard size were implanted in a further ten female CBA mice

and left *in situ* for 10 months, during which time they became enveloped in a thick connective-tissue capsule. The implants were then removed and fresh implants were inserted on the opposite flank of each animal. Seven of these mice developed tumours, but again between months 10 and 20 after implantation, in spite of the more prolonged systemic exposure of the mice to the copolymer. When VCA powder corresponding in weight to four standard films was implanted sc or ip, followed 8 months later by the sc implantation of a single smooth VCA film at a different site, sarcomas developed in eight of the 11 mice given the initial powder treatment sc and in nine of the ten mice given the powder ip. The sarcomas again developed in months 9–20 after the film implantation. The absence of any indication of an increase in the incidence of tumours, apart from sarcomas at the injection site, or any acceleration in their appearance, must lead to the conclusion that no chemical carcinogen or cocarcinogen is released from the powder.

[These observations give some degree of reassurance with regard to one aspect of the current concern over the potential carcinogenic hazard from vinyl chloride monomer.]

3012. Mutagenic activity of vinylidene chloride and chloroprene

Bartsch, H., Malaveille, C., Montesano, R. & Tomatis, L. (1975). Tissue-mediated mutagenicity of vinylidene chloride and 2-chlorobutadiene in *Salmonella typhimurium*. *Nature, Lond.* **255,** 641.

Vinyl chloride monomer has been shown to be carcinogenic in animals and in man, while in animal tests the related compound, vinylidene chloride (VDC), has given evidence that it may have carcinogenic potential (*Food Chemical News* 1974, **16** (29), 2). VDC is commonly used with vinyl chloride in the production of copolymers and may be encountered as a decomposition product of 1,1,1-trichloroethane. Another related chemical, 2-chlorobutadiene (chloroprene; CP) has been used for many years in the manufacture of synthetic rubber. The work cited above was concerned with the mutagenicity of a VDC metabolite.

Salmonella typhimurium strains TA 1530 and TA 100 incubated in a soft agar layer containing an NADPH-generating system and a 9000 **g** tissue supernatant were exposed to VDC or CP at 37°C in the dark. After replacement of the gaseous phase by pure air and further incubation, the number of histidine-revertant colonies/plate was taken as an index of mutagenicity. With liver supernatant from mice pretreated with 0·1% phenobarbitone in the drinking-water for 7 days, the response of the TA 100 strain increased with concentrations of VDC up to 2% in air. A lower response observed with 20% VDC may have been attributable to inhibition of the metabolic activation. The response to 2% VDC showed a linear increase with time up to 4 hr, so this incubation period was used in subsequent studies on liver, kidney and lung tissue from rats and mice. The mutagenic response to VDC was demonstrated using supernatant from mouse kidney, lung and liver fractions, but it was maximal with liver and was increased by the enzyme activation brought about by pretreatment of the animal with phenobarbitone. A lower mutagenic response was obtained with rat- than with mouse-tissue incubations. Addition of sulphur-containing compounds to the medium caused a marked reduction in the response of strain TA 1530 to 2% VDC in a mouse-liver medium.

Exposure of strain TA 100 to up to 8% CP in air in the absence of a system activating metabolism produced a linear increase in mutations with increasing concentration. The addition of mouse-liver supernatant to the incubation doubled this effect and with phenobarbitone pretreatment the response was tripled. This suggests that CP may undergo enzymic conversion to some mutagenic metabolite(s), probably including an epoxide.

NATURAL PRODUCTS

3013. Dealing with alcohol

Korsten, M. A., Matsuzaki, S., Feinman, L. & Lieber, C. S. (1975). High blood acetaldehyde levels after ethanol administration. Difference between alcoholic and nonalcoholic subjects. *New Engl. J. Med.* **292,** 386.

The metabolic arrangements in the body for dealing with acetaldehyde produced by the oxidation of ethanol seem to be less simple than was once supposed, and still present mysteries (*Cited in F.C.T.* 1975, **13,** 668). The paper cited above shows that mechanisms in chronic alcoholics differ from those in normal subjects.

Six men with a history of chronic alcoholism and five controls each received an iv infusion of 15% ethanol in 5% dextrose solution over 3 hr, to produce peak ethanol concentrations in the blood ranging from 43–54 mM. For 8–10 hr after the infusion, blood and urine samples were assayed for ethanol and acetaldehyde content by gas chromatography. In both the alcoholics and controls, blood levels of acetaldehyde remained steady over a wide range of falling ethanol levels, but suffered an abrupt fall when the ethanol level reached a critical value of 17·4–32·6 mM. The mean acetaldehyde plateau level was significantly higher in alcoholics than in controls (42·7 compared with 26·5 μM) and fell abruptly once the blood-ethanol concentration reached 24 mM.

The data suggest that an ethanol-oxidizing system distinct from that involving alcohol dehydrogenase contributes to the oxidation of high blood-ethanol levels, undergoing desaturation at a threshold ethanol concentration. This additional enzyme system may become more active in chronic alcoholism, and together with ethanol-induced mitochondrial damage

may contribute to the known neurological, hepatic and cardiac complications of alcoholism.

3014. A more favourable outlook for coffee

Simon, D., Yen, Stella & Cole, P. (1975). Coffee drinking and cancer of the lower urinary tract. *J. natn. Cancer Inst.* **54,** 587.

Some years ago, a case-control study carried out in Greater Boston, Mass., suggested an association between coffee drinking and the risk of developing cancer of the lower urinary tract (LUT cancer), affecting the renal pelvis, ureter, bladder or urethra (*Cited in F.C.T.* 1972, **10,** 107). This prompted some correspondence, in which Fraumeni *et al.* (*Lancet* 1972, **ii,** 1204) presented evidence indicating that the association was probably indirect or non-causal, and Zeitlin (*ibid* 1972, **i,** 1066) noted an absence of bladder-tumour induction in rats fed a diet containing 5% instant-coffee solids over most of their lifespan.

Since the Boston study lacked information on lifetime coffee- or tea-drinking habits, the use of artificial sweeteners and the presence of other additives in the consumed coffee, a further investigation concerned with these factors was carried out among white women, the group in which the association in question appeared strongest in the earlier study.

Information obtained with the aid of a mailed questionnaire from 135 women with LUT cancer and from 390 controls indicated that the risk of LUT cancer was approximately twice as great for women who drank one or more cups of coffee daily as for those who drank less or none at all. However, the risk did not increase in proportion to the number of cups of coffee consumed or to the duration of the habit. The association of coffee with disease was not affected by the type (decaffeinated, non-decaffeinated, regular or instant) or by the strength of the coffee normally consumed; neither was the use of non-dairy creamers, saccharin or cyclamate associated with any increased risk of disease.

In this more detailed study, therefore, the absence of any dose–response relationship between the development of LUT cancer and coffee-drinking, either in terms of the daily intake or the duration of the habit, supports the view that the observed association is indeed non-causal.

METHODS FOR ASSESSING TOXICITY

3015. Estimation of percutaneous absorption

Franz, T. J. (1975). Percutaneous absorption. On the relevance of in vitro data. *J. invest. Derm.* **64,** 190.

Comparison of *in vitro* and *in vivo* data is essential if predictions are to be made of *in vivo* events from *in vitro* studies. This point is stressed in connexion with percutaneous absorption in the paper cited above.

The testing of 12 organic compounds on excised human abdominal skin is described and compared with the results obtained by other workers from the *in vivo* testing of the same compounds on the skin of the human forearm. A description is given of an *in vitro* system in which an aqueous medium was held in contact with the dermal side of each skin sample while the epidermal surface was kept dry. The ^{14}C-labelled compounds were applied in acetone to the dry surface in an amount ($10\,\mu l/cm^2$) sufficient to spread across the entire surface, and the amount passing through the skin was monitored at intervals by measuring the radioactivity of the aqueous medium.

Absorption-rate patterns were similar in the *in vitro* and *in vivo* situations except in the case of thiourea, for which good agreement was obtained only when an *in vivo* test was performed on abdominal skin. With regard to total absorption, a positive correlation was reported between the *in vitro* and *in vivo* situations; however, whereas the results for compounds that penetrated well showed a fair measure of agreement, for compounds that penetrated less readily the total absorption *in vitro* was some 4–10 times greater than that demonstrated *in vivo*.

The author draws attention to the fact that desquamation may have resulted in some loss of the latter compounds in the *in vivo* situation and that some of the differences between the *in vivo* and *in vitro* results may have been due to differences in the penetrability of abdominal and forearm skin, a point demonstrated in earlier work (*Cited in F.C.T.* 1973, **11,** 707). He concludes that the agreement between the results obtained in the *in vitro* and *in vivo* situations is sufficient for *in vitro* studies to be used for predicting qualitative differences in absorption in the *in vivo* situation (i.e. the ranking of compounds), but not for making a quantitative assessment of the degree of percutaneous absorption occurring *in vivo*.

BIOCHEMICAL PHARMACOLOGY

3016. Coumarin action in oedema reduction

Bolton, Tiffany & Casley-Smith, J. R. (1975). An in vitro demonstration of proteolysis by macrophages and its increase with coumarin. *Experientia* **31,** 271.

Coumarin and related compounds (benzopyrones) are known to be effective in reducing high-protein oedemas, especially lymphoedema. Ultrastructural studies of oedematous tissues have indicated that the coumarin reduces the protein content of the connec-

tive tissues and the lymphatics, and the consequent reduction in the osmotic effect exerted by the protein reduces the fluid content and therefore the oedema. The removal of protein following coumarin treatment is probably brought about by increased metabolism in the connective and lymphatic tissues, since coumarin does not modify the removal of non-metabolizable polyvinylpyrrolidone molecules, which possess diffusion coefficients similar to those of the protein of oedema fluid. Moreover, it has been shown experimentally that the protein reduction is not brought about by an increase in lymph flow, since the action of coumarin is maintained after ligation of the lymphatics, and other work has shown that a decrease in protein outflow from the blood vessels is unlikely to be involved.

To investigate the effect of coumarin on proteolysis, an *in vitro* experiment was undertaken using peritoneal macrophages from the mouse. The cell preparations were incubated with albumin in cultures with or without 25 μg coumarin/ml for 24 hr. The ninhydrin method was used to estimate the protein fragments (soluble in trichloroacetic acid) present in the medium before and after incubation. The results showed that exposure to coumarin produced a roughly twofold increase in the rate of proteolysis effected by the macrophages in 24 hr. This indication that coumarin stimulation of the macrophages leads directly to an increase in their capacity to break down proteins in the oedema fluid received further support from the observation about to be reported by Piller (*Br. J. Path.* 1975, **56**, 554) that coumarin is ineffective in relieving oedema in animals injected with silica, which inactivates macrophages.

3017. Questions of metabolism, safety and effectiveness

Conney, A. H., Coutinho, C., Koechlin, B., Swarm, R., Cheripko, J. A., Impellizzeri, C. & Baruth, H. (1974). From animals to man: Metabolic considerations. *Clin. Pharmac. Ther.* **16**, 176.

Since drugs and other foreign chemicals are often metabolized at widely different rates and by a variety of pathways in different animal species, dosage cannot be used as an accurate basis for extrapolating data from one experimental species to another or to man. To help eliminate the effects of species differences in rates of metabolism, a search for possible correlations between the blood concentration of a drug and/or its metabolites and its pharmacological activity and toxicity in different species might be useful. If such a correlation could be made, the blood concentration achieved in animals given a non-toxic but pharmacologically active dose might provide a useful guide to safe and effective dosage in man. If there are species differences in metabolic pathways for the compound, the determination of blood level and half-life in a range of species may not suffice for extrapolating toxicity or activity data. Such a consideration becomes important, for example, in assessing the carcinogenic potential of *N*-2-fluorenylacetamide, which is *N*-hydroxylated to a more powerful carcinogen in the rat, mouse and dog and in man, but not in the guinea-pig or lemming. Individual variability in the rate of metabolism of a compound may be considerable and may play an important part in determining the safe and effective dose of the compound. For drugs that are metabolized to a toxic intermediate, safe and rapid metabolic tests should be devised to predict in individual patients the levels of activity of hepatic and other tissue enzymes concerned in the formation of the toxic metabolite.

CANCER RESEARCH

3018. The diet of the gut flora

Finegold, S. M., Attebery, H. R. & Sutter, Vera L. (1974). Effect of diet on human fecal flora: comparison of Japanese and American diets. *Am. J. clin. Nutr.* **27**, 1456.

Epidemiological studies have revealed that the incidence of large-bowel cancer in a population group is related to the group's country of residence and not its country of origin. For example, although Japan has a low incidence of the disease, many groups of Japanese migrants to the USA show an increased incidence comparable to that of native Americans (Berg *et al. Hlth Servs Rep.* 1973, **88**, 915). It has been suggested that the incidence of colon cancer is related to diet and to the composition of the bowel microflora, certain members of which may convert faecal steroids to carcinogens (Hill *et al. Lancet* 1971, **i**, 95).

The paper cited above reports a comparative study of the bowel flora from two groups of Japanese-Americans, one eating a traditional Japanese diet and the other an American diet. Numerous selective media were used to investigate the flora in great detail and to isolate organisms that could be important metabolically although present in relatively small numbers.

For most of the bacterial types studied, numbers did not differ to a statistically significant degree between the two population groups. Significantly higher counts of *Streptococcus faecalis* and certain species of Eubacterium and Peptostreptococcus were found in subjects on the Japanese diet, but the importance of these differences in terms of bowel-cancer incidence is difficult to assess. Interestingly, there was no significant difference in the counts of *Clostridium paraputrificum*, a species considered by Hill *et al.* (*loc. cit.*) to be of potential importance in the metabolic conversion of bile salts to carcinogens.

[One reason for the lack of any major difference in gut flora between the two groups is almost certainly the stability of the human intestinal flora and its resistance to change in response to alterations in diet. It would have been interesting therefore had the authors included a group of native Americans in their investigation.]

3019. Malonaldehyde carcinogenesis promotes another hypothesis

Shamberger, R. J., Andreone, Theresa L. & Willis, C. E. (1974). Antioxidants and cancer. IV. Initiating activity of malonaldehyde as a carcinogen. *J. natn. Cancer Inst.* **53,** 1771.

Malonaldehyde, a product of peroxidative fat metabolism, is formed in the tissues of animals on a diet deficient in antioxidants. As malonaldehyde is related structurally to the two isomeric carcinogens, β-propiolactone and glycidaldehyde, it was tested for carcinogenicity by application to the skin of mice.

Groups of 30 female Swiss mice were painted once with 6 or 12 mg malonaldehyde in acetone and this was followed after an interval of 3 wk with daily applications of 0·25 ml 0·1% croton oil on 5 days/wk for 30 wk. β-Propiolactone, glycidaldehyde and 7,12-dimethylbenz[*a*]anthracene (DMBA) were used as positive controls. Keratoacanthomas were present in 44, 40 and 95%, respectively, of the positive controls at wk 30 and in 52% of both groups of malonaldehyde-treated mice.

Daily application of 12 mg malonaldehyde to 30 mice resulted in a high mortality rate, with 12 animals dying in the first 4–6 wk of treatment and six more in the next 3 wk. Metastasizing liver carcinomas were diagnosed in four rats in the latter group and a rectal carcinoma in a fifth. At wk 9, the daily dose was reduced to 0·36 mg and no further tumours were detected up to wk 48. No tumours developed in the control group, 28 of which survived to wk 48. In another group treated throughout the 48-wk test period with 0·36 mg, one mouse developed a keratoacanthoma at wk 43.

In further experiments, the amount of malonaldehyde recoverable from the skin up to 24 hr after application was determined. After 1 hr only 1·9% of a 12-mg dose was detected, indicating that the dose was, as expected, oxidized rapidly to malonic acid. Malonaldehyde was detected in mouse skin that had been painted with one of the carcinogens, benzo[*a*]pyrene, 3-methylcholanthrene and DMBA.

[Several experiments previously reported (*Cited in F.C.T.* 1973, **11,** 328; *ibid* 1974, **12,** 779; Cumming & Walton, *Fd Cosmet. Toxicol.* 1973, **11,** 547; Clapp *et al. ibid*, 1974, **12,** 367) have been concerned with the ability of antioxidants such as butylated hydroxytoluene, butylated hydroxyanisole and selenium to reduce tumour incidence in animals treated with some organ-specific carcinogen, but results have been inconsistent. The above authors' postulate, that reduction of tissue levels of malonaldehyde by antioxidants may be responsible for this reduction in tumour incidence, is yet another hypothesis to add to the list of possible mechanisms, which include the induction of drug-metabolizing systems, reaction with an ultimate carcinogenic metabolite, and mechanisms of lysosomal stabilization.]

3020. Salt and gastric tumours

Tatematsu, M., Takahashi, M., Fukushima, S., Hananouchi, M. & Shirai, T. (1975). Effects in rats of sodium chloride on experimental gastric cancers induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine or 4-nitroquinoline-1-oxide. *J. natn. Cancer Inst.* **55,** 101.

Carcinomas of the glandular or squamous epithelium of the stomach can be induced in both rats and mice by a number of chemical carcinogens. The incidence of tumours in the squamous epithelium of the mouse stomach following administration of 4-nitroquinoline-1-oxide (NQO) can be greatly enhanced by treatment with sodium chloride (NaCl; Kinosita, *Gann Monogr.* 1969, **8,** 263), although attempts to induce stomach cancer by administration of salted foods were unsuccessful (Sato *et al. Bull. Inst. Publ. Hlth. Tokyo* 1959, **8,** 10).

The effect of NaCl on carcinogen-induced stomach tumours has now been studied in rats treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), which induces tumours in the glandular portion of the stomach in this species. Male Wistar rats were divided into five groups of 20 (groups 1, 2, 4, 5 and 6), three groups of ten (groups 7–9) and one of 30 (group 3). Group 1 was given drinking-water containing 50 ppm MNNG and 0·6% NaCl and fed the stock diet supplemented with 10% NaCl. Rats in group 2 were maintained on drinking-water containing 50 mg MNNG/litre and on unsupplemented stock diet but were given 1 ml saturated (29%) NaCl solution by gavage once weekly. Group 3 was treated only with 50 ppm MNNG in the drinking-water. Animals in groups 4–6 received 1 mg NQO dissolved in 20% ethanol by gavage each week. Group 6 was given no other treatment, group 4 was fed the NaCl-supplemented stock diet, and for group 5 the weekly dose of NQO in ethanol was saturated with NaCl (18%). Of the two groups treated only with NaCl, group 7 received 0·6% in the drinking-water and 10% in the diet and group 8 was given 1 ml saturated NaCl solution weekly by gavage. Group 9 was maintained on unsupplemented stock diet and tap-water.

Carcinomas of the glandular part of the stomach developed only in groups 1, 2 and 3, the incidences being 61, 80 and 44%, respectively. The difference between the percentages for groups 2 and 3 was statistically significant. Carcinomas of the squamous part of the stomach appeared only in groups 4 and 5, and in these two groups as well as in group 6 squamous-cell papillomas were common. No tumours were found in either part of the stomach in rats of groups 7–9.

The results are interpreted as indicating that the administration of sodium chloride enhanced the carcinogenic effects of MNNG or NQO. The mechanism by which this enhancement is brought about is still obscure, but it is thought that some part may be played by repeated injury to the gastric mucosa or by a reduction in the viscosity of the gastric mucus and thus in its ability to protect the mucosa.

[Perhaps it is surprising that the experiment is not claimed to demonstrate that sodium chloride has a co-carcinogenic effect and that salted foods should be banned, a restraint possibly due to the popularity of salted foods in certain countries! On a more serious note, the experiments really indicate in yet another system that tissue damage can accelerate the process of carcinogenesis to a marked degree. This has been

clearly demonstrated in the rodent bladder, in subcutaneous tissue and in skin, and the experiments cited above have added the rodent stomach to the list.]

3021. Sulphate involvement in carcinogenic activation

Blunck, Jill M. & Crowther, Carol E. (1975). Enhancement of azo dye carcinogenesis by dietary sodium sulphate. *Eur. J. Cancer* **11**, 23.

Carcinogenic azo dyes exert their tumorigenic effect after metabolic activation of the parent compound. Studies with 2-acetylaminofluorene, a carcinogen from another class of compounds, have indicated that in this case sulphate conjugation of an *N*-hydroxy metabolite may be involved in the activation of the compound and it has been suggested that a similar step may be important in the activation of azo dyes. In order to test this hypothesis, groups of 20 male Sprague–Dawley rats were fed a diet containing 0·06% 3′-methyl-4-dimethylaminoazobenzene (3′MeDAB) alone or together with either 0·84% sodium sulphate or 0·8% acetanilide. Control groups, each of ten rats, were given the basal diet or basal diet containing 0·84% sodium sulphate or 0·8% acetanilide. Initially rats were kept on the allocated diet for 16 wk and then returned to the basal diet for 8 wk. Subsequently they were returned to their experimental diet for several 4-wk periods, with 1 wk on the basal diet between each period. Two rats from each test group and one from each control were killed at wk 24 for liver examination, and further rats (between one and four in each group) were killed at wk 33.

Liver-cell tumours were observed only in the groups given 3′MeDAB alone or with sodium sulphate. In the former group, the first tumour appeared 27 wk after the beginning of the experiment. Of the 11 other rats alive at this stage, five subsequently developed carcinomas. In the group receiving sodium sulphate in addition to the 3′MeDAB, tumours developed in 12 of the 18 rats alive at wk 17, when the first tumour was detected. Acetanilide, which was used to deplete the hepatic sulphate pool, not only inhibited the induction of liver tumours by 3′MeDAB but also prevented the development of cirrhosis and hyperplastic changes.

[The experimental results certainly suggest an enhancement of the carcinogenic activity of 3′-methyl-4-dimethylaminoazobenzene (3′MeDAB) following the addition of sodium sulphate to the diet, but whether this was due to the greater availability of sulphate ions is a different matter. No indication is given about the proportion of sulphate likely to be absorbed or whether any of it appears in the metabolic products of 3′MeDAB. Furthermore sodium sulphate is a good osmotic purgative and one would like to know whether intestinal mobility was affected and whether this in turn affected absorption of the carcinogen. There is much more to learn than meets the eye!]

3022. The role of DNA repair synthesis in tumour promotion

Poirier, Miriam C., De Cicco, B. T. & Lieberman, M. W. (1975). Nonspecific inhibition of DNA repair synthesis by tumor promoters in human diploid fibroblasts damaged with *N*-acetoxy-2-acetylaminofluorene. *Cancer Res.* **35**, 1392.

Tumor promoters enhance the rapidity of onset and the yield of tumours induced by carcinogens, an effect which has been particularly clearly demonstrated in studies involving the application of carcinogens to the skin of mice. Recently it has been shown that some promoters interfere with DNA repair synthesis and it was thought that reduced repair might play some part in the mechanism of their effect on tumour development. Because of the potential importance of this possibility, three promoting agents and their non-promoting analogues were tested in tissue culture, using human diploid fibroblasts of the line designated WI-38. The promoting agents used were croton oil (0·001–0·05%), 12-*O*-tetradecanoylphorbol-13-acetate (1–50 μM) and anthralin (generally considered to be 1,8,9-anthracenetriol, although a more probable structure is 1,8-dihydroxy-9-anthrone; 0·1–100 μM). The non-promoting analogues were phorbol and 1,8-dihydroxyanthraquinone.

In support of the earlier observations, these promoting agents were found to inhibit DNA repair synthesis in WI-38 cells previously damaged by the proximate carcinogen *N*-acetoxy-2-acetylaminofluorene. They inhibited DNA-replicative systems equally effectively, and also inhibited RNA and protein synthesis. For each compound, all these effects were observed over the same range of concentrations and were accompanied by extensive morphological changes suggestive of cytotoxicity. The non-promoting analogues had little effect on these macromolecular processes and caused only slight morphological damage. Parallel studies with phenol (another tumour promoter) and 4-nitrophenol (a non-promoter) showed less consistent effects, which did not conform to the general pattern.

The authors suggest that the effect of tumour promoters on DNA repair synthesis is part of a general response to cellular injury rather than a selective response involving a single metabolic pathway, and they consider that it is unlikely to represent the major mechanism by which promoting agents affect the carcinogenic process.

[Observations of this sort underline the difficulty of interpreting the carcinogenic process in terms of biochemical events. Perhaps a little more light might be shed on the problem if studies of this nature were conducted on the target cells *in vivo*.]

TERATOGENESIS

3023. Hydroxyurea and teratogenicity testing

Wilson, J. G., Scott, W. J., Ritter, E. J. & Fradkin, Rochelle (1975). Comparative distribution and embryotoxicity of hydroxyurea in pregnant rats and rhesus monkeys. *Teratology* **11,** 169.

Hydroxyurea is teratogenic in a number of species, including the rat (*Cited in F.C.T.* 1964, **2,** 521) and the rhesus monkey (Theisen *et al. Teratology* 1973, **7,** A-29). Teratogenic effects in the rat were dose-related, and embryonic levels of the drug correlated roughly with the degree and duration of inhibition of DNA synthesis in the embryo (*Cited in F.C.T.* 1973, **11,** 169). A concentration of 10^{-4} M-hydroxyurea was the lowest to affect DNA synthesis, both in cultured mammalian cells (*ibid* 1967, **5,** 251) and in rat embryos (Rajewsky *et al. Expl Cell Res.* 1971, **66,** 489).

In the study cited above, rhesus monkeys were given a daily iv dose of 100 mg hydroxyurea/kg for 10 days, starting on days 23, 27 or 31 of gestation, and rats were given ip doses of 100, 137 or 175 mg/kg on days 9–12 of gestation. In the latter species, 100 mg/kg had very little effect on the embryos, but at 137 mg/kg the degree of teratogenicity was similar to that in monkeys given 100 mg/kg on days 23–32.

Periodic analyses revealed that following a dose of 100 mg/kg, hydroxyurea still persisted in the maternal plasma of monkeys after 12 hr, although it had virtually cleared within 2 hr in rats. The half-life of hydroxyurea in the plasma was estimated at about 120 min in monkeys, but only about 15 min in rats given 100 or 137 mg/kg. In embryonic tissues, concentrations rose gradually until they exceeded maternal levels, a stage reached after 8 hr in monkeys but after only 1 hr in rats, while the half-life of hydroxyurea in the embryo was estimated as 265 min in monkeys but only 60 min in rats given 100 mg/kg, and 85 min in rats given 137 mg/kg. Hydroxyurea persisted in the chorionic and amniotic fluids of monkeys at levels higher than those in maternal plasma, suggesting that the fluids could have acted as a reservoir from which hydroxyurea continued to enter the embryo. Embryonic levels were calculated to be above 10^{-4} M, the minimum level known to affect DNA synthesis, for 10 hr or more after each injection in the monkey, but for less than 3 hr in rats given 10 mg/kg, and for less than 4 hr in rats given 137 mg/kg.

These results were interpreted as indicating that the embryonic cells of the rat are much more sensitive to the teratogenic effects of hydroxyurea than are those of the monkey. Possible reasons for this could be the placental differences between the two species, or the longer cell cycle in the monkey than in the rat. The finding was thought to have important general implications with regard to selecting an appropriate test species for estimating human teratogenic risks, indicating as it did that there are sizeable differences between rats (currently the most widely used species for such tests) and monkeys, which are thought to be closest to man in teratogenic susceptibility.

Fd Cosmet. Toxicol. Vol. 14, pp. 227–228. Pergamon Press 1976. Printed in Great Britain

LETTER TO THE EDITOR

ACUTE TOXICITY OF AFLATOXIN B_1 FOR BABOONS

Sir,—In 1968 we determined an approximate single dose LD_{50} of aflatoxin for baboons (*Papio anubis*), with the objective of using non-lethal doses of aflatoxin in conjunction with long-term pyridoxine deficiency experiments to ascertain whether various aflatoxin exposures would exert a synergistic effect on the formation of the regenerative nodules in baboon livers, as reported by Foy *et al.* (*Nature, Lond.* 1966, **212**, 150; *ibid* 1970, **225**, 952), and possibly evoke liver cancer. The results of these experiments have been reported elsewhere (*idem, J. natn. Cancer Inst.* 1974, **53**, 1295).

Only an 80%-pure mixture of the four principal aflatoxins (B_1 46·5%, G_1 22·4%, B_2 7·2%, G_2 4·0%) was available at that time in sufficient quantity to dose three baboons at each of four levels. The mixed aflatoxins were dissolved in ethanol–monopropylene glycol (7 : 3, v/v) and administered to animals of 5–6 kg body weight by gastric intubation. Death within an observation period of 7 days after intubation was considered to be due to acute toxicity; all animals surviving this period were biopsied and were still alive 6 months after dosage. According to the notations used by Weil (*Biometrics* 1952, **8**, 249), $K = 3$, $n = 3$, $r = 0, 1, 2, 3$, $d = 0{\cdot}1038$ and $D_a = 1{\cdot}2$ mg/kg body weight. We assumed that the relative toxicities of the four aflatoxin compounds were the same for the baboon as Carnaghan *et al.* (*Nature, Lond.* 1963, **200**, 1101) had reported for ducklings ($B_1 : G_1 : B_2 : G_2 = 1{\cdot}00 : 2{\cdot}15 : 4{\cdot}66 : 9{\cdot}48$) and from our results calculated a single-dose LD_{50} of 1·75 mg/kg body weight with a 95% confidence interval of 0·9–2·6 mg/kg expressed as aflatoxin B_1.

We have recently been able to repeat this acute toxicity study using pure aflatoxin B_1 (from Makor Chemicals, Jerusalem). Three baboons of approximately 5 kg body weight were anaesthetized by intramuscular injection of Sernylan (phencyclidine hydrochloride, from Parke Davis & Co., Hayes, Mddx) in a dose of 1·5 mg/kg and were dosed by gastric intubation with the calculated amount of aflatoxin B_1 dissolved in not more than 2·5 ml dimethylsulphoxide at each of five dosage levels. Two control animals were given 2·5 ml dimethylsulphoxide alone. Surviving animals were biopsied 7 days, 1 month and 3 months after administration of the toxin, a wedge biopsy being removed at laparotomy.

The single-dose LD_{50} for baboons in this experiment, calculated by the Weil procedure, was 2·02 mg/kg with a 95% confidence interval of 1·41–2·89 mg/kg. According to the Weil notation, $K = 3$, $n = 3$, $r = 1, 1, 2, 3$, $d = 0{\cdot}0969$ and $D_a = 1{\cdot}53$ mg/kg. All three animals given the lowest dose of 1·22 mg/kg survived. Four of the animals that died did so between days 4 and 6 but one animal, given the highest dose level, died approximately 45 hr after dosage. The result of this study with pure aflatoxin B_1 thus confirms the earlier study with mixed aflatoxins and suggests that the toxicities of the four major aflatoxins in baboons may be similar to the ratios reported for ducklings.

The animals were housed in separate press-back metal cages with double wire-mesh floors to facilitate the collection of urine and faeces. In the earlier series, aflatoxins B_1, G_1 and M_1 were detected in the urine up to day 5 after dosage, and in the later series B_1 and M_1 were detected over the same period. Continuous urine collection over the 7 days after dosage and quantitative thin-layer chromatographic analysis of extracts of the urine suggested that 5–6% of the ingested dose was eliminated either unchanged or as the fluorescent metabolite M_1.

Histological examination of the liver, either at autopsy or at biopsy 7 days after dosage, showed a considerable variation in hepatic damage within each dose level. At

the higher doses, haemorrhagic necrosis was noted ranging from that confined to the central zone of the lobule to massive destruction of liver cells throughout the lobule. There was a variable intensity of inflammatory reaction throughout the liver parenchyma, but it was most marked in the periportal zone of the lobule and in the portal tract. At lower dose levels, focal necrosis was observed, again mainly in the centrilobular zone, with a diffuse polyzonal inflammatory reaction and some proliferation of bile-duct epithelial cells.

The appearances were similar to those reported in vervet monkeys (van der Watt & Purchase, *Br. J. exp. Path.* 1970, **51**, 183), rhesus monkeys (Deo *et al. J. Path.* 1970, **101**, 47; Svoboda *et al. Am. J. Path.* 1966, **49**, 1023) and macaques (Bourgeois *et al. Lab. Invest.* 1971, **24**, 206), but were in contrast to the pathology described for acute aflatoxicosis in the rat and duckling, in which periportal necrosis is a constant feature (Newberne & Butler, *Cancer Res.* 1969, **29**, 236).

The animals surviving from the earlier study, when mixed aflatoxins were used, were examined recently (7 years after dosage). Abnormal nuclei were still present in some liver cells.

F. G. Peers,
Tropical Products Institute,
56–62 *Grays Inn Road,*
London WC1X 8LU, England

C. A. Linsell,
International Agency for Research on Cancer,
Nairobi Research Centre,
PO Box 46831,
Nairobi, Kenya

Fd Cosmet. Toxicol. Vol. 14, p. 229. Pergamon Press 1976. Printed in Great Britain

MEETING ANNOUNCEMENT

TOXICOLOGY AND OCCUPATIONAL MEDICINE

Plans have been announced by the University of Miami and the Pan American Medical Association (PAMA) for a joint meeting incorporating the University's 9th Inter-American Conference on Toxicology and Occupational Medicine and the Section of Toxicology of PAMA's 50th and Golden Anniversary Congress.

The meeting will be held on 24–29 October 1976 at the Diplomat Hotel, Hollywood, Florida. Further information may be obtained from Dr. R. A. Peñalver, Director, Office of International Medical Education, University of Miami, P.O. Box 52 875, Miami, Florida 33152, USA (telephone: 305-547-6711).

Fd Cosmet. Toxicol. Vol. 14, p. 231. Pergamon Press 1976. Printed in Great Britain

FORTHCOMING PAPERS

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

Effects of chronic oral administration of erythrosine in the mongolian gerbil. By F. X. Collins and E. L. Long.

Short-term toxicity of hydratropic aldehyde in rats. By D. Pelling, I. F. Gaunt, K. R. Butterworth, J. Hardy, A. B. G. Lansdown and S. D. Gangolli.

Long-term toxicity of cyclohexylamine hydrochloride in the rat. By I. F. Gaunt, J. Hardy, P. Grasso, S. D. Gangolli and K. R. Butterworth.

Long-term toxicity of cyclohexylamine hydrochloride in mice. By J. Hardy, I. F. Gaunt, J. Hooson, R. J. Hendy and K. R. Butterworth.

Effects of butylated hydroxyanisole on *Tetrahymena pyriformis*. By J. G. Surak, R. L. Bradley, Jr., A. L. Branen and E. Shrago.

A comparison of the distribution and elimination of oleic and chlorinated oleic acids and their metabolites in rats. By H. M. Cunningham and G. A. Lawrence.

Frequency of polyploid cells in the bone marrow of rats fed irradiated wheat. By K. P. George, R. C. Chaubey, K. Sundaram and A. R. Gopal-Ayengar.

Aflatoxin production on irradiated foods. By E. Priyadarshini and P. G. Tulpule.

Teneur minimale du régime en lindane induisant les monoxygenases microsomales chez le rat. Par M. A. Pélissier et R. Albrecht.

Phototoxicity and photocarcinogenesis: Comparative effects of anthracene and 8-methoxypsoralen in the skin of mice. By P. D. Forbes, R. E. Davies and F. Urbach.

Monographs on fragrance raw materials. By D. L. J. Opdyke.

Contents continued]

Aims and Scope

The Journal provides a wide-ranging service of general articles and abstracts on food and cosmetics toxicology. The Journal is primarily intended for the use of food scientists and technologists, manufacturers and administrators who neither read nor have access to the medical, pharmacological or toxicological literature. In addition, the journal will constitute a medium for the publ cation of reviews and original articles relating to the fields of interest covered by the British Industrial Biological Research Association.

Instructions to Authors

General. Authors from the United Kingdom should send *Original Papers and Reviews* to the Assistant Editor. All other papers and reviews should be sent to the appropriate Regional Editor. All 'Letters to the Editor' should be sent to the Editor and must be signed before they can be considered for publication.

Submission of a paper to the Editor will be held to imply that it reports unpublished original research, that it is not under consideration for publication elsewhere and that if accepted for the Journal, *Food and Cosmetics Toxicology*, it will not be published again, either in English or in any other language, without the consent of the Editor.

Forms of Papers Submitted for Publication. Papers should be headed with the title of the paper, the surnames and initials of the authors and the names and addresses of the institutions where the work was done. A shortened version of the title not exceeding 45 letters and spaces, to serve as a running title, should be supplied.

In general the text should be subdivided as follows: (a) Summary: brief and self-contained, not exceeding 3% of length of paper (b) Introductory paragraphs (c) Experimental (d) Results, presented as concisely as possible (e) Discussion (if any) and general conclusions (f) Acknowledgements and (g) References. Each subdivision should commence on a separate sheet. Manuscripts should be typewritten on *one side of the paper* and *double spaced*. At least *two copies* should be supplied (one original and one, or preferably two, carbon copies). Papers will be accepted in English, French and German.

References. These should be listed on a separate page, in alphabetical order and arranged as follows: author's name and initials, year, title of the journal [abbreviated according to the rules adopted in the *World List of Scientific Periodicals 1963*, 4th Ed., Butterworth & Co. (Publishers) Ltd. London], volume, first page number:

e.g. Hickman, J. R., McLean, D. L. A. & Ley, F. J. (1964). Rat feeding studies on wheat treated with gamma-radiation. I. Reproduction. *Fd Cosmet. Toxicol.* 2, 15.

References to books should include the author's name followed by initials, year, title of book, edition, appropriate page number, publisher and place of publication:

e.g. Dow, E. & Moruzzi, G. (1958). *The Physiology and Pathology of the Cerebellum*. 1st Ed., p. 422. The University of Minnesota Press, Minneapolis.

The names of all the authors of papers to be cited should be given when reference is first made in the text. In cases where there are more than two authors subsequent citations should give the first-named author followed by the words *et al.*:

e.g. (McLaughlin, Bidstrup & Konstam, 1963); (McLaughlin *et al.* 1963).

Where more than one paper by the same author(s) has appeared in any one year, the references should be distinguished in the text and the bibliography by the letters, a, b etc. following the citation of the year:

e.g. 1943a, 1943b or (1943a, b).

Footnotes. These as distinct from literature references should be avoided as far as possible. Where they are essential, reference is made by the symbols * † ‡ § ‖ ¶ in that order.

Illustrations and Diagrams. These should be kept to a *minimum* and they should be numbered and marked on the back with the author's name. Legends accompanying illustrations should be typewritten on a *separate* sheet. Diagrams and graphs must be drawn in Indian ink on good quality paper or tracing linen. The following standard symbols should be used on line drawings since they are easily available to the printers:

Photographs and photomicrographs should be submitted unmounted and on glossy paper. When colour plates are to be printed, payment for blockmaking is the responsibility of the author.

Tables and Figures. These should be constructed so as to be intelligible without reference to the text, each table and column being provided with a heading. The same information should not be reproduced in both tables and figures.

Chemical Nomenclature. The fundamental principles of organic and inorganic chemical nomenclature are laid down in the I.U.P.A.C. 1957 Rules (Butterworths Scientific Publications, London, 1958, 1959). These are given in *Handbook for Chemical Society Authors* (1961), pp. 16–163.

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

Page Proofs. These will be sent to the first-named author for correction.

Reprints. Reprints can be ordered on the form accompanying proofs.

Frequency. The Journal will be published bi-monthly.

Printed in Great Britain by A. Wheaton & Co., Exeter