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Research Section

MUTAGENICITY OF ETHYLENE OXIDE AND PROPYLENE OXIDE AND OF THE GLYCOLS AND HALOHYDRINS FORMED FROM THEM DURING THE FUMIGATION OF FOODSTUFFS

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(*Received 25 July 1979*)

Abstract—A modified Ames test was used, without a liver-microsome activation system, to test and compare the mutagenic activities of ethylene oxide and propylene oxide and of reaction products present in foods after fumigation with one or other of these epoxides. Both epoxides, as well as the 2-chloro-, 2-bromo- and 2-iodoethanols and the 1-chloro-2- and 2-chloro-1-propanol isomers induced dose-dependent increases in the number of revertant mutations in the histidine-dependent *Salmonella typhimurium* strains TA1535 and TA100, the former strain being the more sensitive. None of these compounds was effective in strains TA1537 or TA98, and ethylene glycol, diethylene glycol and propylene glycol showed no mutagenic activity in any of these four Salmonella strains. Of the compounds giving positive results, the weakest mutagenic activity was shown by 2-chloroethanol and the chloropropanols, while the levels of activity shown by the bromo- and iodoethanols were similar to those of the epoxides.

INTRODUCTION

Any evaluation of environmental factors that may have a detrimental effect on the population must take into account not only damage caused by radiation but also that caused by chemicals (Ehling, 1976) and must recognize the possible mutagenicity of these substances as well as the acknowledged dangers of acute and chronic toxicity. It is also important to note that a high proportion of substances found to be mutagenic in established test systems have been shown in animal experiments to possess carcinogenic properties. For example Purchase, Longstaff, Ashby, Styles, Anderson, Lefevre & Westwood (1978) found a high correlation between mutagenicity, as demonstrated by the Ames and cell-transformation tests, and carcinogenicity. Thus a mutagenic substance in the environment must be considered as a possible cancer risk.

In one approach to these problems, the Ames test was used to investigate the mutagenicity both of ethylene oxide and propylene oxide, which are used for the treatment of foodstuffs, and of their reaction products, the glycols and halohydrins. The fumigants themselves and some of their reaction products had already been examined for mutagenicity. Ethylene oxide, for example, had been shown to be mutagenic to barley (Ehrenberg & Gustafsson, 1957) and bacteriophage (Jordy, 1970) and in the dominant-lethal test (Embree, Lyon & Hine, 1977), while propylene oxide was mutagenic to fungal spores of *Neurospora crassa* (Kølmark & Giles, 1956) and to mature Drosophila sperms (Schalet, 1954). A few halohydrins were shown by Carr & Rosenkranz (1978), Malaveille, Bartsch, Barbin, Camus & Montesano (1975), Rosenkranz & Wlodkowski (1974) and Voogd & Van der Vet (1969), to have a mutagenic effect, but the individual results of these various authors could not be compared since, for the most part, different methods were used under different conditions. In this study the mutagenicity of several previously untested reaction products and some already tested substances was examined by the Ames test.

EXPERIMENTAL

Test materials. The mutagenicity of the following substances was investigated: ethylene oxide (purity 99·7%) from J. T. Baker Chemicals BV, Deventer, The Netherlands, propylene oxide (99%) and 2-chloroethanol (99·5%) from Merck-Schuchardt AG, München, 2-bromoethanol (approximately 99%) from EGA-Chemie, 2-iodoethanol (99%) and 1-chloro-2-propanol (97%) from Aldrich-Europe, Beerse, Belgium, a 2-chloro-1-propanol/1-chloro-2-propanol mixture from Fluka AG, Buchs, Switzerland, and ethylene glycol (99%), propylene glycol (98%) and diethylene glycol (98%) from Merck, Darmstadt. β-Propiolactone, from Fluka AG, and benzo[*a*]pyrene-4,5-oxide, a kind gift from Professor F. Oesch, Institute of Pharmacology, Mainz, were used as positive controls in all the tests.

Test organisms and procedure. The histidine-dependent *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 were used. Since reproducible results could not be obtained when ethylene oxide and propylene oxide were tested by the method of Ames, McCann & Yamasaki (1975), the plate incorporation technique was altered by the introduction of a small modification previously used with success by Pelon, Whiteman & Beasly (1977).

The reaction mixture consisted of 2 ml distilled water to which 0·1 ml of the bacterial suspension (10^9 bacteria/ml) and 0·1 ml of the test solution were added. This was then mixed with 2 ml Topagar (14 g Difco agar, 14 g NaCl and 20 ml of a 0·5 mM-biotin–histidine solution in 1000 ml distilled water) and

poured into a Petri dish already containing histidine-free agar [20 g glucose and 15 g Difco agar, in 1000 ml of salt solution from the Vogel–Bonner E-medium (Vogel & Bonner, 1956)]. The test substances were diluted with distilled water, except in the case of ethylene and propylene oxides, which were diluted with cold (0°C) acetone. After incubation for 48 hr at 37°C, the number of revertant colonies, mutating from histidine auxotrophy, to prototrophy, were counted. Liver microsomes were not incorporated into the test mixtures and therefore the possibility of metabolic activation by liver enzymes was excluded. The toxicity of the test substances towards the Salmonella strains was measured by following the death rate over 6 hr at the various dose levels used.

All the experiments were performed between six and ten times. Individual experiments were performed independently and in duplicate.

RESULTS

The results of the modified Ames tests on ethylene oxide and propylene oxide are shown in Fig. 1a, b. Without enzyme activation both epoxides, tested at concentrations in the range 10–200 μmol/plate, caused a clear dose-dependent increase in the number of revertant mutations in the Salmonella strains TA100 and TA1535. However, strain TA100 was much less sensitive to the effects of both compounds than was strain TA1535. It was also apparent that the rates of back-mutation in strain TA1535 showed a much greater scatter with ethylene oxide than with propylene oxide. Reproducibility of these results was found to be good. The positive controls, β-propiolactone and benzopyrene oxide, also showed a small scatter in mutation rates. Under the same test conditions, neither ethylene oxide nor propylene oxide nor any of the other substances investigated had any mutagenic effect on strains TA98 and TA1537.

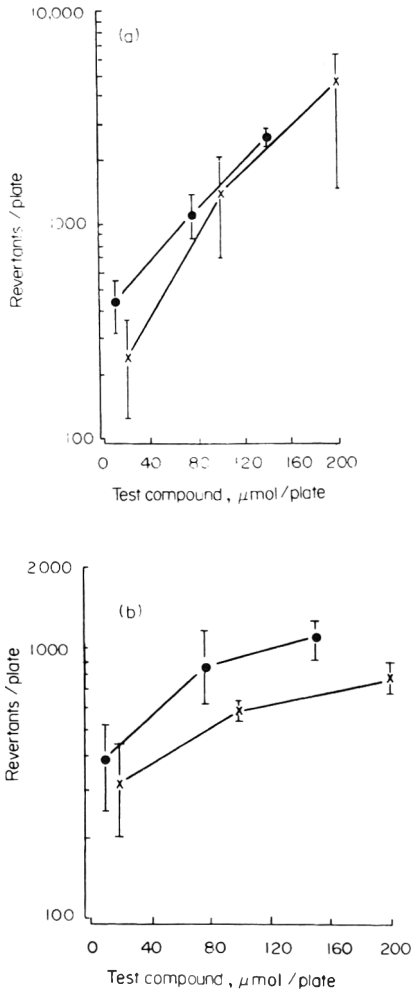

Fig. 1. Dose-response curves of the mutagenic effect of ethylene oxide (×) and propylene oxide (●) to *Salmonella typhimurium* strains (a) TA1535 and (b) TA100.

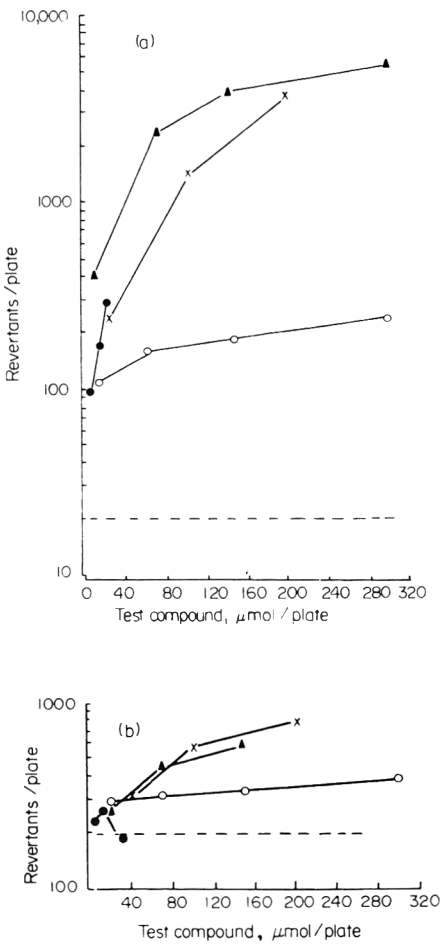

Fig. 2. Dose-response curves of the mutagenic effect of ethylene oxide (×), chloroethanol (○), bromoethanol (▲) and iodoethanol (●) to *Salmonella typhimurium* strains (a) TA1535 and (b) TA100.

When a reliable and reproducible test system to define the mutagenicity of ethylene and propylene oxides had been established, the reaction products known to be present after the fumigation of foodstuffs with these epoxides were tested under the same conditions. Thus ethylene glycol, diethylene glycol, propylene glycol, 2-chloroethanol (ethylene chlorohydrin), 2-iodo- and 2-bromoethanols, 1-chloro-2-propanol and the mixture of 1-chloro-2-propanol and

2-chloro-1-propanol isomers were tested at concentrations between 5 and 300 μmol/plate. The glycols, which result from the reaction of ethylene oxide or propylene oxide with water, caused no increase in the number of revertants. As can be seen from Figs 2 & 3, the haloethanols and halopropanols were mutagenic to strains TA1535 and TA100, and as before, strain TA1535 was more sensitive than TA100.

Comparison of the dose-response relationship for the individual substances shows that 2-bromoethanol, and at certain concentrations also 2-iodoethanol, possess a degree of mutagenic activity similar to that of ethylene oxide. Whether 2-iodoethanol in higher doses would exhibit a stronger effect could not be investigated because of its toxicity. 2-Chloroethanol and the chloropropanols showed the weakest mutagenic activity. This was not due to high toxicity, as no reduction in colony number was detected in control experiments.

DISCUSSION

The formation of halohydrins (2-chloroethanol, 2-bromoethanol and 1-chloro-2-propanol) during the treatment of foodstuffs with ethylene oxide or propylene oxide has already been investigated by many workers. For example, Wesley, Rourke & Darbishire (1965) found 260 ppm 2-chloroethanol in ethylene oxide-sterilized flour and an average of 805 ppm in a spice mixture. Even 28 days after the fumigation of lentils, Scudamore & Heuser (1971) found 65 ppm of both 2-chloroethanol and 2-bromoethanol, while in curry powder 1160 ppm chloroethanol could be measured 182 days after fumigation. Similarly, Heuser & Scudamore (1969) had detected 175 ppm 2-bromoethanol and 640 ppm 2-chloroethanol in flour 28 days after treatment. Traces of 2-chloroethanol (1·8–5·6 ppm) were also demonstrated in chocolate, by Pfeilsticker & Leyendecker (1978). Potato starch that had been treated with propylene oxide was found to contain 12 ppm propylene chlorohydrin, 94% of which was 1-chloro-2-propanol, and also 29 ppm propylene glycol (Steele & Hadziyev, 1976). In addition, Velisek, Davidek, Hajslova, Kubelka, Janicek & Mankova (1978) showed that during the production of protein hydrolysates, which are used to make flavourings, 3-chloro-1-propanol is formed.

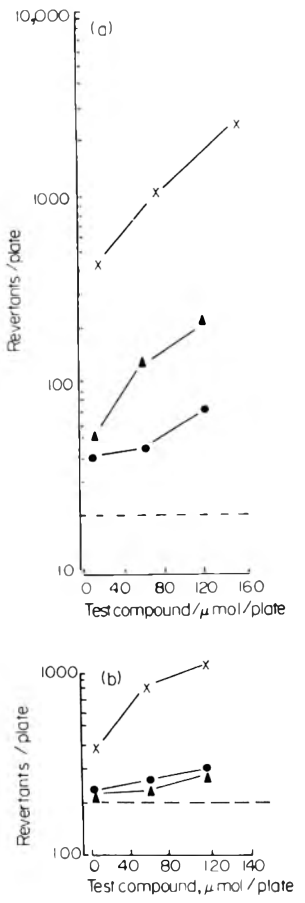

Fig. 3. Dose-response curves of the mutagenic effect of propylene oxide (×), 1-chloro-2-propanol (●) and an isomeric mixture of 1-chloro-2-propanol and 2-chloro-1-propanol (▲) to *Salmonella typhimurium* strains (a) TA1535 and (b) TA100.

Ethylene oxide itself has been detected at a level of 20 ppm after the sterilization of wheat. However, Pfeilsticker & Rasmussen (1968) reported that during storage and the milling procedures any remaining traces of ethylene oxide are removed virtually completely. In the USA, the Environmental Protection Agency (1977) has defined the tolerated level of propylene oxide in foodstuffs such as cocoa and spices as 300 ppm. Thus, in view of the published data, it must be expected that halohydrins will be present in measurable amounts in a variety of foodstuffs.

Comparison of the dose-response relationships demonstrated in our investigations show a lower mutagenic effect for chloroethanol than for 2-iodo- and 2-bromoethanols. This confirms the results of Voogd & Van der Vet (1969), who were able to detect a difference in the mutagenic effect of the halohydrins in *Klebsiella pneumoniae*. The danger of exposing the population to the mutagenic effects of 2-bromo- and 2-iodoethanol in addition to that of 2-chloroethanol should be noted. Information about the occurrence of 2-iodoethanol in materials that have been sterilized with ethylene oxide is not available at present.

2-Chloroethanol possesses considerably less mutagenic activity than ethylene oxide. Moreover, as Malaveille *et al.* (1975) demonstrated using the Ames test, chloroacetaldehyde and to a greater extent chloroethylene oxide are much stronger mutagens than 2-chloroethanol. There are indications that chloroacetaldehyde can be formed from 2-chloroethanol both by liver enzymes and also as a result of bacterial metabolism (Johnson, 1967; van Duuren, 1975). Thus the question arises whether the mutagenicity of 2-chloroethanol is the result of its activation to chloroacetaldehyde by a bacterial enzyme. With regard to the isomer mixture tested, 1-chloro-2-propanol is now known to be responsible for the mutagenic activity, as the other isomer 2-chloro-1-propanol has been shown to be non-mutagenic (Carr & Rosenkranz, 1978). The mutagenicity of bromo- and iodopropanol could not be tested as the substances were not available.

Thus, these experiments show that the halohydrins, which are secondary products formed during the fumigation of foodstuffs with ethylene and propylene

oxides, have a definite mutagenic effect and may therefore present some carcinogenic risk. However, it should be noted that the mutagenicity of the halohydrins was very small compared with that of the positive controls, β-propiolactone and benzopyrene oxide. Nevertheless, in evaluating the danger of the halohydrins to man, it is also important to note that the low mutagenic activity may be offset by the frequency of their occurrence in foodstuffs.

Investigations into the carcinogenic character of these substances have also been performed. Propylene oxide has been proved beyond doubt to be a carcinogen (Dunkelberg, 1979; Walpole, 1958). Early studies on ethylene oxide suggested that it did not possess carcinogenic activity (van Duuren, Orris & Nelson, 1965; Walpole, 1958), but later studies, involving sc application to a large number of mice, showed a definite carcinogenic effect (Dunkelberg, 1979). In considering these studies, however, it has to be remembered that the induction of tumours at the site of sc injection is not necessarily an indication of carcinogenic potency in the same compound when administered by the oral route (Grasso & Golberg, 1966). On the other hand, 2-chloroethanol administered by sc injections to rats (Mason, Cate & Baker, 1971) and mice (Homburger, 1968) was found to be inactive as a carcinogen. 2-Bromoethanol, 2-iodoethanol, 1-chloro-2-propanol and 2-chloro-1-propanol have not been investigated as yet.

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EFFECT OF MALONDIALDEHYDE ON NITROSAMINE FORMATION

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Abstract—Several aldehydes associated with foodstuffs were investigated for their effect on the formation of *N*-nitrosodimethylamine and *N*-nitrosodiethylamine. Whereas glucose, furfural, benzaldehyde and glyoxal had little influence on nitrosamine formation, malondialdehyde, which can be derived from lipids by peroxidation, greatly influenced the reaction, decreasing the formation of nitrosamine at pH 3 and increasing it at pH 4–7. The degree of inhibition or promotion was dependent on the concentration of the aldehyde.

INTRODUCTION

Nitrite is used as a food additive and is produced enzymatically from nitrate present in vegetables. The reaction of nitrite and amines present in food produces carcinogenic nitrosamines (Druckrey, Preussmann, Ivankovic & Schmähl, 1967; Magee & Barnes, 1967) either during food processing (Wasserman, Fiddler, Doerr, Osman & Dooley, 1972) or in the human stomach (Sander & Seif, 1969). This reaction proceeds slowly in an acidic medium and is accelerated by several agents. Thiocyanate and halides accelerate the reaction under strongly acidic conditions (Boyland, Nice & Williams, 1971; Boyland & Walker, 1974a,b; Fan & Tannenbaum, 1973; Yamada, Yamamoto & Tanimura, 1974), while phenolic substances, such as coffee constituents (Challis & Bartlett, 1975), gallic acid (Walker, Pignatelli & Castegnaro, 1975), *p*-cresol (Davies & McWeeny, 1977) and sesamol (Kurechi, Kikugawa & Kato, 1980) promote the reaction under milder conditions through the formation of quinone and nitrosophenol intermediates. Nitrosamines are also formed under neutral conditions in the presence of formaldehyde and chloral (Keefer & Roller, 1973).

The study now reported was undertaken to investigate the effects exerted on nitrosamine formation by several aldehydes related to foodstuffs, including malondialdehyde, an unstable compound that can be derived from lipids by their peroxidation (Kwon & Olcott, 1966; Patton, Keeney & Kurtz, 1951).

EXPERIMENTAL

Materials. The dimethylamine hydrochloride and glyoxal (40% in water) used were commercial products obtained from Kanto Chemical Co., Inc., Tokyo. Diethylamine hydrochloride, anhydrous glucose, formaldehyde (formalin, 37% in water), benzaldehyde, furfural, *N*-nitrosodimethylamine (a standard for gas chromatography) and *N*-nitrosodiethylamine were obtained from Wako Pure Chemical Industries Ltd., Osaka. Malonaldehyde bis(dimethylacetal) ($\lambda_{max}^{CHCl_3}$ 235 nm; ϵ, 0·4) was also a commercial product, supplied by Tokyo Kasei Kogyo, Co. Ltd., Tokyo.

Preparation of malondialdehyde. Malondialdehyde, which was estimated by gas chromatography and ultraviolet absorption was prepared according to the method of Chio & Tappel (1969) as follows. Malonaldehyde bis(dimethylacetal), 1·64 g (10 mmol), was shaken at 40°C with 0·9 ml 1 N-HCl until fully mixed (about 3 min); water was then added to make a final volume of 10 ml and the solution was incubated at 37°C for up to 20 hr. The concentration of malondialdehyde in the solution would have been 1·0 M if the acetal were quantitatively converted. The acetal in the solution was estimated by gas chromatography after quantitative extraction, effected by mixing 10-ml portions of the solution with 5 ml 5 N-NaOH, 2·0 g NaCl and 40 ml chloroform and shaking for 5 min. Estimation of the acetal in the chloroform extract revealed that about 50% of the initial amount remained at the start of incubation while a negligible amount remained after incubation for 1 hr at 37°C. Ultraviolet absorption maxima of the solution were at 243 nm (0·01 N-HCl) and 267 nm (pH 7·0, 0·01 M-phosphate buffer), values close to those reported for malondialdehyde derived from methyl linoleate by aeration (Kwon & Olcott, 1966). The absorbancy at 243 nm (0·01 N-HCl) increased during the first hour of incubation, remained constant for the next hour and thereafter decreased. The absorbancy after incubation for 0 hr and for 20 hr was about 50% of that after incubation for 1–2 hr. Thus gas-chromatographic and ultraviolet analysis indicated that the formation of malondialdehyde in the solution was about 50% at the start of incubation and was almost quantitative after incubation for 1–2 hr, while prolonged treatment caused degradation. The molar extinction coefficients (ϵ) of malondialdehyde determined from the ultraviolet absorption spectra of the solution after the 1-hr incubation were 12,400 ($\lambda_{max}^{0\cdot01\,N\text{-}HCl}$ 243 nm) and 28,500 ($\lambda_{max}^{pH\,7}$ 267 nm).

Since malondialdehyde was unstable, its formation and degradation were dependent not only on the conditions of the preparation but also on the conditions of use. The following three modifications of the malondialdehyde preparation were therefore used for studies of the aldehyde's effects on nitrosamine formation:

Preparation 1: The acetal (10 mmol) was shaken at 40°C with 0·9 ml 1 N-HCl until mixed. After

adjustment to pH 4·5 by the addition of 1 ml glacial acetic acid and the appropriate amount of 1 N-NaOH, the mixture was made up to 10 ml with water. The solution was a 50:50 mixture of the aldehyde and the acetal (total concentration 1 M) and contained chloride and acetate ions.

Preparation 2: The acetal (10 mmol) was shaken with 0·9 ml 1 N-HCl, made up to 10 ml with water, and then incubated at 37°C for 1 hr. The solution then contained about 100% of the aldehyde (1 M) and chloride ions.

Preparation 3: The acetal (10 mmol) was shaken with 0·9 ml 1 N-H_2SO_4 and made up to 10 ml with water, giving a 50:50 mixture of the aldehyde and the acetal (total concentration 1 M) containing sulphate ions.

In order to avoid complexity, the concentration of the aldehyde was expressed as the total of the aldehyde and the acetal, since the proportion of aldehyde and acetal, or the amount of aldehyde, was likely to alter during the acidic treatment associated with the nitrosamine-forming reaction.

Effects of aldehydes on nitrosamine formation. To 50-ml portions of a 0·1 M-sodium citrate solution containing 0·05 M-dimethylamine hydrochloride (or diethylamine hydrochloride) and 0·2 M-sodium nitrite was added one of the following aldehydes in a final concentration between 0·025 and 0·1 M: glucose, furfural, benzaldehyde, glyoxal, malondialdehyde and formaldehyde. In the case of malondialdehyde, each of the three preparations (1 M) described above were used in the required concentration. The solutions were adjusted to the required pH with concentrated hydrochloric acid or 1 N-NaOH and each mixture was placed in a stoppered flask and incubated at 37°C for 3–18 hr. The pH of the reaction mixture was checked at the end of the incubation and was generally found to be unchanged. After a defined period, a 10-ml aliquot was removed from each of the incubation mixtures, mixed with 5 ml 5 N-NaOH and 2 g NaCl and extracted with 40 ml chloroform within 5 min. The chloroform layer was subjected to gas-chromatographic analysis for the nitrosamine and the nitrosamine formation was compared with the control value obtained from the reaction mixture without the aldehyde. The theoretical maximum nitrosamine concentration in Figs 1–3 was 0·05 M.

Confirmation of nitrosamine formation by combined gas chromatography and mass spectrometry. A mixture of 0·05 M-dimethylamine and 0·2 M-sodium nitrite in 0·1 M-citrate buffer (pH 5·0) was incubated at 37°C for 3·5 hr with or without malondialdehyde (preparation 2) and then a 10-ml aliquot was removed from each mixture, mixed with 5 ml 5 N-NaOH and 2 g NaCl and extracted with 10 ml chloroform. The nitrosamine in the chloroform extracts, along with authentic *N*-nitrosodimethylamine (1·6 mg/ml) was confirmed by gas chromatography–mass spectrometry.

Analytical methods. Ultraviolet absorption spectra were measured with a Shimadzu Double Beam Spectrophotometer, UV-200S. A Yanaco Gas Chromatograph Model G80, equipped with a hydrogen flame ionization detector and a glass column (3 mm ID × 3 m) packed with polyethylene glycol 6000 (25%) on 80–100 mesh Chromosorb W AW, was used to determine the *N*-nitrosamines and malonaldehyde bis(dimethylacetal). The chromatograph was operated isothermally at 132°C (column temperature) and 170°C (injection temperature) with a nitrogen carrier-gas flow of 20 ml/min. The chromatograph was run with retention times of 11 min for *N*-nitrosodimethylamine, 16 min for *N*-nitrosodiethylamine and 15 min for malonaldehyde bis(dimethylacetal). The amounts of the nitrosamines and malonaldehyde bis-(dimethylacetal) were determined by comparing the peak area of the samples (5 μl) with that of each authentic standard solution in chloroform (5 μl of 0·4 mg/ml). The nitrosamine formation was confirmed by the use of a Hitachi Double Focusing Mass Spectrometer RMU-7L after gas-chromatographic separation of the chloroform extract using a Hitachi K-53 gas chromatograph with polyethylene glycol 6000 (20%).

RESULTS

The effects of several aldehydes, in 0·05 M concentration, on the formation of *N*-nitrosodimethylamine by reaction of dimethylamine with nitrite for 3·5 hr at 37°C under several conditions of pH are summarized in Table 1. Glucose, furfural, benzaldehyde and glyoxal did not show any significant influence on nitrosamine formation at pH values ranging from 3·0 to 6·0, but 0·05 M-malondialdehyde (preparation 1—a 50:50 mixture of the aldehyde and the acetal) had a striking effect on the reaction. Thus, it prevented nitrosamine formation at pH 3·0 but increased the

Table 1. *Effects of aldehydes in 0·05 M concentration on the formation of N-nitrosodimethylamine from dimethylamine and nitrite in a 3·5-hr incubation at 37°C and various pH values*

pH	Nitrosamine formation (as ratio of control value*) in reaction mixtures containing				
	Glucose	Furfural	Benzaldehyde	Glyoxal	Malondialdehyde†
3·0	1·15	1·10	0·96	1·08	0·63
4·0	1·14	1·13	1·06	1·00	1·18
5·0	1·24	1·14	1·29	1·08	4·53
6·0	—	—	1·29	—	8·64

*The amounts of *N*-nitrosodimethylamine formed in the control incubations were 2·0 mg/ml at pH 3·0, 1·6 mg/ml at pH 4·0, 0·3 mg/ml at pH 5·0 and 0·03 mg/ml at pH 6·0.

†Preparation 1 (see Experimental).

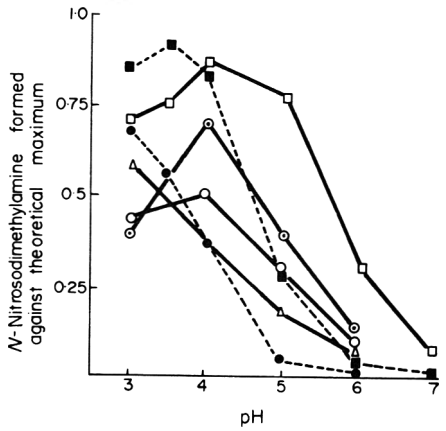

Fig. 1. Formation of *N*-nitrosodimethylamine from a mixture of 0·05 M-dimethylamine and 0·2 M-$NaNO_2$ in citrate buffer incubated at 37°C in the presence of malondialdehyde (preparation 1) in a concentration of 0·025 M (△), 0·05 M (○) or 0·1 M (◎) or in its absence (●; control) for 3·5 hr or in a concentration of 0·05 M (□) or in its absence (■; control) for 18 hr.

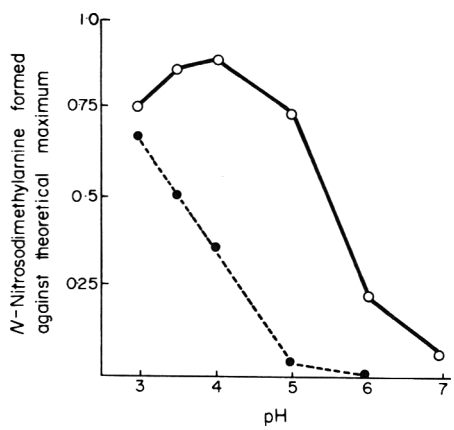

Fig. 2. Formation of *N*-nitrosodimethylamine from a mixture of 0·05 M-dimethylamine and 0·2 M-$NaNO_2$ in citrate buffer incubated at 37°C for 3·5 hr in the absence (●) or presence (○) of formaldehyde (0·05 M).

amounts formed at higher pH values between 4·0 and 6·0. At pH 6·0, the amount of nitrosamine formed was 8·6 times as much as the control value. Both the inhibitory and promoting effects of malondialdehyde were dependent on the concentration (Fig. 1). In a 3·5-hr incubation at 37°C, the presence of 0·025, 0·05 and 0·1 M-malondialdehyde respectively reduced nitrosamine formation at pH 3 to 86, 64 and 58% of the control value and increased the amounts formed at pH 6 to 660, 860 and 1180% of the control amount. Whereas the optimum pH for the formation of the nitrosamine in the control incubation was lower than 3·0, that for the sample with 0·1 M-malondialdehyde shifted to 4·0.

Similar effects of inhibition and enhancement were observed when the incubation with a 0·05 M concentration of the aldehyde was prolonged to 18 hr (Fig. 1). In this case a significant amount of nitrosamine formed at pH 7·0, although a negligible amount of nitrosamine was formed in the neutral control incubation.

To permit a valid comparison between the effects of malondialdehyde and those of formaldehyde previously reported by Keefer & Roller (1973), the effects of 0·05 M-formaldehyde were investigated under the same conditions as were used for malondialdehyde (Fig. 2). In this case, no inhibition occurred at pH 3, nitrosamine formation being increased at each pH from 3 to 7, the increases over control values being 110% at pH 3·0, 170% at pH 3·5, 240% at pH 4·0, 1700% at pH 5·0 and 2000% at pH 6·0. A significant amount of nitrosamine was formed at pH 7·0 in the presence of formaldehyde but not in its absence. The effects of formaldehyde were considerable above pH 4, at which pH the formation of *N*-nitrosodimethylamine reached a maximum. Thus the effects of malondialdehyde and formaldehyde were similar in the pH range 4–7, but the two compounds had opposite effects at pH 3.

The effects of malondialdehyde on the formation of *N*-nitrosodiethylamine by reaction of diethylamine with nitrite at 37°C for 3·5 hr (Fig. 3) were similar to its effects on *N*-nitrosodimethylamine formation.

To confirm the effects of malondialdehyde on nitrosamine formation, preparations 2 and 3 of the agent (see Experimental) and the starting acetal, malonaldehyde bis(dimethylacetal), were tested (Table 2). Preparation 2 contained a maximum concentration of the aldehyde and a negligible amount of the acetal. Preparation 3 was a 50:50 mixture of the aldehyde and the acetal and contained sulphate ions. Both preparations 2 and 3 inhibited nitrosamine formation at pH 3·0 and increased it at pH 5·0, while malonaldehyde bis(dimethylacetal) had little effect.

The identity of *N*-nitrosodimethylamine, determined by gas chromatography, was confirmed by combined gas chromatography–mass spectrometry. Thus, the molecular ion peak (M^+ 74) and the fragment ion peaks of the nitrosamine formed in the incubation with malondialdehyde at pH 5·0 were compared with those of the incubation without the

Table 2. *Effects of 0·05 M concentrations of different preparations of malondialdehyde on the formation of* N-*nitrosodimethylamine in 3·5-hr incubations at 37°C*

Malondialdehyde compound added	Nitrosamine formation (as ratio of control value) at pH 3·0	pH 5·0
Malondialdehyde (preparation 2)	0·78	4·65
Malondialdehyde (preparation 3)	0·97	4·08
Malondialdehyde bis(dimethylacetal)	1·17	1·32

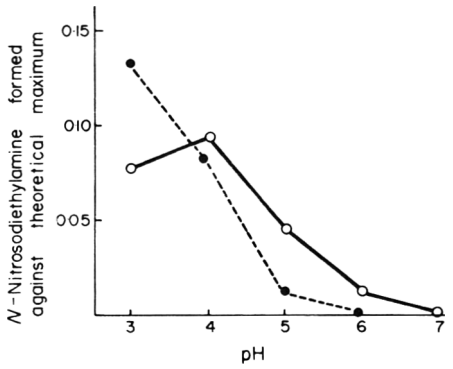

Fig. 3. Formation of *N*-nitrosodiethylamine from a mixture of 0·05 M-diethylamine and 0·2 M-$NaNO_2$ in citrate buffer incubated at 37°C for 3·5 hr in the absence (●) or presence (○) of 0·05 M-malondialdehyde (preparation 1).

aldehyde and with those of authentic *N*-nitrosodimethylamine. The peaks from the test sample were identical to those of the control and the authentic sample.

DISCUSSION

While glucose, furfural, benzaldehyde and glyoxal had little effect on the formation of *N*-nitrosodimethylamine from dimethylamine and nitrite, malondialdehyde and formaldehyde both increased the nitrosamine formed at pH 4 and above. However, the two compounds had opposite effects at pH 3 and the mechanisms of the promoting effect of malondialdehyde do not seem to be the same as those of the formaldehyde effect demonstrated by Keefer & Roller (1973).

It has been shown that the presence of glucose will promote nitrosamine formation when the reaction is terminated by alkalination. Yamamoto, Yamada & Tanimura (1979) reported a 4·6-fold increase in nitrosamine formation when 0·2 M-diethylamine was reacted with 0·2 M-nitrite in the presence of 0·2 M-glucose at pH 3·0 and 25°C for 10 min, with subsequent adjustment of the pH to 13 with 30% NaOH. This increase was attributed to the reaction of the nitrite with free hydroxyl groups available on glucose in acid medium and the subsequent breakdown of the resulting nitrite ester at high pH to liberate active nitrite ions. No such increase was demonstrated in the study reported here, in which incubation at 37°C was terminated after 3·5 hr by alkalination, but the reaction conditions used in the two investigations were not comparable.

In another paper (Kurechi & Kikugawa, 1979) we have reported that lipid-containing food and unsaturated fatty-acid residues efficiently prevented the formation of nitrosamines. It is noteworthy that malondialdehyde, which can be formed by the peroxidation of lipids in foodstuffs and cells (Gardner, 1979), increased the formation of nitrosamines at relatively high pH values. This finding and an earlier report that nitrosamine formation was enhanced by fat in fried bacon (Mottram, Patterson, Edwards & Gough, 1977) suggest that malondialdehyde formed in the fat of adipose tissue might promote the formation of nitrosamines, and point to a possible carcinogen-promoting effect of malondialdehyde.

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EFFET COMPARE DE L'ACIDE LINOLEIQUE ET DE L'ACIDE LINOLEIQUE PEROXYDE SUR L'ACTIVITE D'ENZYMES DE L'ENTEROCYTE ISOLE DE L'INTESTIN DE RAT

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Résumé—Nous avons comparé l'effet de l'acide linoléique (AL) et de l'acide linoléique peroxydé (ALP) sur les propriétés de la membrane et les activités métaboliques d'entérocytes isolés de l'intestin grêle de rats. Nous avons mesuré les quotients métaboliques (QO_2, QG et QL) des entérocytes et quelques activités enzymatiques (invertase, phosphatase alcaline et acide, arylamidase, succinate-deshydrogénase et ATPases) d'un homogénat de ces entérocytes. Aux concentrations utilisées (0,1–1,2 mM) l'AL ne modifie pas le QG ni le QL. A partir de 0,2 mM, cet acide gras diminue significativement le QO_2; cette inhibition atteint 50% à la concentration de 1,2 mM. Comparativement, l'inhibition du QO_2 observée avec l'ALP est beaucoup plus importante et elle se manifeste aussi sur le QG et le QL aux concentrations supérieures à 0,4 mM. Les activités de l'invertase et de la phosphatase alcaline ne sont pas affectées par l'AL et l'ALP. L'arylamidase est légèrement inactivée par les deux acides gras (−33 et −49% respectivement). La succinate-deshydrogénase et l'ATPase $Na^+ + K^+$ sont inhibées par l'AL (−68 et −54%) et fortement inhibées par l'ALP (−91 et −73% respectivement). La peroxydation de l'AL semble donc affecter à la fois l'activité métabolique de ces cellules et quelques activités enzymatiques. Ceci suggère que l'ALP peut modifier les systèmes membranaires des entérocytes en relation avec leurs propriétés physicochimiques. Les effets métaboliques pourraient être en relation avec cet effet membranaire.

Abstract—A comparison was made of the effects of linoleic acid (LA) and of linoleic acid hydroperoxide (LAHP) on the membrane properties and metabolic activities of enterocytes isolated from the small intestine of rats. The metabolic quotients (QO_2, QG and QL) of the enterocytes were measured and the activities of some enzymes (including sucrase, alkaline and acid phosphatase, arylamidase, succinate dehydrogenase and ATPases) were determined in a homogenate of these cells. At the concentrations studied (0·1–1·2 mM) LA altered neither the QG nor QL, but in concentrations at or above 0·2 mM, this fatty acid reduced the QO_2 significantly, the inhibition reaching 50% at the 1·2 mM concentration. By comparison, the QO_2 inhibition observed with LAHP was much more marked, and there was also a significant depression of QG and QL at concentrations above 0·4 mM. Sucrase and alkaline-phosphatase activities in the homogenates were not affected by LA or LAHP. Arylamidase was slightly inactivated by the two fatty acids (−33 and −49%, respectively). Succinate dehydrogenase and $Na^+ + K^+$ ATPase were inhibited by LA (−68 and −54%) and strongly inhibited by LAHP (−91 and −73%, respectively). The peroxidation of linoleic acid therefore appears to alter the metabolic activity of these cells as well as some enzymatic activities. This suggests that LAHP may alter the membrane systems of the enterocytes in line with their physico-chemical properties. The metabolic effects could be related to this membrane effect.

INTRODUCTION

Les membranes riches en acides gras polyinsaturés sont très sensibles à l'oxydation. Ainsi la peroxydation des membranes mitochondriales ou microsomales provoque une perte rapide de l'activité métabolique de ces organites (McKnight, Hunter et Oehlert, 1965; Wills, 1969).

Par ailleurs, les peroxydes exogènes sont des inhibiteurs puissants du métabolisme respiratoire (Desai et Tappel, 1963; Flaschenträger, 1926; Matsuo, 1962; O'Brien et Frazer, 1966); ainsi, la glycolyse et la respiration des cellules de tumeurs ascitiques sont très sensibles à la présence de linoléate de méthyle peroxydé (Baker et Wilson, 1966; Shuster, 1955).

Bergan et Draper (1970) ont montré que les lipoperoxydes présents dans la lumière intestinale sont partiellement absorbés. Cependant la muqueuse digestive subit les conséquences de l'action directe de ces substances; ainsi après administration d'une dose léthale, on note une ulcération avec destruction de la muqueuse stomacale et un œdème au niveau de l'intestin (O'Brien et Frazer, 1966). Les régimes contenant des lipoperoxydes provoquent aussi une irritation de la muqueuse digestive (Artman, 1969; Ogata, Ohtake, Kawano, Kitagawa, Tominaga et Akiyama, 1955) qui diminuerait l'absorption des nutriments.

L'entérocyte semble particulièrement exposé à l'action des lipoperoxydes exogènes apportés par les ali-

ments; on sait cependant que les entérocytes seraient capables de dégrader les lipoperoxydes puisque ces composés n'apparaissent pas dans la lymphe (Andrews, Griffith, Mead et Stein, 1960; Glavind et Tryding, 1960) mais on ignore tout de leur action sur le métabolisme de ces cellules.

Dans cette perspective, nous nous sommes proposés d'étudier l'effet comparé de l'acide linoléique (AL) et de l'acide linoléique peroxydé (ALP) sur l'activité métabolique d'entérocytes isolés et sur l'activité de diverses enzymes présentes dans un homogénat ou dans une fraction microvésiculaire de la bordure en brosse.

METHODES EXPERIMENTALES

Isolement des entérocytes, préparation des homogénats cellulaires et des microvésicules de la bordure en brosse. Les entérocytes sont isolés de l'intestin grêle de rats de race Wistar, mâles, d'un poids moyen de 200 g, selon une technique décrite précédemment (Mitjavila, Mitjavila et Derache, 1973). L'activité de diverses enzymes a été déterminée dans un homogénat de la muqueuse intestinale et dans une fraction microvésiculaire de la bordure en brosse. A cet effet, l'intestin grêle est retourné et lavé plusieurs fois dans une solution de NaCl 0,9%. La muqueuse est prélevée à l'aide d'une spatule et une prise de 1 g est mise en suspension dans 100 ml de tampon Tris, 2 mM, à pH 7,4, contenant 50 mM de mannitol et homogénéisée à l'aide d'un Waring Blendor pendant 30 sec à grande vitesse. L'homogénat est ensuite filtré à travers un filtre en nylon de 40 μm de porosité pour éliminer le mucus et les grosses particules. Toutes ces opérations sont effectuées à la température de 4°C. Les microvésicules provenant de la bordure en brosse, sont préparées à partir de cet homogénat (Schmitz, Preiser, Maestracci, Ghosh, Cerda et Crane, 1973). Le culot final contenant les microvésicules est mis en suspension dans 20 ml de tampon Tris–mannitol.

Peroxydation de l'acide linoléique (AL). Les solutions micellaires d'AL, 5 mM, sont préparées soit dans un tampon phosphate ($Na_2HPO_4.12H_2O$) 10 mM, soit dans un tampon Tris 2 mM, à pH 9, contenant 2 mM de taurocholate de sodium. Ces solutions sont peroxydées en ajoutant 10000 UI/ml de lipoxydase (linoléate:oxygen oxidoreductase EC 1.13.11.12, activité—150 000 U/mg). Les trois produits (AL, taurocholate et lipoxydase) proviennent de Sigma Chemical Company, St. Louis, MO, USA. La peroxydation est suivie par mesure de la DO à 234 nm et par la consommation d'oxygène à l'électrode de Clark. Après la peroxydation, l'enzyme est inhibée par chauffage à 70°C pendant 4 min; les solution sont ajustées au pH désiré et les tampons complétés pour les études concernées.

Mesure du métabolisme cellulaire. Le métabolisme des entérocytes a été déterminé pendant 30 min par la mesure des QO_2, QG et QL (μmol d'oxygène ou de glucose consommés ou d'acide lactique produit par mg de protéines et par heure), selon la méthode décrite par Mitjavila *et al.* (1973), en utilisant un tampon KRP glucosé exempt d'ions Ca^{2+} et Mg^{2+}, susceptibles de précipiter les acides gras. Le glucose restant après incubation a été déterminé par la méthode à la glucose oxydase à l'aide d'un autoanalyseur Beckman. L'acide lactique formé a été dosé selon la méthode de Horn et Bruns (1956) et les protéines par la méthode de Lowry, Rosebrough, Farr et Randall (1951).

Activités enzymatiques. Les activités enzymatiques (invertase, phosphatase alcaline, arylamidase, succinate-deshydrogénase et ATPase $Na^+ + K^+$) ont été déterminées à 37°C pendant 15 min après addition du substrat, selon des méthodes décrites précédemment (Mitjavila, Lacombe, Carrera et Derache, 1977). En ce qui concerne la détermination de l'activité des ATPases, le tampon phosphate 10 mM est remplacé par un tampon Tris 2 mM contenant 50 mM de mannitol. Le milieu d'incubation contient 0,2 ml d'une solution micellaire d'AL ou d'ALP à des concentrations variables dans le tampon approprié. On ajoute successivement 0,5 ml de tampon d'incubation dont les caractéristiques sont détaillées lors de la description de la méthode correspondante (Mitjavila *et al.* 1977), 0,1 ml d'homogénat ou de préparation de microvésicules et 0,2 ml de substrat.

Absorption de l'AL et de l'ALP par les entérocytes. L'utilisation de l'acide linoléique radioactif ([1-^{14}C]-linoleic acid; NEN Chemical GmbH, Dreieich, Federal Republic of Germany; activité spécifique—50 mCi/mmol) nous a permis de suivre l'absorption comparée de cet acide gras et de son hydroperoxyde par les entérocytes isolés. A cet effet, 5 μCi d'AL radioactif sont incorporées à 100 ml d'une solution micellaire d'AL, 1 mM (tampon KRP sans Ca^{++} ni Mg^{++} à pH 9, contenant du taurocholate de sodium). Une partie de cette solution est peroxydée comme indiqué ci-dessus. Le milieu d'incubation est constitué de 3 ml de la solution micellaire radioactive d'AL et d'ALP et de 1 ml de suspension cellulaire. A intervalles réguliers, 0,25 ml du milieu d'incubation sont prélevés et passés sur un filtre de cellulose régénérée d'une porosité de 0,45 μm. Les cellules retenues sur le filtre sont lavées plusieurs fois avec du tampon phosphate, puis placées dans des flacons de comptage contenant 1 ml de soude N, pendant 24 heure. Sur une prise aliquote on détermine d'une part la teneur en protéines, d'autre part l'AL et l'ALP absorbés par mesure de la radioactivité en scintillation liquide.

RESULTATS

Absorption de l'AL et de l'ALP

L'absorption en fonction du temps de l'AL et de l'ALP par les entérocytes isolés est présentée dans le Tableau 1. On peut remarquer la fixation rapide du produit radioactif par ces cellules. Avec l'AL on note une stabilisation aux alentours de 300 nmol/mg de protéines; par contre, la rétention après incubation de l'ALP plafonne rapidement et correspond à environ 115 nmol/mg de protéines.

Mesure du métabolisme cellulaire

La Figure 1 montre l'action comparée de l'AL et de l'ALP en quantité croissante sur le métabolisme d'entérocytes isolés. On remarque que l'AL n'exerce sur le métabolisme global des cellules épithéliales incubées qu'une action inhibitrice partielle (QO_2 inhibé de 50% à la concentration la plus élevée), le QG et le QL

Tableau 1. *Acide linoléique (AL) et acide linoléique peroxydé (ALP) absorbés par les entérocytes isolés en fonction du temps*

Temps (min)	Retention par les cellules (nmol/mg de protéines cellulaires)	
	AL	ALP
1	81,19 ± 7,148	86,86 ± 10.903
5	224,40 ± 16.540	111,39 ± 10,843
10	280,25 ± 13,756	119,64 ± 12,795
20	305,23 ± 11,796	127,50 ± 5,770
30	322,61 ± 7,821	115,90 ± 17,390

Chaque valeur représente la moyenne ± l'erreur type de la moyenne de quatre expériences.

n'étant pratiquement pas modifiés. L'activité métabolique cellulaire est par contre fortement inhibée par le dérivé peroxydé de l'AL. L'inhibition affecte à la fois la respiration, la consommation du glucose et la production d'acide lactique. A la concentration maximum de 1,2 mmol/litre, le peroxyde réduit le QO_2 de 82%, le QG de 86% et le QL de 84%.

Activités enzymatiques

Les résultats obtenus lors des incubations en présence d'AL ou d'ALP sont consignés dans le Tableau 2. Il est intéressant de noter que certaines enzymes sont complètement absentes dans la préparation de microvésicules de la bordure en brosse; tel est le cas de la succinate-deshydrogénase localisée dans les mitochondries et de la phosphatase acide localisée dans les lysosomes. Quant à l'ATPase $Na^+ + K^+$, moins de 1% de l'activité totale de l'homogénat est retrouvée dans les microvésicules; nous avons donc négligé cette enzyme. L'ATPase Mg^{2+} est présente dans la fraction vésiculaire, son activité spécifique étant augmentée de 2,5 fois. Par ailleurs, la phospha-

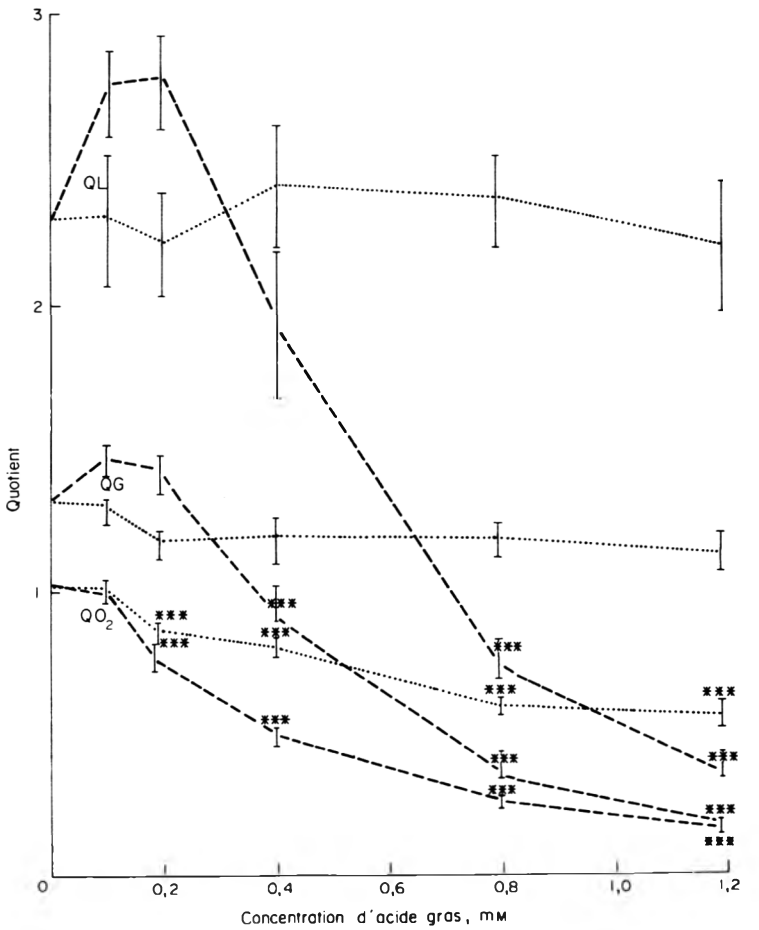

Fig. 1. Activité métabolique des entérocytes incubés en présence d'acide linoléique (-----) ou d'acide linoléique peroxydé (– – –). QL—μmol d'acide lactique formé/mg protéines/heure; QG—μmol de glucose consommé/mg protéines/heure; QO_2—μmol d'oxygène consommé/mg protéines/heure. Chaque point représente la moyenne ± l'erreur-type sur la moyenne de cinq expériences. Différences significatives; ***$P < 0.001$.

Tableau 2. *Activité enzymatique d'un homogénat et de la bordure en brosse d'entérocytes incubés en présence d'acide linoléique (AL) et d'acide linoléique peroxydé (ALP)*

Traitement	Dosage de l'acide gras (mM)	Activité enzymatiques (µmol/mg prot./15 min)						
		Invertase	Arylamidase	Phosphatase alcaline	ATPase Mg^{2+}	Succinate deshydrogenase	ATPase $Na^{+} + K^{+}$	Phosphatase acide
				Homogenat				
Témoin	0	2,05 ± 0,075	2,38 ± 0,255	30,8 ± 0,37	5,73 ± 0,748	0,705 ± 0,025	2,35 ± 0,33	0,552 ± 0,0341
AL	0,125	2,19 ± 0,134	2,22 ± 0,322	31,8 ± 0,26	5,05 ± 0,900	0,424 ± 0,042***	1,19 ± 0,18***	0,526 ± 0,0127
	0,250	2,06 ± 0,891	2,04 ± 0,199	33,0 ± 0,32	4,41 ± 0,851	0,307 ± 0,035***	1,13 ± 0,21***	0,485 ± 0,0164
	0,500	2,05 ± 0,168	1,82 ± 0,161	32,0 ± 0,87	4,23 ± 0,786	0,260 ± 0,033***	1,21 ± 0,21***	0,452 ± 0,0086*
	1,00	2,19 ± 0,150	1,60 ± 0,142*	32,6 ± 0,84	4,32 ± 0,860	0,225 ± 0,024***	1,08 ± 0,23***	0,414 ± 0,0288*
ALP	0,125	2,07 ± 0,178	2,07 ± 0,206	33,0 ± 0,33	6,02 ± 0,951	0,407 ± 0,029***	1,53 ± 0,60***	0,433 ± 0,0042*
	0,250	2,17 ± 0,159	1,81 ± 0,107	32,0 ± 0,21	4,33 ± 0,924	0,248 ± 0,033***	0,87 ± 0,20***	0,370 ± 0,0153**
	0,500	2,04 ± 0,201	1,50 ± 0,194*	32,5 ± 0,27	4,21 ± 0,868	0,136 ± 0,013***	0,50 ± 0,18***	0,338 ± 0,0172**
	1,00	2,19 ± 0,157	1,22 ± 0,147*	32,0 ± 0,35	3,90 ± 0,716	0,062 ± 0,012***	0,64 ± 0,28***	0,255 ± 0,0107***
				Bordure en brosse				
Témoin	0	15,9 ± 1,34	13,26 ± 0,430	232,0 ± 1,73	14,32 ± 1,40	—	—	—
AL	0,125	13,3 ± 1,35	12,37 ± 0,125	234,0 ± 1,00	9,62 ± 0,679*	—	—	—
	0,250	14,9 ± 1,06	11,51 ± 0,163***	232,0 ± 1,77	9,64 ± 0,705*	—	—	—
	0,500	15,8 ± 1,06	11,07 ± 0,380***	231,0 ± 1,00	9,50 ± 0,651*	—	—	—
	1,00	14,8 ± 1,34	9,25 ± 0,267***	231,0 ± 1,00	10,28 ± 0,841*	—	—	—
ALP	0,125	15,9 ± 1,40	11,18 ± 0,135**	242,0 ± 1,77	11,62 ± 0,984	—	—	—
	0,250	14,9 ± 1,57	9,99 ± 0,125**	239,0 ± 1,53	10,67 ± 1,04	—	—	—
	0,500	15,9 ± 1,16	9,46 ± 0,275***	239,0 ± 1,53	10,50 ± 1,13	—	—	—
	1,00	15,7 ± 1,66	8,02 ± 0,430***	241,0 ± 3,76	10,15 ± 0,866*	—	—	—

Chaque valeur représente la moyenne ± l'erreur type sur la moyenne de six expériences. Différences significatives: $*P < 0,05$; $**P < 0,01$: $***P < 0,001$.

tase alcaline, l'invertase et l'arylamidase sont des enzymes caractéristiques de la bordure en brosse et leur activité spécifique se trouve concentrée entre 6 et 8 fois dans les microvésicules par rapport à l'homogénat total. De ces trois enzymes, seule l'arylamidase est particulièrement inhibée et il est important de remarquer qu'à concentrations égales, le pourcentage d'inhibition obtenu avec l'ALP est toujours supérieur à celui de son précurseur. Cette différence d'activité entre les deux acides gras est encore plus marquée lorsqu'on compare les résultats de la phosphatase acide. L'enzyme la plus sensible à l'action de ces inhibiteurs est la succinate-deshydrogénase, son activité spécifique étant déjà très significativement diminuée à la concentration la plus faible de peroxyde. A la concentration la plus élevée (1,0 mmol/litre) le dérivé peroxydé inhibe complètement son activité. L'ATPase $Na^+ + K^+$ est aussi fortement inhibée et montre une plus grande sensibilité envers l'acide gras peroxydé.

DISCUSSION

L'action de l'ALP sur le métabolisme cellulaire est d'autant plus importante que ce dérivé peroxydé est moins absorbé par les entérocytes isolés que son précurseur. En effet, dans nos conditions expérimentales il existe une différence très nette entre le taux d'absorption de l'AL et de l'ALP, ceci confirme les résultats obtenus *in vivo* par Bergan et Draper (1970). Si l'on accepte que le transfert entre le milieu d'incubation et les cellules se fait en fonction du coefficient de partage de l'acide gras entre l'eau et les lipides cellulaires, il est logique que l'ALP se concentre moins que son précurseur dans les lipides cellulaires, puisque la présence de la fonction hydroperoxyde augmente légèrement l'hydrosolubilité. Par ailleurs, la peroxydation entraine un déplacement des doubles liaisons en position *cis-cis* vers un système de doubles liaisons conjuguées en position *cis-trans* (Dolev, Rohwedder et Dutton, 1967) ce qui s'accompagne d'une modification de la structure stérique de la molécule et qui peut contribuer à expliquer cette différence de comportement.

L'effet inhibiteur des acides gras saturés ou insaturés sur le métabolisme cellulaire est bien connu (Bennett et Connon, 1957; Edwards et Ball, 1954). Cependant, l'AL est sans action sur le QG et le QL malgrè sa rapide absorption. Il est donc vraisemblable que le mécanisme de transport actif faisant intervenir l'ATPase membranaire ainsi que le système enzymatique intervenant dans la glycolyse soient peu affectés. Par contre, le métabolisme oxydatif des entérocytes représenté par le QO_2 est partiellement inhibé par l'AL. Il semble donc que dans la membrane mitochondriale il existe un site particulièrement sensible à l'AL.

Dans des conditions normales, il existe dans les entérocytes une protéine, la FABP (Fatty Acid Binding Protein) qui fixe spécifiquement les acides gras libres et élimine l'effet tensioactif de ces derniers à l'intérieur de la cellule. Il se peut que cette protéine soit insuffisante ou inactivée dans nos conditions expérimentales, soit encore que l'AL subisse une peroxydation au cours de l'incubation. En effet, nos résultats montrent que le peroxyde est nettement plus actif que son précurseur; ainsi pour inhiber de 50% le QO_2, il faut une concentration de 0,4 mM d'ALP ou 1,2 mM d'AL. Or, *in vivo*, l'activité de la muqueuse intestinale irait plutôt dans le sens d'une déperoxydation (Glavind et Tryding, 1960). Bien que la FABP possède une activité plus grande pour les acides gras non saturés à longue chaîne (Ockner, Manning, Poppenhausen et Ho, 1972) tant *in vivo* que *in vitro* (Ockner et Manning, 1974), il est vraisemblable que cette affinité soit plus faible lorsque l'acide gras est peroxydé, ce qui expliquerait l'inhibition plus importante du QO_2.

Les acides gras à longue chaîne et en particulier les insaturés sont considérés comme des inhibiteurs enzymatiques puissants (O'Brien et Green, 1967), la succinate-deshydrogénase de divers tissus en serait très sensible (Edwards et Ball, 1954). Cette enzyme fait partie des molécules protéiques responsables du transport des électrons et de l'hydrogène, disposées dans une séquence enchaînée et dont la structure est maintenue par des phospholipides. Les acides gras agiraient en désorganisant ces structures, inactivant ainsi l'enzyme (Bennett et Connon, 1957). Par une méthode manométrique, Capalna (1968) trouve que l'activité de la succinate-deshydrogénase est inhibée tant par l'AL que par l'ALP. Dans nos conditions expérimentales le peroxyde est beaucoup plus actif mais il est évidemment difficile de comparer les résultats lorsque la méthode de peroxydation du substrat et la préparation enzymatique sont très différentes.

Avec l'acide gras peroxydé les inhibitions du QG et du QL sont comparables; ceci exclut toute action sur les enzymes impliquées dans la glycolyse. Néanmoins, Shuster (1955) et Baker et Wilson (1966) trouvent que le linoléate de méthyle oxydé inhibe la glycolyse et la respiration des cellules de tumeurs ascitiques. Nos résultats seraient plus favorables à une diminution du transport du glucose. Divers types d'inhibiteurs du transport du glucose sont connus; ainsi, le mode d'action des acides gras peroxydés serait différent de celui des découplants de la phosphorylation oxydative, comme le 2,4-dinitrophénol (Israel et Salazar, 1967), lesquels en même temps augmentent la consommation d'oxygène. Par ailleurs, une action du type de l'oligomycine serait marquée par une augmentation de la production de l'acide lactique au détriment de l'utilisation de l'acide pyruvique dans le cycle de Krebs (Kimmich, 1970), ce n'est pas le cas dans nos expériences. Une inhibition du type ouabaïne (Israel, Kalant et Laufer, 1965) est vraisemblable étant donné la grande sensibilité de l'ATPase $Na^+ + K^+$. Cette inhibition pourrait s'expliquer par la nature très hydrophobe des sites actifs de cette enzyme. Par contre les enzymes situées dans la surface externe de la bordure en brosse des entérocytes, donc probablement très hydrophiles, tels que l'invertase ou la phosphatase alcaline ne sont pas inhibées ni par l'AL ni par l'ALP, alors que l'arylamidase qui comporte forcément une région lipophile du fait de sa structure transmembranaire (Louvard, Maroux, Vannier et Desnuelle, 1975) est partiellement inhibée par les acides gras utilisés.

Les modifications au niveau moléculaire apportées par la fonction hydroperoxyde (diminution de la liposolubilité et apparition de doubles liaisons conjuguées en position *cis-trans*) sont certainement en relation avec les effets métaboliques et membranaires exercés par l'acide linoléique peroxydé sur les entérocytes.

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EFFECT OF THE PLANT GROWTH REGULATOR, 2-CHLOROETHYLPHOSPHONIC ACID, ON SPONTANEOUS AND CHEMICALLY-INDUCED LUNG TUMORIGENESIS IN STRAIN A MICE

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Abstract—Both the carcinogenicity and the co-carcinogenicity of the plant growth regulator, 2-chloroethylphosphonic acid, were assessed in the strain A mouse pulmonary adenoma bioassay. 2-Chloroethylphosphonic acid suppressed the spontaneous development of lung tumours in strain A mice and inhibited the development of lung tumours in response to urethane, an active carcinogen in the pulmonary adenoma bioassay. At physiological pH, 2-chloroethylphosphonic acid breaks down into ethylene, phosphate and chloride. The ethylene thus generated may be responsible for the anticarcinogenic effects of the plant growth regulator, since this gas has been shown to inhibit both cell division and DNA synthesis in plants.

INTRODUCTION

Within the last few decades organohalides have become common in our environment (Burchfield & Storrs, 1977). Compounds of this class have been used in medicine as antitussives, diuretics, antihistamines, analgesics, sedatives, tranquillizers, anticancer agents and antimalarials. Organohalides have also been manufactured for use as dry-cleaning fluids, degreasing agents, fuel additives, refrigerants, aerosol propellants and plastics. In agriculture, organohalides have been used as pesticides, fungicides, herbicides and plant growth regulators.

Because some organohalides have been linked to cancer in both human and animal studies (Burchfield & Storrs, 1977), the exposure of humans to this class of compounds has become a matter for concern. Over the last few years a series of carcinogenicity studies on selected organohalides has been conducted in our laboratory. These have included studies designed to correlate carcinogenic activity with chemical structure (Poirier, Stoner & Shimkin, 1975; Theiss, Shimkin & Poirier, 1979) and investigations of the carcinogenicity of organohalides to which humans are exposed (Theiss, Stoner, Shimkin & Weisburger, 1977).

An organohalide which is widely used in agriculture is 2-chloroethylphosphonic acid. This chemical is a plant growth regulator registered in the USA for use on apples, blackberries, blueberries, cantaloupes, cherries, cranberries, figs, filberts, lemons, peppers, pineapples, tangerines, tangerine hybrids, tobacco, tomatoes and walnuts. 2-Chloroethylphosphonic acid first came into commercial use in 1970 and today it is the second most frequently used plant growth regulator in agriculture.

The wide use of this organohalide prompted us to undertake a study of both its carcinogenic and its co-carcinogenic activity. Carcinogenicity was assessed by the strain A mouse pulmonary adenoma bioassay. Co-carcinogenicity was determined by measuring the effect of 2-chloroethylphosphonic acid on the response of strain A mice to urethane, an active carcinogen in the pulmonary adenoma bioassay system.

EXPERIMENTAL

Materials. 2-Chloroethylphosphonic acid was obtained as a commercial preparation (Ethrel) from Amchem Products, Inc., Fremont, CA. Urethane was obtained from Matheson, Coleman & Bell, Norwood, OH and strain A/St male and female mice were supplied by the L.C. Strong Research Foundation, San Diego, CA.

Effect of 2-chloroethylphosphonic acid on spontaneous pulmonary carcinogenesis. The first step in this investigation was to determine a maximum tolerated dose of 2-chloroethylphosphonic acid by a preliminary toxicity study. Groups of five mice were given ip injections of various amounts of 2-chloroethylphosphonic acid diluted in saline three times a week for 3 wk. The mice were observed for a further 2 wk to detect delayed toxicity. The maximum tolerated dose was defined as the highest dose at which no deaths occurred.

Next, the effects of 2-chloroethylphosphonic acid on spontaneous pulmonary tumorigenesis were assessed. Groups of 20 (10 male and 10 female) 6-wk-old mice were given ip injections of 2-chloroethylphosphonic acid at the maximum tolerated dose (80 mg/kg) or at 40 mg/kg or 20 mg/kg three times a week for a total of 24 injections, when possible. Controls were injected with saline. The mice were killed 24 wk after the first injection and the excised lungs

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were placed in Tellyesniczky's fluid (70% ethanol–formalin–glacial acetic acid; 20:1:1, by vol.). The lungs were examined under a Spencer dissecting microscope (×10; Spencer Lens Co., Buffalo, NY) and the surface adenomas were counted. A few of these surface nodules were examined histologically to confirm that they had the typical morphological appearance of an adenoma. The frequency of lung tumours in each treated group was compared with the control group by Student's *t* test.

Effects of 2-chloroethylphosphonic acid on the development of pulmonary tumours in response to urethane. In this experiment, groups of 20 (10 male and 10 female) 16-wk-old strain A mice were given a single sc injection of either 0·25 or 1·0 mg urethane/g and repeated ip injections of 2-chloroethylphosphonic acid (50 mg/kg thrice weekly for a total of 24 injections). In four groups, 2-chloroethylphosphonic acid treatment was begun 7 days before urethane injection. In a second set of four groups, treatment with 2-chloroethylphosphonic acid was started on the day of the urethane injection, and in a third set it was initiated 7 days after urethane injection. Sixteen weeks after the urethane injection, the mice were killed and the number of adenomas appearing on the surface of the lungs was counted. The mean number of lung tumours in each 2-chloroethylphosphonic acid-treated group was compared to the corresponding group that received urethane alone by Student's *t*-test.

RESULTS

Effect of 2-chloroethylphosphonic acid on spontaneous pulmonary carcinogenesis

The pulmonary adenoma response of strain A mice to 2-chloroethylphosphonic acid is shown in Table 1. At the low and middle doses of this chemical it was possible to administer 24 injections and animal survival was good. However, at the highest dose of 2-chloroethylphosphonic acid, only 14 injections could be given and only 11 of the mice survived treatment. Decreased numbers of lung tumours were found in the treated groups compared to the control group and these decreases were statistically significant. The decrease in lung tumours was dose-related, increasing from a 69% inhibition of lung-tumour formation at the lowest dose of 2-chloroethylphosphonic acid to 100% inhibition at the highest dose.

Effect of 2-chloroethylphosphonic acid on the development of pulmonary tumours in response to urethane

A dose of 50 mg 2-chloroethylphosphonic acid/kg was used to assess the effect of this chemical on the development of lung tumours in response to urethane (Table 2). At the lower urethane dose, 2-chloroethylphosphonic acid slightly inhibited the development of lung tumours when treatment with this chemical was begun either 7 days before or on the same day as urethane administration. At the higher urethane dose, 2-chloroethylphosphonic acid produced a statistically significant suppression of lung tumorigenesis when treatment was started 7 days before or on the same day as urethane administration. Under these conditions, 2-chloroethylphosphonic acid produced a 40–42% inhibition of lung tumorigenesis in response to urethane. When 2-chloroethylphosphonic acid treatment was begun 7 days after urethane injection, it had no significant effect on the carcinogenicity of urethane, either at the lower or higher dose of the carcinogen.

DISCUSSION

The finding that 2-chloroethylphosphonic acid actually suppressed the spontaneous lung-tumour incidence indicates that this compound is not carcinogenic under the conditions of this bioassay and that it may even possess anticarcinogenic activity. 2-Chloroethylphosphonic acid also suppressed the effects of urethane on lung tumorigenesis; the incidence of lung tumours was less in the groups given 2-chloroethylphosphonic acid (dosing starting before or at the same time as urethane injection) than in those given urethane alone. The finding that beginning 2-chloroethylphosphonic acid treatment 7 days after urethane administration did not significantly suppress lung-tumour development suggests that the anticarcinogenic effect of 2-chloroethylphosphonic acid is due to its interference with the ability of urethane to induce pulmonary adenomas rather than to a suppression of the growth of the induced tumours. Thus it appears that 2-chloroethylphosphonic acid possesses anticarcinogenic activity towards both spontaneous and carcinogen-induced tumours.

While these data do not provide evidence for the possible mechanisms by which 2-chloroethylphosphonic acid produces anticarcinogenic effects, it is interesting to speculate on the mode of action of this plant growth regulator. It has been demonstrated that, above pH 5, 2-chloroethylphosphonic acid decomposes to phosphate, chloride, and ethylene gas and the ethylene is thought to be responsible for the growth-regulating effects of the compound (Cooke & Randall, 1968; Warner & Leopold, 1969). Ethylene is considered to be a plant hormone that influences nearly all aspects of plant growth and development

Table 1. *Effects of 2-chloroethylphosphonic acid on the development of pulmonary adenomas in strain A mice*

Treatment	Dose/ip injection (mg/kg)	No. of injections given	Total dose (mg/kg)	No. of mice surviving/total no. in group	No. of lung tumours	
					Per mouse (mean ± SEM)	% of control
Saline (control)	0	24	0	44/50	0·36 ± 0·06	—
2-Chloroethylphosphonic acid	20	24	480	19/20	0·11 ± 0·02**	31
	40	24	960	18/20	0·06 ± 0·01*	17
	80	14	1120	11/20	0·00 ± 0·00**	0

Values marked with asterisks differ significantly (Student's t test) from the control value (*$P < 0·05$; **$P < 0·01$).

Table 2. *Effects of 2-chloroethylphosphonic acid on the development of pulmonary adenomas in response to urethane in strain A mice*

Treatment (mg/g)	No. of mice surviving/total no. in group	No. of lung tumours		No. of mice surviving/ total no in group	No. of lung tumours		No. of mice surviving/ total no in group	No. of lung tumours	
		Per mouse (mean ± SEM)	% of control		Per mouse (mean ± SEM)	% of control		Per mouse (mean ± SEM)	% of control
		Day −7†			**Day 0†**			**Day +7†**	
Urethane (0·25)	40/40	0·98 ± 0·17	—	40/40	0·98 ± 0·17	—	40/40	0·98 ± 0·17	—
Urethane (0·25) + 2-CEPA (0·05)	30/30	0·77 ± 0·16	79	30/30	0·83 ± 0·17	85	30/30	1·00 ± 0·14	102
Urethane (1)	39/40	4·85 ± 0·68	—	39/40	4·85 ± 0·68	—	39/40	4·85 ± 0·68	—
Urethane (1) + 2-CEPA (0·05)	30/30	2·90 ± 0·36*	60	27/30	2·8 ± 0·41*	58	30/30	4·13 ± 0·50	85

2-CEPA = 2-Chloroethylphosphonic acid

†Dosing with 2-chloroethylphosphonic acid was started 7 days before sc injection with urethane (day −7), or on the same day as urethane injection (day 0) or 7 days after urethane injection (day +7). In each case 2-chloroethylphosphonic acid was injected ip three times a week for 8 wk.

Values marked with an asterisk differ significantly (Student's *t*-test) from those of the corresponding group that was given urethane alone (*$P < 0·05$).

(Pratt & Goeschl, 1969; Lieberman, 1979). Inhibition by ethylene of the growth of the apical-hook region of pea seedlings results from ethylene-induced inhibition of DNA synthesis (Apelbaum & Burg, 1972). It is the capacity of this gas to suppress DNA synthesis that may account for its anticarcinogenic effects.

The length of the binding time of urethane to DNA has been related to the level of DNA synthesis in the target organ by the demonstration that the binding of urethane to mouse-liver DNA persisted longer in partially hepatectomized than in intact mice (Lawson & Pound, 1973). Also, the ability of caffeine to inhibit the carcinogenic response of mouse lung to urethane has been ascribed to a decrease in the binding time of urethane to lung-tissue DNA as a consequence of caffeine-induced suppression of DNA synthesis in the lung (Theiss & Shimkin, 1978). Since 2-chloroethylphosphonic acid evolves ethylene at physiological pH, it is possible that this ethylene may inhibit the development of lung tumours in response to urethane in a way similar to that of caffeine. Ethylene-induced suppression of DNA synthesis in lung tissue could also play a role in the inhibition by 2-chloroethylphosphonic acid of the spontaneous development of pulmonary adenomas (Theiss & Shimkin, 1978).

Thus it would appear that 2-chloroethylphosphonic acid does not pose a carcinogenic threat to man and may actually confer some protection against environmental carcinogens. Elucidation of the mechanism by which this plant growth regulator produces anticarcinogenic effects may lead to the development of a means to counteract to some extent the carcinogenic effects of chemicals found in the environment.

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STABLE LEAD, ^{210}Pb AND ^{210}Po IN THE LIVER AND KIDNEYS OF CATTLE. II. ANIMALS FROM AN AREA NEAR AN ABANDONED LEAD MINE

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Abstract—The concentrations of ^{210}Pb, ^{210}Po and stable lead in the livers and kidneys of 35 animals from an area close to an abandoned lead mine were determined. The median liver concentrations were 12·9 pCi ^{210}Pb, 35·8 pCi ^{210}Po and 0·58 mg stable lead/kg wet tissue, and the median kidney concentrations were 19·5 pCi ^{210}Pb, 152·4 pCi ^{210}Po and 1·20 mg stable lead/kg wet tissue. The specific activity of ^{210}Pb was 20·0 nCi/g Pb in both the livers and the kidneys. The median values for the ratios of the concentrations in the kidneys to those in the livers were 1·47 for ^{210}Pb, 4·15 for ^{210}Po and 2·04 for stable lead. The median ratio of ^{210}Po to ^{210}Pb was 2·50 in the livers and 8·18 in the kidneys. Significant correlations were found between the liver and the kidney levels of ^{210}Pb, ^{210}Po and stable lead and between ^{210}Po and ^{210}Pb in the kidneys, ^{210}Po and ^{210}Pb in the livers, and ^{210}Pb and stable lead in the livers. All the data and their relationships were compared with corresponding data from a control group of cattle from an area which was apparently unaffected by pollution from traffic or industry.

INTRODUCTION

In general, the presence of the radionuclides ^{210}Pb and ^{210}Po in our environment is due to the continuous release of the gaseous parent radionuclide ^{222}Rn from the earth's crust into the atmosphere. In addition, however, there are anthropogenic sources of ^{210}Pb and ^{210}Po, associated for example with phosphate fertilizers, power plants burning fossil fuels, lead production, cement manufacture (Moore, Martell & Poet, 1976) and fallout from nuclear weapon tests (Jaworowsky, 1963 & 1966). Similarly, stable lead is fairly abundant in the earth's crust and is, therefore, present in all parts of the environment, including the biosphere. Again, there is a variety of additional anthropogenic sources associated for example with industrial processing, the combustion of fossil fuels, the use of insecticides containing lead or the use of lead additives in petrol.

It is generally agreed that food constitutes a major source of intake for man of the radionuclides ^{210}Po and ^{210}Pb (Holtzman, 1963; Ladinskaya, Parfenor, Popov & Fedorova, 1973) and of stable lead (Lucas, 1975). In this connection the concentrations of ^{210}Po, ^{210}Pb and stable lead in the kidneys and livers of cattle are important; the kidneys in particular are known to be rich in Pb (Kreuzer, Kracke, Sansoni & Wissmath, 1978). Investigations of the content of ^{210}Pb, ^{210}Po and stable lead in the kidneys and livers of cattle are of interest not only because both organs are part of the human diet but also because of the possibility that they may serve as a bio-indicator of a local environmental pollution by these metals.

In contrast to our previous work (Bunzl, Kracke & Kreuzer, 1979), in which we determined the ^{210}Pb, ^{210}Po and stable lead concentrations in the livers and kidneys of cattle from an area which seemed to be unaffected by anthropogenic sources of these metals (control-group), in the present study we investigated a large group of cows from an area near an abandoned lead mine. We hoped to discover whether higher concentrations of the above metals would be found in the organs of these animals compared with the control group. In addition, we selected a group of animals large enough to determine whether the statistically significant correlations between the amounts of these metals in the liver and kidneys observed for the control-group would also be found for the group near the lead mine.

EXPERIMENTAL

The lead mine is situated in the west of the FRG at the northern spur of the Eifel, about 3 km to the west of the small town of Mechernich. Since its first exploitation by the Celts, more than 3 million tonnes of lead were mined until 1957, when the mine was closed down. The ore deposit covers an area about 10 km long, 1 km wide and has a thickness of between 30 and 70 m. The average metal content of the ore is between 0·6 and 1% Pb and 1·1 to 2·3% Zn. The

metallic beds consist of sediment layers of finely dispersed sandstone and coarse conglomerates, belonging to the middle 'Buntsandstein'. The chief mineral ores are galena (PbS), Cerussite ($PbCO_3$) and zinc blende (ZnS). The dressing of the ore was achieved by a wet process, subsequent to disintegration of the conglomerates. The remaining, very finely dispersed flotation sand is dumped on large waste heaps. Because resuspension of this sand, which still contains some lead (c. 0·2%), by the wind cannot be avoided completely, and because many pastures are frequently flooded by brooks considerably contaminated with lead, increased levels of Pb in the grass (on average 136 mg/kg dry weight) and in the soil (7550 mg/kg dry soil) of our sampling area were reported by Glabasnia (1978).

All the 35 animals (mostly cows and a few bulls) came from farms located within about 7 km to the north of the mine. A considerable part of the agricultural land there is used as pasture for cattle. All animals were fed in stalls during the winter but grazed on pasture during the summer. The samples were taken between autumn 1975 and summer 1976. The liver and kidneys were taken from the carcass of each animal and 100–200 g were cut from the *lobus caudatus* of each liver and the apical pole of each right kidney.

The ^{210}Pb, ^{210}Po and stable lead were determined by procedures reported previously (Bunzl *et al.* 1979).

RESULTS AND DISCUSSION

Concentrations of ^{210}Pb, ^{210}Po and stable lead in the liver and kidneys

The frequency distributions of ^{210}Pb, ^{210}Po and stable lead as well as of the specific activity of ^{210}Pb are shown in Fig. 1 for the livers and in Fig. 2 for the kidneys. To determine whether these frequency distributions were compatible with a normal or log-normal distribution, the chi-squared goodness-of-fit test at the 5% level was applied to the data groups. Only the concentrations of ^{210}Pb and ^{210}Po in the kidneys and the specific activities of ^{210}Pb in livers and kidneys were found to be distributed according to a log-normal frequency distribution, while for all of the other histograms neither a normal nor a log-normal distribution was applicable at the selected significance level. Similar results were obtained for the control group (Bunzl *et al.* 1979) except that in this case log-normal distributions were also observed for the concentrations of ^{210}Pb, ^{210}Po and stable lead in the livers. Because of these asymmetric frequency distributions, which were all somewhat skewed to the right, we characterized the average concentrations of the metals in the two organs not only by their arithmetic means and standard deviations but also by their medians and corresponding 95% confidence limits. These values, and the range of each data group are listed in Table 1.

The medians of the concentrations of ^{210}Pb, ^{210}Po and stable lead in the organs observed for the animals near the lead mine differed considerably from the corresponding values for the control group. To test whether these differences were statistically significant we applied the U-test of Mann & Whitney (Sachs, 1974) for the comparison of medians. Significant differences were found only in the case of the ^{210}Pb levels in livers and kidneys (99·9% level, two-tailed test) which were significantly lower in the animals from the area near the lead mine compared with the control group; no significant differences in the ^{210}Po concentrations were observable. Very significant increases in the concentrations of stable lead were found in the organs of the animals near the lead mine compared to those of the control group. This result is not surprising, because comparatively high values of lead had previously been reported in grass (average 136 mg Pb/kg dry weight) and soil samples (7550 mg/kg dry soil) from the area near the lead mine (Glabasnia, 1978). The same author also reports an average value of 910 pCi ^{210}Pb/kg dry weight in

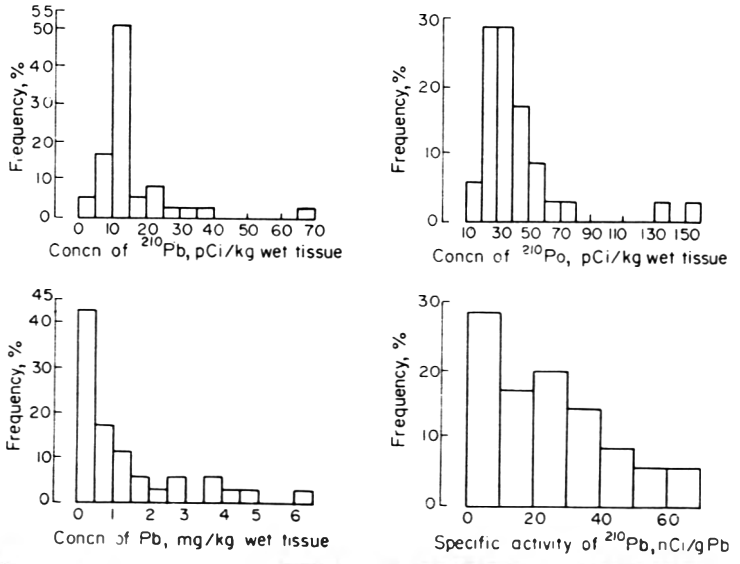

Fig. 1. Frequency distributions for the concentrations of ^{210}Pb, ^{210}Po and stable lead, and the specific activity of ^{210}Pb in the livers of cattle, from an area near an abandoned lead mine (n = 35).

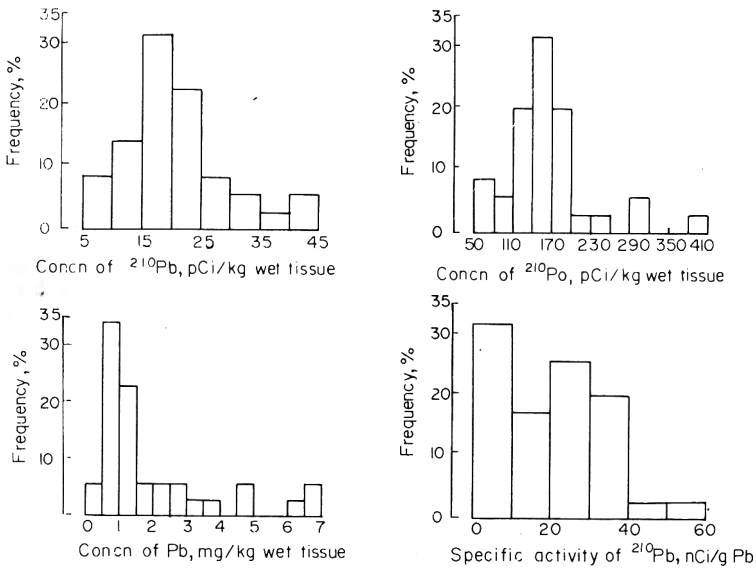

Fig. 2. Frequency distributions for the concentrations of ^{210}Pb, ^{210}Po and stable lead, and the specific activity of ^{210}Pb in the kidneys of cattle from an area near an abandoned lead mine (n = 35).

grass. Representative concentrations of Pb, ^{210}Pb or ^{210}Po in grass and soil samples from the area of the control animals are not available. As a result of both the smaller ^{210}Pb- and the higher stable lead concentrations in the organs of the animals from the area near the lead mine we observed very low values for the specific activity of ^{210}Pb (see Table 1) when compared to the control group. As demonstrated for the control group, the median specific activities of ^{210}Pb in livers and kidneys did not differ significantly (U-test of Mann & Whitney).

Table 1 also shows that the kidneys always have higher median concentrations of ^{210}Pb, ^{210}Po and stable lead than the livers. To calculate the average ratio of the concentrations of these metals in the kidneys to those in the livers we first found the ratio for each animal and then the median and 95% confidence limits of each data group (Table 2). The arithmetic means and the standard deviations of these ratios are also given. The U-test of Mann and Whitney was again applied to compare the values of the median ratios with the corresponding values of the control group. We found that the animals from the area near the lead mine showed significantly different (99·9% level, two-tailed test) median ratios only in the case of ^{210}Pb. No difference was observable (95% level, two-tailed test) in the case of ^{210}Po or stable lead.

Also shown in Table 2 are the median ratios of ^{210}Po:^{210}Pb in the livers and in the kidneys. Although the value of this ratio observed in the livers is

Table 1. *Concentrations of ^{210}Pb, ^{210}Po and stable lead and the specific activity of ^{210}Pb in the livers and kidneys of cattle from an area near an abandoned lead mine*

Organ	Values for cattle grazing near lead mine*					Median for control group
	Median	95% Confidence limits of median	Mean	Standard deviation	Range	
			^{210}Pb (pCi/kg wet tissue)			
Liver	12·9	11·4–14·0	15·62	11·52	4·0–67·5	18·0
Kidney	19·5	16·4–21·0	20·68	8·39	8·4–42	44·0
			^{210}Po (pCi/kg wet tissue)			
Liver	35·8	29·2–40·0	42·66	29·45	13·9–159	33·3
Kidney	152·4	135–169	162·7	65·9	57·8–387·8	178·5
			Pb (mg/kg wet tissue)			
Liver	0·58	0·4–1·2	1·34	1·51	0·2–6·2	0·12
Kidney	1·20	0·9–1·6	1·94	1·80	0·4–6·8	0·34
			Specific activity of ^{210}Pb (nCi/g Pb)			
Liver	20·0	12·8–28·0	25·5	18·75	0·8–69	161
Kidney	20·0	12·6–23·8	19·65	13·74	1·7–50	132

*The number of cattle in the group near the lead mine was 35.

Table 2. *Ratios of concentrations (kidney: liver) of ^{210}Pb, ^{210}Po and stable lead and ratios of ^{210}Pb: ^{210}Po in cattle from an area near an abandoned lead mine*

Metal or organ	Values of ratios for cattle grazing near lead mine*					Median ratio for control group
	Median	95% Confidence limits of median	Mean	Standard deviation	Range	
			Kidney: liver			
^{210}Pb	1·47	1·16–1·74	1·61	0·76	0·31–3·18	2·37
^{210}Po	4·15	3·71–5·10	4·54	1·93	0·65–10·2	5·05
Pb	2·04	1·56–2·65	2·11	1·03	0·27–4·00	2·89
			^{210}Po: ^{210}Pb			
Liver	2·50	2·13–3·11	3·29	2·37	1·14–12·1	2·18
Kidney	8·18	7·28–8·94	8·46	3·15	3·54–15·9	3·95

*The number of cattle in the group near the lead mine was 35.

higher than the corresponding value for the control group, application of the U-test showed that there was no statistically significant difference (95% level, two-tailed test) between the two values. In the kidneys, however, a highly significant difference (99·9% level, two-tailed test) in the ^{210}Po: ^{210}Pb ratio was found by comparison with the control group.

Correlations

In the case of the control group several statistically significant correlations between the concentrations of ^{210}Pb, ^{210}Po and stable lead in the livers and kidneys were observed by us. To find out whether similar correlations existed for the animals from the area near the lead mine, we applied the Spearman rank correlation coefficient (Sachs, 1974) rather than the linear correlation coefficient, because the data to be correlated were not normally distributed. The resulting rank correlation coefficients are summarized in Table 3 for the animals from the area near the lead mine. The coefficients of the control group are also given to allow comparison. In the last two columns of Table 3 the significance levels of the observed rank correlation coefficients are given. Where the significance of the rank correlation coefficient is below 90%, a correlation could not be established.

Table 3 shows that strong correlations between kidney and liver values were observed for ^{210}Pb, ^{210}Po and for stable lead. Similar correlations were observed for the control group, except that no correlation could be established there in the case of ^{210}Pb.

A highly significant correlation was found between ^{210}Po and ^{210}Pb levels in kidneys; a similar correlation had been noted in the control group. However, the correlation between ^{210}Po and ^{210}Pb levels in the livers was very weak and not observed in the control group. No correlation could be established between the levels of ^{210}Pb and stable lead in kidneys, although a highly significant correlation was found in this case for the control group. In the livers the correlation between ^{210}Pb and stable lead was fairly weak, both for the control group and for the group from the lead-mining area. This is illustrated in Fig. 3, where the experimentally obtained concentrations of ^{210}Pb in livers and kidneys are plotted against the corresponding concentrations of stable lead in these organs. The equal frequency ellipses (Sokal & Rohl, 1969), enclosing 95% of the observations in the control group of animals are also shown. It is clear that the animals from the lead-mining area differ from the control group essentially in their much higher concentrations of stable lead in both livers and kidneys and in their

Table 3. *Spearman rank correlation coefficient and significance levels of correlations between ^{210}Pb, ^{210}Po and stable lead in the livers and kidneys of cattle from an area near an abandoned lead mine and for a control group*

Test-system	Spearman rank correlation coefficient		Significance level of correlation (%)	
	Group near lead mine	Control group	Group near lead mine	Control group
^{210}Pb in kidney/^{210}Pb in liver	0·389	−0·039	95	<90
^{210}Po in kidney/^{210}Po in liver	0·387	0·808	95	99·9
Pb in kidney/Pb in liver	0·751	0·497	99·9	99
^{210}Po in kidney/^{210}Pb in kidney	0·605	0·417	99·9	99
^{210}Po in liver/^{210}Pb in liver	0·271	0·0748	90	<90
^{210}Pb in kidney/Pb in kidney	−0·134	0·678	<90	99·9
^{210}Pb in liver/Pb in liver	0·383	0·244	95	90
^{210}Po in kidney/Pb in kidney	0·0254	0·359	<90	95
^{210}Po in liver/Pb in liver	0·0366	0·402	<90	95

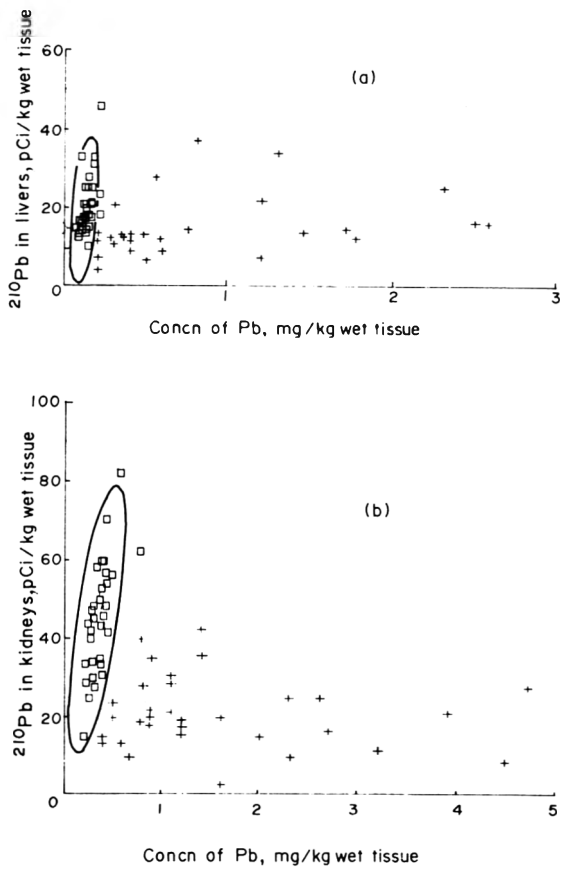

Fig. 3. Concentrations of ^{210}Pb and stable lead in (a) livers and (b) kidneys of animals from an area near an abandoned lead mine (+), and of a control group (□) from an area that seems to be unaffected by traffic or industrial pollution. The equal frequency ellipses, enclosing 95% of the observations are shown for the control group. (Lead levels above 3 and 5 mg Pb/kg wet tissue for the liver and kidney, respectively, were excluded.)

somewhat smaller ^{210}Pb concentrations in these organs. In the case of ^{210}Po and stable lead in both kidneys and livers, no correlation was observed for the animals from the area near the lead mine, in contrast to the control group, where a weak correlation was established.

Because most of the animals were about 2 yr old, no attempt was made to correlate the concentrations of ^{210}Pb, ^{210}Po or stable lead in the organs with the age of the cattle.

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THE PROTECTIVE POTENCY OF MARINE ANIMAL MEAT AGAINST THE NEUROTOXICITY OF METHYLMERCURY: ITS RELATIONSHIP WITH THE ORGAN DISTRIBUTION OF MERCURY AND SELENIUM IN THE RAT

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Abstract—The meats of two marine animals, that were known to contain high levels of both methylmercury and selenium, were compared with respect to their protective potencies against the neurotoxicity of methylmercury. The organ distributions of selenium and mercury in rats given diets containing these meats was also compared. Weanling rats were fed for 12 wk on a diet containing 17·5 ppm methylmercuric chloride and one of two levels of selenium (0·3 or 0·6 ppm) originating from the meat of sebastes (*Sebastes iracundus*) or of the sperm whale (*Physeter catodon*), or from sodium selenite. The protection conferred by sebastes meat was roughly equal to that of the selenite regarding growth rate but was considerably greater with respect to the neurological signs. By both criteria, sperm-whale meat was less effective than sebastes meat and selenite in providing protection. The organ distribution pattern of selenium and mercury showed that selenium in the blood, brain and spinal cord was positively correlated with neurological protection, while total mercury and methylmercury in those organs were negatively correlated with neurological protection. The level of protection (delay of the neurological manifestations by nearly 7 wk) corresponding with the increase in selenium levels in the nervous system (from about 1/50 to 1/10 of the methylmercury levels on a molar basis) indicated that the protective mechanism was not simply direct conjugation of selenium with methylmercury. It has previously been noted that tuna fish containing high levels of selenium provide protection against methylmercury toxicity and it may be that all marine animals rich in both selenium and methylmercury afford this protection.

INTRODUCTION

Fish traditionally constitutes a primary source of animal protein in an average Japanese diet. Recently an increasing amount and variety of deep sea fish has been consumed and many of them are known to contain high levels of methylmercury (Kamimura, 1975; Nishigaki, Tamura, Maki, Yamada, Shimamura, Ochiai & Kimura, 1974). Although there is some evidence indicating that fish with high levels of methylmercury also contain high levels of selenium (Itano, Sasaki, Okamura & Tatsukawa, 1977; Kari & Kauranen, 1978; Nakagawasai, Yamanaka & Kikuchi, 1976; Nishigaki *et al.* 1974), and that selenium in tuna and marlin can modify the toxicity of methylmercury (Friedman, Eaton & Carter, 1978; Ganther, Goudie, Sunde, Kopecky, Wagner, Sang-Hwang Oh & Hoekestra, 1972; Nishigaki, Tamura, Maki, Shimamura, Ohi, Seki, Minowa, Mizoguchi & Yagyu, 1977; Ohi, Nishigaki, Seki, Tamura, Maki, Konno, Ochiai, Yamada, Shimamura, Mizoguchi & Yagyu, 1976) there have been no reports of the protective potency of selenium in the meat of deep sea fish or higher marine animals known to contain high levels of both methylmercury and selenium.

The first objective of our study was to examine the potencies of the meat of a deep sea fish, the sebastes (*Sebastes iracundus*) and a higher marine mammal, the sperm whale (*Physeter catodon*) both of which are known to contain high levels of methylmercury and selenium in terms of protection against methylmercury toxicity. Our second objective was to

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Table 1. *Methylmercury and selenium and added animal protein concentrations in experimental diets*

Group	Selenium (ppm)	Form of selenium	Added protein (% of diet)		
			Casein	Sebastes	Sperm whale
IA	0·3	In sebastes		15	
IB	0·6	In sebastes		30	
IIA	0·3	In sperm whale			15
IIB	0·6	In sperm whale			30
IIIA	0·3	Sodium selenite	15		
IIIB	0·6	Sodium selenite	30		
IVA	—*		15		
IVB	—*		30		

All the diets contained 17·5 ppm methylmercury chloride.
*The concentration of selenium in the basal diet was 0·1 ppm.

examine the organ distribution pattern of mercury and selenium in relation to the protective effect against neurotoxicity expressed as delay in early (tail rotation) and late (crossing and/or paralysis of hind legs) neurological signs.

EXPERIMENTAL

Materials and methods. Male weanling Wistar rats weighing 56 ± 5 g were given one of the diets detailed below and water *ad lib.* for 12 wk. Each group comprised 20 animals; they were weighed twice a week and observed daily for deaths and signs of morbidity, such as roughened hair, or neurological signs expressed as tail rotation, paralysis and/or crossing of hind legs. The 'tail rotation' (TR) is a vigorous, sustained circular movement of the tail which an animal with methylmercury poisoning displays when grasped around the trunk and can be demonstrated about 2 wk earlier than the paralysis or crossing of hind legs (CP) in this experimental design, and hence was used as an early neurological sign of methylmercury poisoning. A detailed description of this neurological sign has been reported elsewhere (Ohi, Nishigaki, Seki, Tamura, Mizoguchi, Yagyu & Nagashima, 1978).

Preparation of diets. The composition and the selenium concentrations of the ingredients in the basal diet have been described elsewhere (Ohi *et al.* 1976). The composition of the experimental diets is given in Table 1. Selenium levels of lyophilized samples of sebastes and sperm whale ranged from 1·5 to 2·1 (1·9 ± 0·2) and 1·7 to 2·2 (2·0 ± 0·2) ppm respectively; both the sebastes and the sperm whale meat were adjusted to give concentrations of 2·0 ppm selenium before diet preparation. The increased levels of protein (sebastes, sperm whale or casein) in the diets were made at the expense of corn which contained very low levels of naturally-occurring selenium (0·05 ppm). The levels of selenium in Group IV diets when no selenite was added were demonstrated to be 0·1 ± 0·01 ppm. Methylmercuric chloride was dissolved in acetone and mixed with the diets before pelleting.

Analytical method. On day 70 the animals were killed, and the brain, spinal cord, liver, kidney and blood were frozen soon after dissection and stored at −25°C until the time of analysis for total mercury, methylmercury and selenium. Analytical conditions of selenium and mercury have been described elsewhere (Ohi, Nishigaki, Seki, Tamura, Maki, Maeda, Ochiai, Yamada, Shimamura & Yagyu, 1975); but briefly, extraction of methylmercury followed essentially the

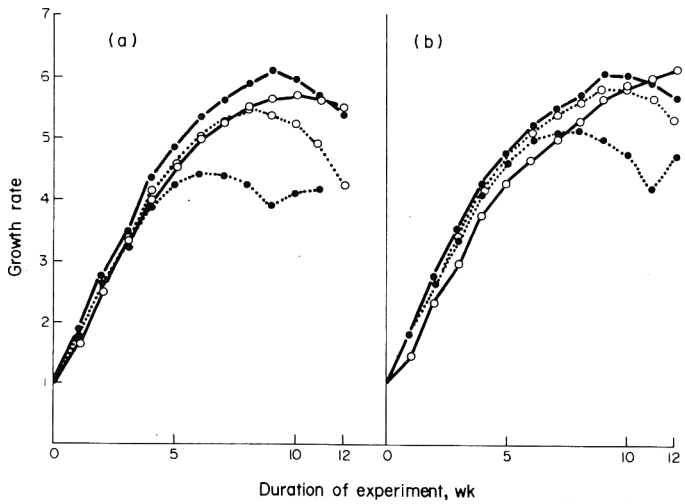

Fig. 1. Growth rates of (a) 15% animal protein groups (IA, —○—; IIA, ···○···; IIIA, —●—; IVA, ···●···) and (b) 30% animal protein groups (IB, —●—; IIB, ···○···; IIIB, —●—; IVB ···●···) on the right. Note IVA were extinct before the wk 12. The dips in IVA and B were due to the elimination of animals for chemical analyses. (The units of growth rate are multiples of the initial weight.)

method described by Westoo (1968); gas chromatography with electron capture detection was used for analysis. Total mercury was analysed by flameless atomic absorption spectrophotometry of vaporized mercury after digesting the specimen in sulphuric nitric acid with vanadium pentoxide (Dietz, Sell & Bristol, 1973). For selenium determination the specimen was digested in nitric perchloric acid and the selenium diaminonaphthalene complex was measured by fluorimetry (Watkinson, 1966).

RESULTS

The protective potency of sebastes and sperm whale against methylmercury toxicity

Growth rate. Figure 1 shows the growth rate of each group; methylmercury toxicity was manifested as growth suppression, weight loss and shorter survival period (IVA). Each of sebastes, sperm whale and selenite improved growth rate and delayed the onset of weight loss but sperm whale appeared less effective than the others at the dietary meat level of 15%. The protective effect of dietary casein with an increase of from 15 to 30% was manifested as the difference in growth rate and survival between IVA and IVB.

Neurological manifestations. Figures 2 and 3 demonstrate the cumulative incidence of the early (TR) and late (CP) neurological signs as S-shaped curves, with TR becoming evident for 2–3 wk prior to the onset of CP. The protective potency of a diet was represented by the shift of a curve towards the right in relation to IVA, the first group to display each of the neurological signs, and could be expressed in terms of

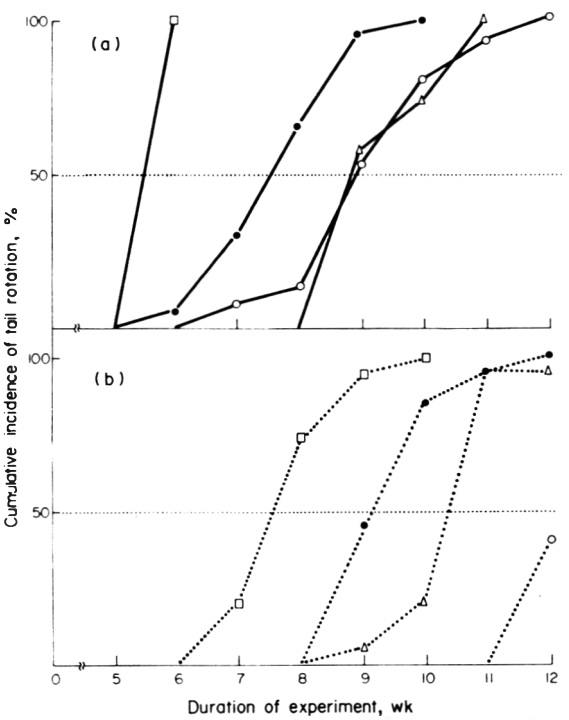

Fig. 2. Cumulative incidence of tail rotation for (a) 15% animal protein groups (IA, —○—; IIA, —●—; IIIA, —△—; IVA, —□—) and (b) 30% animal protein groups (IB, ···○···; IIB, ···●···; IIIB, ···△···; IVB, ···□···).

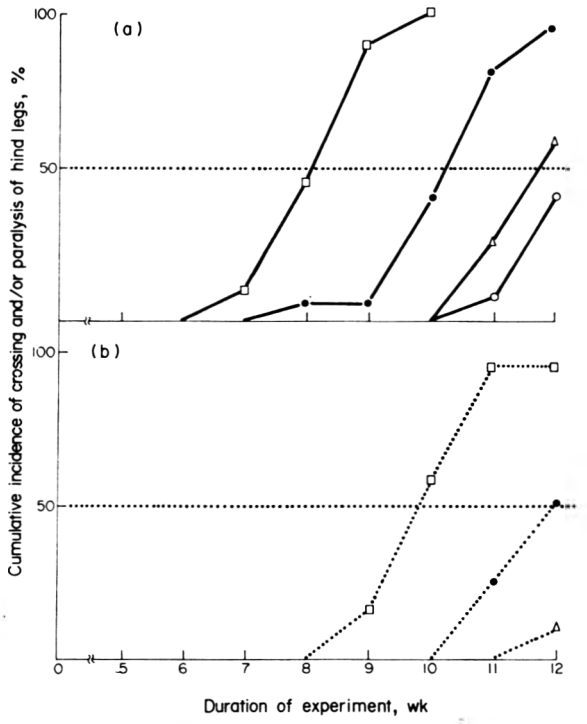

Fig. 3. Cumulative incidence of crossing and/or paralysis of hind legs for (a) 15% animal protein groups (IA, —○—; IIA, —●—; IIIA, —△—; IVA, —□—) and (b) 30% animal protein groups (IIB, ···●···; IIIB, ···△···; IVB, ···□···).

number of weeks delay in reaching a 50% cumulative incidence (Table 2). In the groups with 0·3 ppm dietary selenium, the protective effect of selenite selenium was roughly equivalent to that of sebastes. However, when the selenium level was increased to 0·6 ppm, sebastes appeared more protective. The protection afforded by the sperm whale meat was less than that provided by sebastes or selenite at both selenium levels.

It is interesting that protective potency appears to vary depending on the index chosen and the source of selenium. In a preceding study we observed protection conferred by tuna being equal to that by selenite with respect to growth rate but about one half that of selenite regarding neurotoxicity (Ohi *et al.* 1976), while in this study sebastes appeared considerably more potent than selenite in delaying neurotoxic signs but of roughly equal potency with respect to growth rate.

The relationship between the organ distribution of mercury and selenium and protective effects

Table 2 summarizes the results of analyses of selenium, total mercury and methylmercury in the brain, spinal cord, blood, liver and kidney and protective effects expressed as delay in 50% cumulative incidence of TR and CP (TR50, CP50). The concentration of selenium in the brain and spinal cord ranged roughly from 1/50 to 1/10 of that of mercury on a molar basis while the corresponding increase in protection was as great as 6·8 weeks' delay in the TR50 (IB). Increases in the selenium content in the brain,

Table 2. *Organ distribution of selenium, total mercury and methylmercury, molar ratio of selenium and total mercury and methylmercury and protective effects expressed as delay of 50% incidence of neurological signs*

		Concentration (ppm) or ratio of substance(s) in column 2						
Group	Material(s)	Brain	Spinal cord	Blood	Kidney	Liver	TR50* (wk)	CP50* (wk)
IA	Hg	59·5 ± 13·2	27·7 ± 2·9	722 ± 109	284 ± 45	143 ± 21		
	MeHg	51·9 ± 10	24·6 ± 3·5	551 ± 136	195 ± 18	136 ± 22		
	Se	1·23 ± 0·07	0·78 ± 0·04	2·9 ± 0·2	27·8 ± 4·4	5·9 ± 0·8	3·5	4·2
	Se:Hg	0·057	0·078	0·011				
	Se:MeHg	0·065	0·087	0·015				
IB	Hg	49·3 ± 5·8	22·3 ± 4·4	585 ± 71	335 ± 11	130 ± 28		
	MeHg	40·2 ± 3·9	18·9 ± 4·7	493 ± 66	266 ± 9	121 ± 24		
	Se	1·41 ± 0·09	0·75 ± 0·03	3·2 ± 0·1	27·0 ± 3·7	5·5 ± 0·2	6·8*	–†
	Se:Hg	0·078	0·086	0·015				
	Se:MeHg	0·096	0·108	0·018				
IIA	Hg	69·3 ± 6·7	33·0 ± 12·3	972 ± 96	298 ± 34	221 ± 51		
	MeHg	54·2 ± 6·6	28·6 ± 11·1	779 ± 44	211 ± 38	213 ± 53		
	Se	0·76 ± 0·11	0·52 ± 0·03	1·9 ± 0·2	24·8 ± 2·6	7·9 ± 1·3	2·0	2·2
	Se:Hg	0·031	0·045	0·005				
	Se:MeHg	0·038	0·050	0·007				
IIB	Hg	42·8 ± 5·9	18·3 ± 3·3	660 ± 87	306 ± 48	139 ± 22		
	MeHg	35·3 ± 3·9	15·4 ± 2·3	505 ± 79	219 ± 40	135 ± 18		
	Se	0·88 ± 0·03	0·57 ± 0·05	2·4 ± 0·1	22·2 ± 4·2	5·8 ± 0·7	3·7	4·0
	Se:Hg	0·055	0·082	0·010				
	Se:MeHg	0·068	0·101	0·013				
IIIA	Hg	46·7 ± 6·0	24·3 ± 2·9	679 ± 88	300 ± 38	151 ± 24		
	MeHg	39·6 ± 5·2	13·3 ± 2·8	662 ± 88	205 ± 21	141 ± 26		
	Se	0·62 ± 0·07	0·51 ± 0·08	1·9 ± 0·08	29·4 ± 2·3	7·0 ± 1·3	3·3	3·7
	Se:Hg	0·037	0·051	0·008				
	Se:MeHg	0·043	0·105	0·008				
IIIB	Hg	45·7 ± 8·4	24·2 ± 4·9	699 ± 136	301 ± 52	164 ± 23		
	MeHg	38·6 ± 2·9	14·8 ± 3·2	626 ± 138	197 ± 43	141 ± 31		
	Se	0·77 ± 0·09	0·46 ± 0·02	2·1 ± 0·2	34·4 ± 8·2	6·2 ± 0·5	4·8	–†
	Se:Hg	0·047	0·052	0·008				
	Se:MeHg	0·054	0·085	0·009				
IVA	Hg	71·5 ± 4·3	35·9 ± 8·6	1170 ± 68	302 ± 51	263 ± 53		
	MeHg	58·6 ± 2·3	24·3 ± 5·8	767 ± 26	209 ± 39	246 ± 51		
	Se	0·46 ± 0·04	0·35 ± 0·03	1·13 ± 0·10	11·4 ± 2·0	4·4 ± 0·95	0	0
	Se:Hg	0·018	0·023	0·003				
	Se:MeHg	0·018	0·039	0·004				
IVB	Hg	67·4 ± 3·8	33·1 ± 2·7	1164 ± 83	289 ± 29	280 ± 47		
	MeHg	55·2 ± 5·0	23·8 ± 3·0	767 ± 26	218 ± 29	239 ± 42		
	Se	0·56 ± 0·07	0·44 ± 0·07	1·40 ± 0·40	20·2 ± 3·8	5·1 ± 0·6	2·0	1·7
	Se:Hg	0·022	0·038	0·003				
	Se:MeHg	0·028	0·051	0·005				

TR50 = Delay in onset of 50% cumulative incidence of tail rotation
CP50 = Delay in onset of 50% cumulative incidence of paralysis or crossing of hind legs
Hg = Total mercury MeHg = Methylmercury
Se:Hg = Ratio of selenium to total mercury
Se:MeHg = Ratio of selenium to methylmercury

* Extrapolated from Figs 2 & 3.
† CP50 not available nor could it be extrapolated at the end of the experimental period.

spinal cord and blood appeared to accompany better neurological protection.

Table 3 shows the relationship between the protective effect expressed as the delay in the neurological signs and selenium, and total mercury and methylmercury levels in the brain, spinal cord and blood. In the brain and spinal cord the selenium level is positively correlated with the neurological protection while mercury levels show a tendency to be negatively correlated to the protective effects. The blood concentrations of these substances had the closest correlations to the neurological protections. The selenium levels relative to those of mercury in these organs were more highly correlated with the protective effect than the absolute levels.

DISCUSSION

Our observation in this study regarding protection by sebastes and sperm whale against methylmercury toxicity is consistent with the observations made on tuna (Ganther *et al.* 1972; Ganther & Sunde, 1974; Ohi *et al.* 1975). Although there may be multiple protective factors in these marine animals, one feature common to them is an elevated level of selenium. This suggests that selenium, present usually in excess of molar levels of naturally-occurring methylmercury in pelagic fish (Itano *et al.* 1977; Kari & Kauranen, 1978; Nakagawasai *et al.* 1976; Nishigaki *et al.* 1974), is generally capable of modifying methylmercury toxicity. This might also give a clue to explaining the historical fact that there has been no known incidence of methylmercury poisoning among the great consumers of fish in Japan, e.g. tuna wholesalers and fishermen, and crew members of tuna-fishing boats. This is, of course, true only when the fish are not polluted artificially.

There seem to be at least two possible explanations of the difference in protective potency between sebastes and sperm whale against the neurotoxicity of methylmercury. Firstly, the availability and/or potency of the selenium occurring in sebastes is greater than that in the sperm whale; secondly, factor(s) other than selenium provide additive (or synergistic) protection. The former is suggested by the observation that different selenium compounds have different protective potencies against mercurial toxicity (Alexander & Norseth, 1979; Fang, 1977; Komiya, Otaka, Kawauchi & Sakurai, 1978) and by our present finding that increased levels of selenium in the nervous system were generally associated with better protection. It is conceivable that the selenium compounds in sebastes and sperm whale have different chemical structures and thus differ in their potency and/or availability at the critical site. The second explanation is based on evidence of naturally-occurring modifying agents of mercury toxicity in the biosphere such as vitamin E, tellurium, arsenic and zinc (Chang, Gilbert & Sprecher, 1977 & 1978; El-Begearmi, Ganther & Sunde, 1974; Groth, Stettler & MacKay, 1976; Imagawa, Fukino & Yamane, 1976; Welsh & Soares, 1976). Analyses for these factors were not done in this study with the exception of arsenic which is known to be present in marine animals at relatively high concentrations. Our finding that sebastes contained more arsenic (5·05 ppm) than the sperm whale (1·33 ppm) is in accordance with the observation that arsenic was protective synergistically with selenium against mercurial toxicity (El-Bergearmi *et al.* 1974).

The protective mechanism(s) of selenium against methylmercury neurotoxicity is unclear in terms of the accumulation of these elements in the nervous system. On the one hand selenium appeared to increase the accumulation of methylmercury in the brain (Alexander & Norseth, 1979; Chen, Lacy & Whanger, 1975; Magos & Webb, 1977; Ohi *et al.* 1975; Stillings, Logally, Bauerfeld & Soares, 1974; Stoewsand, Bache & Lisk, 1974) and on the other to facilitate the demethylation (and elimination) of mercury (Fukino, Aida & Yamane, 1976; Iwate, Okamoto & Ohsawa, 1973; Ohi *et al.* 1976). These seemingly contradictory observations were probably due to differences in the timing of analyses and the mode of administration; Iwata *et al.* (1973) and Fukino *et al.* (1976) observed an initial increase in methylmercury uptake by the brain followed by subsequent enhancement of elimination of mercury. Our finding in this study that the neurological protection was generally accompanied by an increase in the levels of selenium and a decrease in those of mercury in the brain and spinal cord was in accordance with the latter observations.

On the other hand methylmercury has been noted to increase the concentrations of selenium in the nervous system of experimental animals (Fang, 1977; Johnson & Pond, 1974; Magos & Webb, 1977; Ohi *et al.* 1975 & 1976). Furthermore a recent study by means of X-ray microanalysis revealed accumulation of 'selenium bound to mercury' in the brains of patients with both acute and chronic Minamata disease (Shirabe, Eto & Takeuchi, 1980). Thus, a part of the protective action of selenium against the neurotoxicity of methylmercury seems to be attributable to the capacity of selenium to form a biologically inactive conjugate with mercury on a one molar basis (Groth *et al.* 1976; Koeman, Peeters, Koudstaal-Hol, Tjioe & de Goeij, 1973; Kosta, Byrne & Zelenko, 1974). However, the magnitude of protection noted in our study is far greater than could be explained on the basis of selenium conjugating directly with methylmercury, the increase in the molar ratios of selenium to methylmercury in the brain (disregarding possible contributions of other factors) from roughly 1/50 to 1/10 corresponded to a delay in the TR50 of nearly 7 wk (Table 2). Assuming that methylmercury shows considerable neurotoxicity in its unmodified form, its toxic effects would hardly be reduced by the inactivation of a mere 2–10% through conjugation with selenium. Thus, from a quantitative angle this observation seems inexplicable, unless we postulate either the mediation of the protective qualities of selenium by another highly efficient protective mechanism or the neurotoxicity of methylmercury being primarily attributable to demethylated mercury rather than methylmercury *per se*. The observation that intracranial administration of mercuric chloride in the rat produced neurological signs similar to those induced by methylmercury seems to suggest the latter possibility (Venable & Mills, 1977) and warrants further investigation.

Table 3. *Correlation between neurological protection (TR50, CP50) and levels of selenium, total and methylmercury in the brain, spinal cord and blood, selenium-mercury ratio (on a molar basis)*

Statistical parameters	Total mercury in			Methylmercury in			Selenium in			Ratio of selenium to total mercury in			Ratio of selenium to methylmercury in		
	Brain	Spinal cord	Blood	Brain	Spinal cord	Blood	Brain	Spinal cord	Blood	Brain	Spinal cord	Blood	Brain	Spinal cord	Blood
Correlation with TR50															
r	−0·76	−0·79	−0·86	−0·76	−0·53	−0·81	0·77	0·69	0·85	0·91	0·80	0·89	0·91	0·83	0·84
P	<0·05	<0·02	<0·01	<0·05	>0·1	<0·02	<0·05	<0·1	<0·01	<0·01	<0·02	<0·01	<0·01	≤0·01	<0·01
Correlation with CP50															
r	−0·80	−0·84	−0·93	−0·75	−0·51	−0·83	0·76	0·83	0·90	0·92	0.92	0·92	0·90	0·90	0·87
P	<0·1	<0·05	<0·01	<0·1	>0·1	<0·05	<0·1	<0·05	<0·05	<0·01	<0.01	<0·01	<0·02	<0·02	<0·02

TR50 = Delay in onset of 50% cumulative incidence of tail rotation CP50 = Delay in onset of 50% cumulative incidence of paralysis or crossing of hind legs
r = coefficient of correlation *P* = probability

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NITROSAMINES IN NEW MOTOR-CARS

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Abstract—Volatile nitrosamines, *N*-nitrosodimethylamine (NDMA), *N*-nitrosomorpholine (NMOR), *N*-nitrosodiethylamine (NDEA) and possibly *N*-nitrosodibutylamine (NDBA), have been found as airborne contaminants inside new (1979 model) motor-cars. NDMA was found in the front passenger area of 37 of the 38 motor-cars at levels ranging from 0·07 to 0·83 $\mu g/m^3$ (mean 0·29 $\mu g/m^3$), NMOR was present at levels between a trace and 2·5 $\mu g/m^3$ (mean 0·65 $\mu g/m^3$) in 16 vehicles and 0·04–0·39 μg NDEA/m^3 (mean 0·11 $\mu g/m^3$) was detected in 17 of the cars. Traces of NDBA (<0·01 $\mu g/m^3$) were also tentatively identified in three of the vehicles. The exposure of man to volatile *N*-nitrosamines from motor-cars is estimated to be similar to that from bacon and beer.

INTRODUCTION

Volatile *N*-nitrosamines have been found as contaminants associated with the production of tyres and rubber products (Fajen, Carson, Rounbehler, Fan, Vita, Goff, Edwards, Fine, Reinhold & Biemann, 1979), of tanned leather (Rounbehler, Krull, Goff, Mills, Morrison, Edwards, Fine, Fajen, Carson & Reinhold, 1979a) and in the atmosphere of a warehouse containing fabrics for motor-cars and furniture (Fine, 1979a). Therefore it is possible that *N*-nitrosamines may be encountered in the air when the finished products are used in confined areas. In order to test this hypothesis we monitored volatile *N*-nitrosamine levels in the interiors of new motor-cars.

In sampling air for *N*-nitrosamines, great care must be taken to avoid false analytical results due to either nitrosamine formation from airborne precursors *in situ*, or the inability of the sorbent to retain the *N*-nitrosamines quantitatively. The trapping techniques that have been used in the past, including activated charcoal (Bretschneider & Matz, 1974), cryogenic trapping (Fine, Rounbehler, Belcher & Epstein, 1976), Tenax-GC (Pellizzari, Bunch, Bursey, Berkley, Sawicki & Krost, 1976) and dilute alkali (Fisher, Reiser & Lasoski, 1976; Rounbehler *et al.* 1979a) have all been shown to have limitations (Rounbehler, Reisch, Coombs & Fine, 1980; Rounbehler, Reisch & Fine, 1979b). We therefore used the ThermoSorb/N sampling system, which has been demonstrated both to be free of *in situ* nitrosamine formation and to be capable of retaining 100% of any pre-loaded nitrosamines (Rounbehler *et al.* 1979b & 1980).

EXPERIMENTAL

Chemicals. All the solvents were glass-distilled and were obtained from Burdick & Jackson (Muskegon, MI). KOH (ACS grade pellets) was purchased from Fisher Scientific Co. (Medford, MA). All the materials used for gas chromatography (GC) were purchased from Supelco (Bellefonte, PA). Nitrosamine standards were supplied by Thermo Electron Corp. (Waltham, MA).

Apparatus. Prototype ThermoSorb/N cartridges (Thermo Electron Corp.) were tested when they were received. The cartridges were constructed of medical-grade polyethylene with 100-mesh stainless-steel screens at the inlet and outlet and had standard Luer fittings to facilitate solvent elution of the trapped materials. The sorbent bed (20 mm deep and 15 mm ID) contained a mixture of magnesium silicate and an amine trapping (complexing) agent and nitrosation inhibitor. Aqueous alkali traps were constructed from 250 mm long and 38 mm ID glass vacuum traps (Sargent Welch, Skokie, IL) and contained 45 ml 1 N-KOH. Air was drawn through the ThermoSorb/N samplers by individual, battery-powered air pumps (Model C115, Bendix Corp. Rochester, NY). A mass flow meter (Hastings Model ALL 10K, Toledyne, Hampton, VA) was used to calibrate the rotometers on the air pumps before and after each test.

GC analyses were carried out using an isothermal GC (Model 661, Thermo Electron Corp.), interfaced to a Thermal Energy Analyser (TEA; Model 502LC, Thermo Electron Corp.). The GC column was a 500 cm long and 0·32 cm OD stainless-steel tube, packed with 5% Carbowax 20M and 0·5% KOH on Chromosorb W 100/120 (Analabs, New Haven, CT). The GC was operated isothermally at 160°C for *N*-nitrosodimethylamine (NDMA) and at 210°C for *N*-nitrosomorpholine (NMOR), with a helium carrier gas flow of 35 ml/min. The TEA oven temperature was 500°C.

The high-performance liquid chromatograph (HPLC) was constructed by combining a high-pressure pump (Model 110, Altex Corp., Berkeley, CA) with an injector (Model 7120, Rheodyne, Berkeley, CA), a stainless-steel column (4 × 39 mm) packed with 10 μm Lichrosorb Si60 (E. Merck Labs, Elmsford, NY) and a TEA-detector (Model TEA-502LC, Thermo Electron Corp.). The HPLC–TEA was operated isocratically using 2 ml/min of *n*-hexane–acetone (95:5, v/v).

Procedure. With the exception of vehicle J28, all the cars were new and were tested on the premises of

new-car dealers in the greater Boston area. The air flow rate of the sampling pumps was usually set at 2–3 litres/min, and the sample size varied from 151 to 798 litres (Table 1). In ten of the 38 cars tested, air samples were collected simultaneously in both the front and rear seats; in the remaining cars, samples were collected only in the front seat. Control samples of the ambient air were also taken at each location. The samples were collected and analysed on the same day.

The cartridges were eluted with methanol (usually 1·25 ml) and a 10-μl aliquot of the eluate was introduced onto the GC–TEA and/or HPLC–TEA (Rounbehler *et al.* 1979b,c). The KOH from the alkali traps was extracted with 3 × 15 ml dichloromethane (DCM), dried over sodium sulphate and then concentrated on a Kuderna-Danish evaporator (Kontes, Vineland, NJ) at 52°C to a volume of 1 ml with 0·5 ml of isooctane added as a keeper. The concentrate was then analysed by GC–TEA and HPLC–TEA.

In vehicle C19, six samples were taken simultaneously, five with ThermoSorb/N cartridges and one in an impinger containing 1 N-KOH. The total volume of air sampled was 4357 litres. After analysis the individual samples were combined and then added to 90 ml water in a separation funnel. Nonpolar compounds were extracted with 2 × 15 ml pentane and discarded. The aqueous layer was then extracted with 5 × 15 ml DCM, dried over sodium sulphate and concentrated to a final volume of 1·2 ml on a Kuderna-Danish evaporator at 53 C. The final concentrate was quantitatively analysed by GC–TEA, HPLC–TEA and GC–high-resolution mass spectrometry (HRMS) with peak matching on the parent ion.

Vehicle J28, a 1-month-old estate car made in the USA, was tested more extensively. Air samples were taken under the bonnet near the engine (with the engine running), in and around the spare-tyre well, under the dashboard, with the heater on and off, and in the rear seat with the engine on and off. A single air sample of 2900 litres was taken in the spare-tyre well; this sample was quantitatively analysed by GC–TEA, HPLC–TEA and GC–HRMS with peak matching on the parent ion, and by GC–low-resolution MS.

High-resolution GC–MS peak matching (Gough, Webb, Pringuer & Wood, 1977) was carried out by scanning at the exact mass of NDMA ($C_2H_6N_2O$-74·0480; resolution 7000) and NMOR ($C_4H_8N_2O_2$-116·0585; resolution 7000). Detection of the exact mass fitting the composition for the molecular ion of NDMA or NMOR, combined with coincident GC-retention time, were used to confirm the presence of these two nitrosamines.

GC–low-resolution MS was carried out using a modified Hitachi RMU6 instrument (Perkin Elmer, Norwalk, CT) and the data were processed on an IBM 1800 computer. Samples were monitored by repetitive scanning of the mass spectrometer to obtain mass spectra of each GC eluate, or when lower levels of material were anticipated, the instrument was set to resolve the mass equal to the molecular ion of interest (single ion monitoring).

RESULTS

Table 1 lists the 38 motor-cars that were tested, in order of their total nitrosamine content (NDMA + NMOR + NDEA). *N*-Nitrosamines were detected in none of the control samples of ambient air. The identity of NDMA, NMOR and NDEA was determined by coincident retention time on GC–TEA, and, for 16 of the samples, by parallel HPLC–TEA analyses (Fine, 1979b). For those samples analysed by both GC–TEA and HPLC–TEA, quantitation was within the range of experimental error (±10%). Although both GC–TEA and HPLC–TEA gave a chromatographic peak at the correct retention time for NDMA, the quantitation of this nitrosamine by HPLC was always larger than by GC. This may indicate the presence of a co-eluting, interfering compound in HPLC–TEA. Therefore, because the parallel GC–TEA and HPLC–TEA procedures were not quantitative, and in the absence of mass-spectral confirmation, the identification of NDBA remains tentative. The identification and quantitation of NDMA and NMOR in the samples from two vehicles (C19 and J28) were also confirmed by GC–HRMS with peak matching. In addition, an air sample taken from the spare-tyre well of J28 contained enough NMOR to obtain a complete spectrum by GC–low-resolution MS. The same levels of NDMA and NMOR were detected in the air sample taken from C19 using the ThermoSorb/N cartridge as in that collected using the impinger containing KOH.

NDMA was found in 37 out of the 38 vehicles at levels up to 0·83 $\mu g/m^3$. NMOR was present in only 16 vehicles, but the level in one car was as high as 2·5 $\mu g/m^3$. NDEA was found at lower levels in 17 vehicles. When similar cars with different interiors were tested (three of model C19 and two each of A21, B4, C6, E8 and G13) the variation was as large as the variation from model to model. In the ten cars in which air samples were taken simultaneously in the front and rear seats, the NDMA levels in the front were always lower than in the rear (Table 2). In contrast, no significant trend was evident in NMOR levels in the front and rear seats.

The results of the more thorough testing of one vehicle (Model J28) are shown in Table 3. The NMOR level in the spare-tyre well was almost 70 times greater than the NMOR level on the rear seat when the engine was not running. When the spare tyre was removed, the NMOR level dropped from 33 to 17 $\mu g/m^3$ and the NDMA level from 5·2 to 1·6 $\mu g/m^3$. The spare tyre and an air-sampling pump were placed in a plastic bag, and the air inside the bag was immediately sampled using a ThermoSorb/N cartridge. The equivalent of 30 μg NMOR/m^3 and 7·9 μg NDMA/m^3 were found in this air. Therefore it appears that in this car, the spare tyre was the major source of NMOR. It should be noted, however, that several cars that did not have the spare tyre inside the passenger compartment had higher levels of NMOR than did the vehicle of model J28. This indicates that there must be other sources of NMOR.

NMOR and NDMA levels in 2-hr air samples taken in the rear seat of model J28 were about four and ten times lower, respectively, when the engine was running than when it was not. Presumably this was because the car was equipped with a positive air-venting system. This result implies that in some recent models of car, human exposure to nitrosamines is probably limited to the first few minutes of each trip.

Table 1. *Atmospheric N-nitrosamine levels in the front passenger areas of new cars*

Vehicle of		Country of manufacture	Size or class	Interior finish	Volume of air sampled (litres)	NDMA* ($\mu g/m^3$)	NMOR* ($\mu g/m^3$)	NDEA* ($\mu g/m^3$)
Company	Model							
C	19	USA	Luxury	Red cloth	156	0·41†	2·5†	
C	5	USA	Luxury	Red vinyl	312	0·49	1·7	0·18
C	19	USA	Luxury	Red cloth	164	0·29†‡	1·9†‡	
C	6	USA	Mid-size estate	Green vinyl	356	0·61	1·2	0·16
D	7	Germany	Luxury	Blue leather	798	0·83		0·14
G	13	Japan	Economy	Plaid cloth	275	0·33†	0·52†	
D	12	Germany	Luxury estate	White leather	528	0·77		0·06
D	7	Germany	Luxury	Bamboo leather	554	0·43		0·39
A	1	USA	Luxury	Blue vinyl	217	0·30†	0·31†	0·09†§
A	2	USA	Luxury	Red vinyl	252	0·30†	0·25†	0·08†§
A	21	USA	Luxury	Grey leather	202	0·45†	0·03†	0·12†§
J	28	USA	Luxury estate	Tan cloth seats	332	0·24†‡	0·35†‡	
A	21	USA	Luxury	Blue leather	235	0·30	0·20†	0·06†
E	8	Germany	Luxury	Blue leather	514	0·40		0·14
G	13	Japan	Economy estate	Blue vinyl	252	0·25†	0·26†	
I	23	USA	Luxury	Cordovan vinyl	324	0·23†	0·27†	
I	22	USA	Mid-size	Black cloth	246	0·42†		
H	18	USA	Economy	Blue vinyl	192	0·37†		
B	3	USA	Economy	Black vinyl	356	0·23	0·12	0·02
I	24	USA	Mid-size	Grey vinyl	286	0·19†		0·15†
C	6	USA	Mid-size	Cream vinyl	299	0·33		
C	20	USA	Luxury	Green vinyl	186	0·32†		
D	10	Germany	Luxury	Bamboo leather	720	0·30		
E	8	Germany	Luxury	Tan leather	770	0·24		0·06
I	25	USA	Economy	Plaid cloth	300	0·20		0·05
H	15	USA	Mid-size estate	Blue vinyl	163	0·22†		
B	3	USA	Small economy	Tan vinyl	357	0·09	0·12	
J	26	USA	Mid-size	Red cloth	473	0·15		0·05
C	19	USA	Luxury	Green vinyl	178	0·19		
F	11	UK	Luxury	Brown leather	682	0·13		0·05
H	16	USA	Large mid-size	Blue vinyl	160	0·17†		
G	14	Japan	Economy	Black vinyl	195	0·16		
F	9	UK	Luxury	Brown leather	762	0·12		0·04
G	13	Japan	Economy estate	Brown vinyl	252	0·16		
B	4	USA	Mid-size	Blue vinyl	338	0·08	0·07	
J	27	USA	Economy	Tan vinyl	636	0·12		
B	4	USA	Estate	Blue vinyl	234	0·07		
H	17	USA	Mid-size	Green vinyl	151	ND		

NDMA = *N*-Nitrosodimethylamine NMOR = *N*-Nitrosomorpholine NDEA = *N*-Nitrosodiethylamine
ND = Not detected (detection limit = 0·02 $\mu g/m^3$)

*The samples were analysed by gas chromatography–thermal energy analysis (GC–TEA).

†The nitrosamine levels were confirmed by high-performance liquid chromatography–thermal energy analysis (HPLC–TEA).

‡The nitrosamine levels were confirmed by mass spectrometry (MS).

§*N*-Nitrosodibutylamine confirmed by GC–TEA and HPLC–TEA.

Table 2. *NDMA and NMOR levels in air samples taken in the front and rear passenger areas of new motor-cars*

Vehicle of		NDMA ($\mu g/m^3$) in air samples from the		NMOR ($\mu g/m^3$) in air samples from the	
Company	Model	Front	Rear	Front	Rear
C	5	0·49	0·55	1·7	1·8
C	6	0·61	0·73	1·2	1·1
J	28	0·24	0·47	0·35	0·48
D	7	0·43	0·46	0·39	0·35
E	8	0·40	0·44	ND	ND
C	6	0·33	0·34	ND	ND
J	26	0·15	0·16	ND	ND
B	4	0·08	0·09	0·07	0·10
J	27	0·12	0·20	ND	ND
B	4	0·07	0·09	ND	ND

NDMA = *N*-Nitrosodimethylamine NMOR = *N*-Nitrosomorpholine
ND = Not detected (detection limit = 0·02 $\mu g/m^3$)

Table 3. *Nitrosamine levels in air samples collected at different locations within a motor-car (Model J28)*

Location of air samples	Engine	NDMA ($\mu g/m^3$)	NMOR ($\mu g/m^3$)
Front seat	Off	0·24	0·35
Rear seat	Off	0·47	0·48
Rear seat	On	0·05	0·11
Under the dashboard	Off	0·34	0·37
In front of air flow from the heater	On	ND	ND
Under the bonnet, near the engine	On	ND	ND
Spare-tyre* well, with tyre present	Off	5·2	33
Spare-tyre* well, with tyre removed	Off	1·6	17
Inside a plastic bag containing the spare tyre	—	7·9	30

NDMA = *N*-Nitrosodimethylamine NMOR = *N*-Nitrosomorpholine
ND = Not detected
*The spare tyre was of the collapsible type.

DISCUSSION

Both NMOR and NDMA have been shown to be potent carcinogens in a wide variety of animals, even when they have been given at extremely low doses (IARC Working Group, 1978). Shank & Newberne (1976) reported an increased incidence of liver carcinomas and angiosarcomas and of lung angiosarcomas in rats fed a diet containing 5 μg NMOR/g. Anderson, Priest & Budinger (1979) administered NDMA in drinking-water (0·01 μg/ml; 10 ppb) to pregnant tumour-prone, strain A mice and then to their offspring and observed an increase in lung adenomas in the offspring. Moiseev & Benemansky (1975) exposed male rats to air containing 220 μg NDMA/m^3 for 25 months, and found malignant tumours of the liver and kidney in 83% of the test animals but in only 13% of the controls. However, it is not possible to extrapolate these animal data to man, as the nitrosamines have not been clearly identified as human carcinogens.

It is interesting to compare the nitrosamine exposure from new cars to that from two other widely distributed sources, beer and cooked bacon. A can of beer bought in the USA contains about 2·8 μg NDMA/litre (average of 18 samples analysed by Goff & Fine, 1979), or about 1 μg. A strip of cooked bacon in which *N*-nitrosopyrrolidine is present at the 5 μg/kg level, half that permitted by the USDA (United States Department of Agriculture, 1978), contains about 0·1 μg. Sitting in a car in which the total (NDMA + NDEA + NMOR) nitrosamine level was 1 $\mu g/m^3$ would result in an exposure of 0·1 μg in only 10 min, or about 1 μg in 100 min. Thus, on average, the intake from new cars is probably similar to the intake from beer and is probably higher than the intake from bacon. However, because of the wide variations between the nitrosamine levels in cars of different models, and even between individual cars of the same model, it is likely that the source of the nitrosamine contamination can be readily identified and the problem eliminated.

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TRANSFER OF POLYCHLORINATED DIBENZOFURANS TO THE FOETUSES AND OFFSPRING OF MICE

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Abstract—A diet containing 0·6 ppm of a mixture of polychlorinated dibenzofurans (PCDFs) having 4, 5 or 6 chlorine atoms was fed to mice for 18 days after mating or for 14 days after delivery. Dams, foetuses and offspring were analysed for PCDFs by gas chromatography. PCDFs were transferred to the foetuses across the placenta and to the offspring through milk. The amounts of PCDFs transferred through milk were much larger than the amounts transferred across the placenta. A greater level of PCDFs was accumulated in the livers of the dams than in other tissues. There were differences between the PCDFs in their levels of accumulation in the tissues of the mice.

INTRODUCTION

Commercial polychlorinated biphenyls (PCBs) are known to be contaminated with polychlorinated dibenzofurans (PCDFs; Bowes, Mulvihill, Simoneit, Burlingame & Risebrough, 1975; Nagayama, Kuratsune & Masuda, 1976; Roach & Pomerantz, 1974; Vos, Koeman, van der Maas, ten Noever de Brauw & de Vos, 1970) which are much more toxic than PCBs (Goldstein, Hass, Linko & Harvan, 1978; Vos & Koeman, 1970). Using various kinds of animals, many researchers have studied the tissue distribution of PCBs (Allen, Norback & Hsu, 1974; Burse, Kimbrough, Villanueva, Jennings, Linder & Sovocool, 1974; Curley, Burse, Grim, Jennings & Linder, 1971; Platonow, Liptrap & Geissinger, 1972) and polychlorinated dibenzo-*p*-dioxin (Allen, Van Miller & Norback, 1975; Firestone, Flick, Ress & Higginbotham, 1971; Norback, Engblom & Allen, 1975; Van Miller, Marlar & Allen, 1976; Williams, Cunningham & Blanchfield, 1972), but little is known about the biological behaviour of PCDFs *in vivo* (Goldstein, McKinney, Lucier, Moore, Hickman & Bergman, 1974; McKinney, Chae, Gupta, Moore & Goldstein, 1976; Morita & Oishi, 1977). PCDFs have been identified in the tissues of patients with 'Yusho' (Kuroki & Masuda, 1978; Nagayama, Masuda & Kuratsune, 1977) but the transfer of PCDFs from dams to foetuses and offspring in experimental animals has not been reported. We therefore investigated the transfer of PCDFs to the foetuses and offspring of mice that were fed these chemicals.

EXPERIMENTAL

Chemicals. Ethanol, *n*-hexane, potassium hydroxide, anhydrous sodium sulphate, water and silica gel were purified as described previously (Masuda, Kagawa & Kuratsune, 1974; Masuda, Kagawa, Tokudome & Kuratsune, 1978). Carbon tetrachloride and methylene chloride, both reagent grade, were fractionally redistilled. Alumina for column chromatography (about 300 mesh, Wako Pure Chemical Industry Ltd., Osaka) was activated by heating at 150°C for 12 hr before every use.

The PCDFs used were prepared by chlorination of dibenzofuran (Nishizumi, Kuratsune & Masuda, 1975), and, by gas chromatography–mass spectrometry (D-100, JEOL, Tokyo), they were determined to consist of 48% tetrachlorodibenzofurans (tetra-CDFs), 49% penta-CDFs and 3% hexa-CDF.

Animals and diets. Male and female ddN mice were supplied by the animal centre of Kyushu University. Test diet containing PCDFs was prepared as described in a previous paper (Masuda *et al.* 1978). Pellets of the test diet were analysed for PCDFs by gas chromatography (see below), and the mean level derived from a triplicate determination was 0·6 ppm. The gas chromatogram of the PCDFs in this test diet is shown in Fig. 1. The normal diet was Oriental MF (Oriental Kobo Co., Chiba), and no PCDFs were detected in it by gas chromatography (detection limit = 0·001 ppb).

Experimental procedure. The female mice, 10 wk old and weighing 20–25 g, were divided into five groups of 15–20 mice and mated. Insemination was confirmed by the presence of a vaginal plug. Two groups of pregnant mice were caged individually and were fed the test diet until day 18 of pregnancy. Then the mice in one group were killed and those in the other group were given the normal diet and allowed to raise their offspring for 2 wk. The third group was fed the normal diet up to day 18 of pregnancy, then fed the test diet and allowed to nourish the offspring for 2 wk. The last two groups were controls, fed only the normal diet throughout the experiment. One control group was killed on day 18 of pregnancy and another group gave birth and raised their offspring for 2 wk. The consumption of PCDFs by each group was calculated. The foetuses, liver and the rest of the body

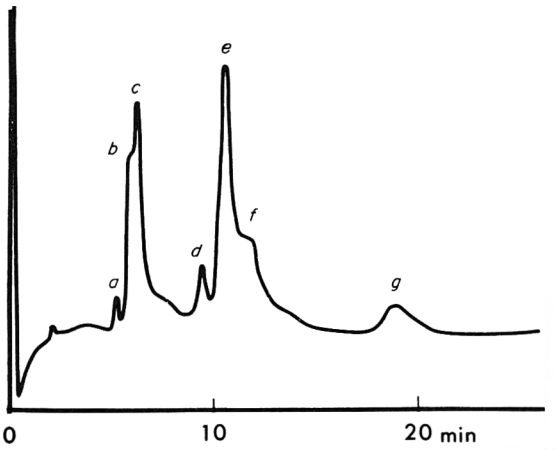

Fig. 1. Gas chromatogram of the PCDFs in the test diet. Peaks *a*, *b* and *c* correspond to tetra-CDF, *d*, *e* and *f* to penta-CDF and *g* to hexa-CDF. Column, 2% SE-30; detector, electron capture detector.

(excluding the digestive tract) of each dam killed on day 18 of gestation were analysed for PCDFs. The dams that reared their offspring were killed 2 wk after delivery. The liver and the rest of the body (excluding the digestive tract) of each dam were analysed for PCDFs. Half of the offspring were killed for whole-body analysis 1 wk after birth and the rest were killed and analysed at 2 wk of age. All the samples were stored separately in clean bottles and were kept at −20°C until they were analysed.

Analytical methods. Procedures for the extraction and separation of samples were essentially the same as those described in a previous paper (Nagayama *et al.* 1977). The samples were first homogenized with 100–200 ml *n*-hexane and 20 g anhydrous sodium sulphate in a Waring blender. The separated *n*-hexane solutions were combined and evaporated to dryness, yielding fatty residues which were saponified with 1 N-KOH in 50–100 ml ethanol. For liver samples, the saponification was carried out without extraction. The *n*-hexane extracts of the KOH solution were combined, concentrated, and then chromatographed on a column of silica gel (2 g) eluted with 150 ml *n*-hexane. The *n*-hexane eluate was further fractionated on a column of alumina (3 g) eluted successively with 60 ml *n*-hexane–carbon tetrachloride (80:20, v/v) and 25 ml *n*-hexane–methylene chloride (80:20, v/v). The latter eluate, which was expected to contain PCDFs, was subjected to gas chromatography in a Beckman GC 72-5 fitted with an electron-capture detector and a glass column (2 mm × 2 m) containing Chromosorb W AW-DMCS (100–120 mesh) coated with 2% SE-30. Individual and total PCDFs were quantified by comparing the heights of the gas-chromatographic peaks with those of the synthesized PCDFs, the assumption being made that all the PCDFs had the same peak-height sensitivity.

RESULTS

All the mice grew well throughout pregnancy, and on day 18 of pregnancy the mean body weights of the groups given test and normal diet were 39·4 and 39·5 g, respectively. There was no significant difference

Table 1. *Levels of PCDFs in dams, foetuses and offspring after oral administration of PCDF isomers for 18 days during pregnancy*

PCDF isomer*	Total intake of PCDFs by dams killed on day 18 of pregnancy (μg)	Levels of PCDFs† (dams killed on day 18 of pregnancy) in			Total intake of PCDFs by dams killed 2 wk after delivery (μg)	Levels of PCDFs† (dams killed on day 14 after delivery) in			
						Offspring aged (wk)		Dams	
		Whole body	Liver	Foetuses		1	2	Whole body	Liver
a	1·4 ± 0·06	ND	32 ± 3·8 (5·4)	ND	1·6 ± 0·10	ND	ND	ND	ND
b	8·1 ± 0·32	ND	ND	ND	9·0 ± 0·57	ND	ND	ND	ND
c	11·4 ± 0·46	2·3 ± 0·58 (0·44)	270 ± 32 (5·5)	0·17 ± 0·02 (0·007)	12·5 ± 0·79	0·31 ± 0·06 (0·05)	T	T	1·8 ± 0·2 (0·03)
d	2·9 ± 0·12	0·23 ± 0·04 (0·18)	70 ± 11 (5·7)	ND	3·1 ± 0·20	0·15 ± 0·04 (0·10)	0·20 ± 0·04 (0·27)	T	53 ± 3·4 (4·0)
e	13·2 ± 0·53	1·7 ± 0·37 (0·28)	220 ± 40 (3·9)	0·11 ± 0·03 (0·004)	14·5 ± 0·91	0·21 ± 0·07 (0·03)	T	ND	5·7 ± 4·1 (0·1)
f	4·9 ± 0·20	0·78 ± 0·16 (0·35)	300 ± 42 (14·5)	ND	5·4 ± 0·34	0·71 ± 0·22 (0·29)	1·2 ± 0·15 (0·89)	0·15 ± 0·03 (0·06)	230 ± 11 (10·0)
g	1·4 ± 0·06	T	64 ± 16 (10·7)	ND	1·6 ± 0·10	0·16 ± 0·10 (0·28)	0·29 ± 0·04 (0·76)	T	43 ± 12 (6·4)
Total	43·2 ± 1·73	5·06 ± 1·1 (0·26)	950 ± 140 (5·2)	0·30 ± 0·04 (0·003)	47·7 ± 3·0	1·6 ± 0·4 (0·07)	1·7 ± 0·22 (0·14)	0·21 ± 0·03 (0·01)	330 ± 21 (1·6)

ND = Not detected (<0·01 ppb) T = 0·01–0·1 ppb

*The lettering corresponds to the gas chromatographic peaks in Fig. 1.
†Values are means ± SEM for PCDF concentration (ppb), the figures in brackets being the mean weight of PCDFs in the given tissue expressed as a percentage of the total intake. There were nine dams in the group killed on day 18 of pregnancy and ten in the group killed on day 14 after delivery. No detectable levels of PCDFs were found in tissue samples from the control groups.

in body-weight gain between the groups. The mean numbers of offspring produced by each dam in the groups given the test and control diets were 5·5 and 5·7, respectively. For the groups of mice that were fed the test diet during pregnancy or after delivery or fed the normal diet throughout the experiment, the mean body weights of each pup at 1 wk of age were 4·0, 3·9 and 4·1 g, respectively, and at 2 wk of age were 8·6, 7·8 and 8·1 g, respectively. There were no significant differences in body-weight gain among the three groups. No abnormalities in appearance were observed in any group.

The tissue samples from the control groups contained no detectable amount of PCDFs. Table 1 summarizes the results of the PCDF analyses of the dams that were fed PCDFs for 18 days of pregnancy and then killed before, or 2 wk after, delivery. The amount of PCDFs that was transferred to the foetuses was small; only 0·003% of the total amount ingested by the dams was found in the foetuses, compared with 0·26% in the whole-body homogenates of the dams. In contrast the mean PCDF accumulation in the livers of the dams during pregnancy was 5·2% of the total intake. Even so, more than 90% of the PCDFs administered were eliminated from the body during the 18 days of pregnancy.

The transfer of PCDFs to the offspring by suckling was much greater than that to the foetuses. After 1 wk, 0·07% of the PCDF intake had been transferred to the offspring, and after 2 wk the level was 0·14%. Consequently the levels of PCDFs remaining in the bodies of the dams decreased from 5·06 ppb (μg/kg; 0·26% of the total intake) before delivery to 0·21 ppb (0·01%) after 2 wk of nursing the offspring. However, during the same period, the concentration of PCDFs in the livers of the dams decreased more slowly, from 950 ppb (5·2%) to 330 ppb (1·6%). The quantity of PCDFs accumulated in the livers was markedly higher than in other parts of the bodies of the dams. Assuming that the dissected digestive tract contained a similar level of PCDFs to the rest of the body, more than 90% of PCDFs in the dam were found in the liver.

Table 2 lists the concentrations and percentages of PCDFs in the offspring and in the dams that were fed PCDFs for 2 wk after delivery. As was expected, the concentration and percentage of PCDFs in the offspring of dams in this group were higher than those in the corresponding offspring of the dams that were fed PCDFs during pregnancy. This difference was most prominent in the 2-wk-old offspring, in which there was about a tenfold difference between the two groups.

Of the three tetra-CDFs, represented by GC peaks *a*, *b* and *c* (Fig. 1), *c* was retained in the bodies of the mice to a much greater extent than *a* and *b*. It was detected in the bodies of dams and offspring, especially in the liver of dams, even when the feeding of PCDFs had been discontinued for 2 wk (Table 1). In contrast tetra-CDFs *a* and *b* were not found in the dams and offspring (Tables 1 and 2) except in the livers of dams that were being fed PCDFs; the livers of these dams contained the tetra-CDF represented by GC peak *a*.

The penta-CDF represented by peak *e* was the most abundant PCDF in the test diet. Its concentrations in the bodies of dams (excluding the livers) and in the offspring were higher than those of the other penta-CDFs, represented by peaks *d* and *f*, while the mice were being fed PCDFs (Tables 1 and 2). However, when the feeding of PCDFs was discontinued the concentrations of penta-CDFs *d* and *f* that were retained in the tissues were greater than the concentration of *e* except in the 1-wk-old offspring. The loss of isomer *e* from the livers of the dams was almost complete 2 wk after PCDF-feeding ceased, whereas much smaller proportions of *d* and *f* had been lost. Overall, the penta-CDFs *d* and *f* were more readily retained in the tissues of the mice than were the other tetra- and penta-CDF isomers. The extent of retention of the hexa-CDF, represented by GC peak *g*, was similar to that of the penta-CDFs represented by peaks *d* and *f*.

DISCUSSION

The PCDFs tested on mice were transferred to the foetuses across the placenta in small amounts but were transferred to the offspring in the milk in much

Table 2. *Levels of PCDFs in dams and offspring of mice after oral administration of PCDF isomers for 14 days following delivery*

PCDF isomer*	Total intake of PCDFs (μg)	Levels of PCDFs† in Offspring aged (wk) 1	Offspring aged (wk) 2	Dams Whole body	Dams Liver
a	2·3 ± 0·13	ND	ND	ND	68 ± 8·7 (6·9)
b	12·9 ± 0·71	ND	ND	ND	ND
c	17·9 ± 3·10	3·7 ± 0·68 (0·5)	5·4 ± 0·96 (1·4)	0·6 ± 0·12 (0·06)	430 ± 22 (5·6)
d	4·4 ± 0·24	T	0·5 ± 0·08 (0·6)	T	130 ± 11 (6·7)
e	21·0 ± 1·14	3·4 ± 0·48 (0·4)	7·3 ± 1·02 (1·6)	0·5 ± 0·07 (0·05)	330 ± 17 (3·7)
f	7·7 ± 0·42	1·4 ± 0·24 (0·4)	3·7 ± 0·50 (2·2)	0·3 ± 0·04 (0·08)	460 ± 26 (13·8)
g	2·3 ± 0·13	T	0·7 ± 0·15 (1·5)	T	78 ± 20 (7·9)
Total	68·3 ± 3·75	8·5 ± 1·30 (0·3)	18·1 ± 2·50 (1·2)	1·4 ± 0·22 (0·04)	1490 ± 75 (5·1)

ND = Not detected (<0·01 ppb) T = 0·01–0·1 ppb

*The lettering corresponds to the gas chromatographic peaks in Fig. 1.

†Values are means ± SEM for PCDF concentration (ppb), the figures in brackets being the mean weight of PCDFs in the given tissue expressed as a percentage of the total intake. The no. of dams in the group was ten. PCDFs were not detected in tissue samples from the control group.

larger quantities. This transfer is similar to that previously reported for PCBs (Masuda *et al.* 1978; Masuda, Kagawa, Kuroki, Tokudome & Kuratsune, 1979). However, variation in the transferability of isomers was very large for PCBs. The amounts of PCDFs transferred to foetuses and to 1- or 2-wk-old offspring were 0·003, 0·07 and 0·14% of the total intake, respectively, while the corresponding figures for tetra-, penta- and hexa-PCB isomers were 0·006–0·14, 0·05–35 and 0·1–53%, respectively (Masuda *et al.* 1979). The degree of transfer of the PCDFs was similar to that of 2, 5, 3', 4'-tetrachlorobiphenyl, which was less transferable than some of the other PCB isomers.

Some of the PCDFs tested were readily accumulated in the liver of the dams, although most of them were easily eliminated from the dams, foetuses and offspring of mice. The levels of PCDFs found in the liver, whole body and foetuses of mice were, respectively, 5·2, 0·26 and 0·003% of the total intake by the dams that were fed PCDFs for 18 days during pregnancy. The corresponding figures for mice that were fed Kanechlor-500 were 0·8–3·2, 5·9–15·6 and 0·1–0·2%, respectively (Masuda *et al.* 1978). Accumulations of PCDFs in the human liver have also been observed in patients with 'Yusho' (Nagayama *et al.* 1977). The concentrations of PCBs and PCDFs in the livers of these patients were similar, but in the adipose tissue the level of PCBs was more than a hundred times higher than that of PCDFs.

The retention of the different PCDFs in the tissues of mice varied (Tables 1 and 2). Peaks *c* and *f* of the gas chromatogram (Fig. 1) have recently been determined to be 2,3,7,8-tetra-CDF and 2,3,4,7,8-penta-CDF by comparing their retention times with those of the authentic specimens. It is interesting that these PCDFs were retained in the tissues of mice, especially in the liver, and were identified in the tissues of patients with Yusho (Kuroki & Masuda, 1978). Since they are considered to be among the most toxic PCDFs (Poland, Glover & Kende, 1976), further investigations on their biological behaviour are desirable.

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TOXICOLOGY OF GLUCOSINOLATES, RELATED COMPOUNDS (NITRILES, *R*-GOITRIN, ISOTHIOCYANATES) AND VITAMIN U FOUND IN CRUCIFERAE

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Abstract—The cruciferous glucosinolates (epiprogoitrin, sinigrin and sinalbin), nine of their derivatives (nitriles, isothiocyanates, *R*-goitrin) and vitamin U were not teratogenic to the rat foetus when administered sc to the dams on days 8 and/or 9 of gestation. However, 1-cyano-3,4-epithiobutane, 3-methylsulphinylpropyl isothiocyanate and allyl isothiocyanate caused embryonal death and decreased foetal weight, whereas five other derivatives only decreased foetal weight. Under identical conditions, the reference teratogen, trypan blue, caused a significant incidence of malformations and resorptions. 3-Methylsulphinylpropyl isothiocyanate was the most toxic compound, followed by allyl isothiocyanate, 1-cyano-3,4-epithiobutane (nephrotoxic) and *S*-1-cyano-2-hydroxy-3-butene. Some nitriles caused hyperplasia of the thyroid, an effect which often was more pronounced in nonpregnant than in pregnant rats.

INTRODUCTION

The long search for the cause of neural tube defects in humans has been described in a review by Leck (1974). In the 1960's, potato blight was suspected as the cause of foetal anencephaly and spina bifida cystica in England and Wales. However, this hypothesis was rejected because potato-free diets did not prevent these malformations (Renwick, 1974). Births recorded from 1965 to 1972 in Sweden (Sandahl, 1977) showed a significant increase in the incidence of anencephaly in foetuses conceived in March and of spina bifida in July. In South Africa, Singer, Nelson & Beighton (1978) reported an increase in central nervous system defects in foetuses conceived in May.

Certain plant foods are the prime suspects for the seasonal rise in foetal malformations due to their seasonal abundance and availability. In addition, seasons and climate are known to influence the chemical composition of plants.

Vegetables and seeds of the Cruciferae family, such as crambe, kale, mustard, rape, cabbage, turnips, etc., are rich in glucosinolates (Ahmed, 1972; VanEtten, 1969). Many feeds containing a high concentration of rape or crambe seed meal have been shown to decrease feed intake and growth rate and to cause enlarged liver, kidneys, thyroid and adrenal glands (Bell, Benjamin & Giovannetti, 1972; Clandinin & Bayly, 1960; Hussar & Bowland, 1959; Kennedy & Purves, 1941; Kirk, Mustakas, Griffin & Booth, 1971; Manns, Bowland, Mendel & Zivkovic, 1963; Nordfeldt, Gellerstedt & Falkmer, 1954; Srivastava, Philbrick & Hill, 1975; VanEtten, Daxenbichler & Wolff, 1969a; VanEtten, Gagne, Robbins, Booth, Daxenbichler & Wolff, 1969b). These adverse effects of the feeds were attributed to the high content of glucosinolates and their derivatives, which include goitrin, isothiocyanates and nitriles. Very little is known about the toxicity of the individual compounds.

In this study, the potential teratogenicity and acute toxic actions of certain glucosinolates and derivatives are described.

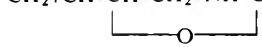
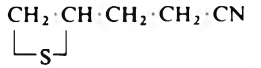
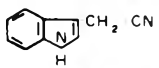
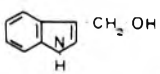
EXPERIMENTAL

The chemicals used in this study and tested for toxic and teratogenic action are listed in Table 1. Listed below each glucosinolate (epiprogoitrin, sinigrin and sinalbin) are its decomposition products. Owing to unavailability, glucobrassicin was not tested; but its derivatives, 3-indolylacetonitrile and indole-3-methanol were tested. Vitamin U was included due to its coexistence with glucosinolates in Cruciferae, and trypan blue was used as the reference teratogenic compound. The chemistry of these glucosinolates and their derivatives has been described by Cole (1975, 1976), Daxenbichler, VanEtten, Brown & Jones (1964), Daxenbichler, VanEtten & Spencer (1977), Daxenbichler, VanEtten & Wolff (1966, 1968), Kirk & MacDonald (1974), VanEtten (1969) and Virtanen (1965).

Pregnant Holtzman rats weighing 250–300 g were used to test the potential teratogenic effect of the compounds listed in Table 1. The animals were maintained on commercially available Teklad* 4% mouse/rat diet (obtained from Mogul Corp., Winfield, IA)

*The mention of this product does not imply recommendation over similar products that may be available.

Table 1. *Compounds screened for toxicity and teratogenicity*

Chemical	Structure	Source
Epiprogoitrin	$CH_2{:}CH{\cdot}CHOH{\cdot}CH_2{\cdot}C(S\text{-glucose}){:}N{\cdot}OSO_3{\cdot}Na/K$	*Crambe abyssinica* (crambe) seed
S-1-Cyano-2-hydroxy-3-butene	$CH_2{:}CH{\cdot}CHOH{\cdot}CH_2{\cdot}CN$	*C. abyssinica* seed
R-5-Vinyl-2-oxazolidinethione (*R*-goitrin)		*C. abyssinica* seed
1-Cyano-3,4-epithiobutane		*Brassica rapa* L. (tender green mustard) seed
Sinigrin	$CH_2{:}CH{\cdot}CH_2{\cdot}C(S\text{-glucose}){:}N{\cdot}OSO_3K{\cdot}H_2O$	Pfaltz & Bauer, Stamford, CT
Allyl isothiocyanate	$CH_2{:}CH{\cdot}CH_2{\cdot}N{:}C{:}S$	Aldrich Chemical Co., Milwaukee, WI
Sinalbin hydrate	$OH{\cdot}C_6H_4{\cdot}CH_2{\cdot}C(S\text{-glucose}){:}N{\cdot}OSO_3\text{-sinapin}$	Aldrich Chemical Co.
p-Hydroxyphenylacetonitrile	$OH{\cdot}C_6H_4{\cdot}CH_2{\cdot}CN$	ICN Pharmaceuticals, Inc., Cleveland, OH
1-Cyano-3-methylsulphinylpropane (iberin nitrile)	$CH_3{\cdot}SO{\cdot}CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}CN$	*Lesquerella gordonii* seed
3-Methylsulphinylpropylisothiocyanate (iberin)	$CH_3{\cdot}SO{\cdot}CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}NCS$	*L. gordonii* seed
3-Indolylacetonitrile		Aldrich Chemical Co.
Indole-3-methanol		Aldrich Chemical Co.
Vitamin U (cabagin)	$CH_3{\cdot}S(CH_3){\cdot}CH_2{\cdot}CH_2{\cdot}CH(NH_2){\cdot}COOH$	ICN Pharmaceuticals Inc.
Trypan blue (reference teratogenic compound)		Allied Chemical Corp., Morristown, NJ

with 1% iodized salt, and two rats were placed in each suspended wire-bottomed cage with an automatic flushing system to clean the cages. The compounds were dissolved in either propylene glycol or distilled water and administered sc or orally in volumes of 0·5–1·0 ml/kg. One dose/day was administered on day 8 and/or 9 of gestation, although trypan blue was also given on day 7 of gestation. Normally, six pregnant (sperm positive) rats were used per dose. Six pregnant rats from each shipment of 24–30 were treated sc with either saline or propylene glycol and served as the control group.

The treated and control rats were weighed daily, and the weight gains or losses were calculated as percentages of the body weight on day 3 of gestation. On day 20 of gestation (11–12 days after treatment), all rats were anaesthetized with sodium pentobarbital, and the foetuses and resorbed embryos were counted. Each foetus was weighed and examined for external malformations. For examination of the skeletal malformations, the viscera and skin of one third of the foetuses were removed; and, sequentially, the foetuses were fixed overnight in 95% alcohol, placed in 1% KOH for 8 hr and then placed in 0·01 alizarin red, according to Dawson (1926). The internal malformations in two-thirds of the foetuses were examined after they had been fixed in Bouin's fluid and serially sectioned (Wilson, 1965). Also on day 20, following removal of the foetuses, the organs (liver, kidneys, adrenals and thyroids) of the dams were removed, weighed and fixed in formalin solution for histological examination.

In order to find the approximate lethal doses of the test compounds that were available in greater supply, relatively high single doses were administered sc to male Sprague–Dawley rats (50-210 g). These treated rats were weighed daily and observed for 12 days and killed. The same organs were removed, weighed and fixed in formalin solution for histological examination.

Periodic acid–Schiff and haematoxylin were used as the histological stains. Student's *t* test was used to determine the significance of weight gains, foetal weights, litter size and organ weight changes caused by the test compounds. The chi-square test was used to determine the significance of the difference in the incidence of resorbed and malformed foetuses.

RESULTS

Embryotoxicity and teratogenicity

Of the 13 compounds tested, none was teratogenic to rat foetuses. However, seven compounds decreased foetal weight at certain doses (Table 2). Also, three of them at high doses increased embryonic death (resorption). In comparison with control foetuses, those with significantly lower weights were less developed, as indicated by the lower number of calcified small bones of the feet, tail and sternum. Generally, the control foetuses had six small calcified bones forming the sternum; but underweight foetuses had three to five bones calcified, and some of the bones were split or abnormally shaped.

Epiprogoitrin and *S*-1-cyano-2-hydroxy-3-butene increased or decreased foetal weight depending on dosage (Table 2). Three nitriles, 3-indolylacetonitrile, *p*-hydroxyphenylacetonitrile and 1-cyano-3-methylsulphinylpropane (iberin nitrile), increased foetal weights. From nine pregnant rats treated with iberin nitrile (200 mg/kg, sc) on days 8 and 9 of pregnancy, only one embryo out of 87 had skeletal malformations, namely, talipes and curled tail. Alizarin stain revealed that this malformed, underweight foetus (2·14 g) had less calcified tail and feet bones than did control foetuses.

Table 2 shows that single-dose treatments of the reference teratogenic compound trypan blue (100 mg/kg, sc) given on day 7 of pregnancy caused the highest rate of embryonal death (resorption) and the lowest rate of malformations. Trypan blue injection on day 8 resulted in higher incidence of malformations of the head (cranioschisis, meningocele, hydrocephalus, unilateral-microphthalmia, -anophthalmia, -anotia, -agnathia). Injection on day 9 resulted in a higher incidence of malformations in the hind quarters (spina bifida, curled tail, talipes, omphalocele, hernia etc.). When the same total amount of 100 mg trypan blue/kg was given in 2 days (50 mg/kg/day), on days 8 and 9 of gestation, the incidence of malformation was comparable to that caused by a single high dose (100 mg/kg) given either on day 8 or 9 of gestation. The high yield of malformed foetuses caused by one or two sc doses of trypan blue on day 8 and/or 9 of pregnancy indicate that these injection days were suitable for tests to screen the potential teratogenic action of the compounds listed in Table 1.

Effects on the body weight of pregnant rats

Control pregnant rats had a weight gain of 50·13 ± 9·2% from day 3 to day 20 of pregnancy. Nonpregnant female controls with almost identical initial body weights gained 9·4 ± 3·9% during the same period. Two sc injections of epiprogoitrin, sinigrin, sinalbin hydrate, 1-cyano-3-methylsulphinylpropane or vitamin U on days 8 and 9 of gestation had no significant effect on the weight gain of pregnant rats (Table 2). 3-Indolylacetonitrile and indole-3-methanol depressed weight gains from 2 to 3 days following injections. *S*-1-Cyano-2-hydroxy-3-butene, *R*-goitrin, 1-cyano-3,4-epithiobutane, allyl isothiocyanate, and *p*-hydroxyphenylacetonitrile depressed weight gains from 5 to 12 days following sc injections. This depression of weight gain was associated with a decrease in food intake. In addition, 1-cyano-3,4-epithiobutane caused mild diarrhoea of short duration.

Effects on relative organ weights

Sinigrin (sc), sinalbin and trypan blue increased the relative liver weight of pregnant rats, whereas epiprogoitrin and *R*-goitrin had the same effect in male rats (Table 3). *S*-1-Cyano-2-hydroxy-3-butene decreased the relative liver weight of pregnant rats. 1-Cyano-3,4-epithiobutane was nephrotoxic and increased the kidney weight to a greater extent in pregnant rats than in male rats. 1-Cyano-3,4-epithiobutane, *S*-1-cyano-2-hydroxy-3-butene and iberin increased the adrenal weights of pregnant rats; and *R*-goitrin increased the adrenal weights of nonpregnant rats. In this series of test compounds, only *p*-hydroxyphenylacetonitrile increased thyroid weight significantly in pregnant rats; sinigrin (orally) decreased the thyroid

Table 2. *Effects of glucosinolates and related compounds on dams and foetuses, after sc or oral administration on day 7, 8 and/or 9 of gestation*

Test compounds	Dose (mg/kg body weight) and (day† of administration)	No. of dams	Days† of weight loss in dams	No. of live foetuses/dam‡	Total no. of live foetuses	Percentage increase or decrease in foetal weight compared with control§	Percentage of foetuses that were resorbed	Percentage of foetuses with malformations
Control	—	54	—	9·39 ± 2·9	507	—	8·15	1·77
Epiprogoitrin	159 (8,9)	5	0	9·6 ± 3·8	48	+14·35**	5·88	0
	320 (8,9)	8	0	10·5 ± 2·2	84	−9**	7·69	1·19
S-1-Cyano-2-hydroxy-3-butene	76 (8,9)	7	9–17	10·1 ± 1·6	71	+6·4**	11·25	1·4
	150 (8)	4	9–20	9·5 ± 2·6	38	−15·5**	7·32	0
	175 (8)	5	10–20	9·0 ± 4.9	45	−18·0**	13·46	2·2
R-Goitrin	100 (8,9)	6	0	9·0 ± 3·6	54	−5·6**	3·57	0
	200 (8)	12	9–15	9·92 ± 2·2	119	−0·12	9·85	2·52
1-Cyano-3,4-epithiobutane	44 (8,9)	5	10–16	10·6 ± 1·6	53	−20**	5·35	0
	95 (8)	10	9–20	5·0 ± 4·34**	50	−27**	48·4**	2
Sinigrin	322 (8,9)	12	0	8·92 ± 4·5	107	−5·6**	7·69	1·18
	322‖ (8,9)	10	0	10·0 ± 2·5	100	−5·74**	3·85	3·0
Allyl isothiocyanate	50 (8,9)	6	0	10·0 ± 2·0	60	−11**	3·22	0
	100 (8,9)	8	11–17	9·87 ± 3·6	79	+3·9	19·4**	1·27
Sinalbin hydrate	300 (8)	6	0	8·83 ± 4·7	53	−3·5	5·36	1·75
	300 (8,9)	3	0	11·3 ± 2·1	34	+1·4	2·85	2·94
p-Hydroxyphenylacetonitrile	100 (8,9)	4	0	11·25 ± 1·5	45	+4·25	4·25	4·44
	200 (8)	6	9–14	9·33 ± 4·37	55	+6·2*	5·17	5·45
1-Cyano-3-methylsulphinylpropane (iberin nitrile)	100 (8,9)	6	0	11·7 ± 1·75	70	+3·6	0	0
	200 (8,9)	9	0	9·66 ± 3·4	87	+4·58*	2·24	3·44
3-Methylsulphinylpropyl isothiocyanate (iberin)	100 (8)	5	0	7·0 ± 6·55	35	−1·1	28·6**	0
3-Indolylacetonitrile	200 (8)	6	9–10	10·8 ± 3·43	65	+6·3**	6·75	0
Indole-3-methanol	200 (8,9)	4	0	6·75 ± 5·05	27	−9·2**	10·0	0
	300 (8,9)	3	9–11	12·7 ± 0·58	38	−0·76	2·56	0
Vitamin U	1000 (8,9)	6	0	12·3 ± 1·03	74	0	2·64	2·7
Trypan blue (reference teratogen)	100 (7)	6	0	7·17 ± 3·7	43	−6·7**	34·8**	11·67*
	100 (8)	6	0	8·33 ± 1·4	50	−15·1**	25·4**	36·0**
	100 (9)	6	0	9·5 ± 1·22	57	−16·8**	26·0**	31·6**
	50 (8,9)	6	0	4·5 ± 1·05*	27	−9·0	59·6**	29·6**

†Day of gestation.
‡Values are means ± SD.
§The mean (± SD) weight of control foetuses was 4·18 ± 0·47 g.
‖This dose was given orally; all other doses were given sc.
All foetal data were collected on day 20 of gestation. Values marked with asterisks differed significantly (Student's *t* test or chi-square test) from the control value: $*P < 0.05$; $**P < 0.01$.

Table 3. *Effects of glucosinolates and related compounds on relative organ weights of pregnant and nonpregnant females and male rats killed 11–12 days after sc or oral administration*

Compound	Dose (mg/kg body weight) × no. of doses†	Condition	No. of rats	Initial body weight	Relative organ weights			
					Liver	Kidney	Adrenal	Thyroid
Epiprogoitrin	320 × 2	P	5		NS	NS	NS	NS
	320 × 2	M	3		+15·9**	NS	NS	NS
S-1-Cyano-2-hydroxy-3-butene	175 × 1	P	5		−29**	NS	+57**	+14·75
	175 × 1	NP	7		NS	NS	NS	NS
	200 × 1	M	3		NS	NS	NS	NS
R-Goitrin	200 × 1	P	6		NS	NS	NS	NS
	200 × 1	NP	8		NS	NS	+13·8**	+11·7
	200 × 1	M	5		+12·2**	NS	NS	+10·4
1-Cyano-3,4-epithiobutane	95 × 1	P	5		NS	+78·7**	+53·6**	NS
	95 × 1	M	6		NS	+13·1*	+24·7*	NS
Sinigrin	322 × 2	P	7		+17·1*	NS	−14*	−15
	322 × 2	NP	2		−11	NS	−10	−22·3*
	322 × 2‡	P	7		NS	NS	NS	−23*
	322 × 2‡	NP	2		NS	−10*	−13	−28**
	322 × 2‡	M	3		NS	NS	+10·9	+10·3
Sinalbin hydrate	300 × 1	P	6		+14·3**	NS	NS	−10
	300 × 2	P	3		NS	NS	+10·3	+13·2
	300 × 2	NP	3		NS	NS	NS	NS
p-Hydroxyphenylacetonitrile	200 × 1	P	6		NS	NS	+12·4	+17·4*
	100 × 2	P	4		NS	NS	NS	−10
	200 × 1	M	3		NS	NS	NS	NS
3-Methylsulphinylpropyl isothiocyanate (iberin)	100 × 1	P	5		NS	NS	+15·25*	+14
	100 × 1	NP	7		NS	NS	NS	NS
	88 × 1	M	4		NS	NS	+16·5**	NS
Trypan blue: day 8,9	50 × 2	P	6		+10·6*	+17*	+14·4	+23·8*
day 7	100 × 1	P	6		+17·5*	+21·8**	NS	+23·4**
day 8	100 × 1	P	6		+22·8**	+17·2**	NS	NS
day 9	100 × 1	P	6		+24·3**	+15·4**	NS	NS
			Controls§					
		P	54	268 ± 14·42	35·24 ± 14·4	5·03 ± 0·6	180·4 ± 21·5	51·2 ± 10·1
		NP	15	267 ± 19	32·0 ± 1·8	6·71 ± 0·55	240·8 ± 23	65·3 ± 10·8
		M	6	202 ± 5·5	37·7 ± 2·5	7·93 ± 0·41	189·3 ± 23	67·0 ± 4·81

P = Pregnant NP = Nonpregnant M = Male NS = Value not significant and under 10%

†Pregnant rats were injected on day 8 and/or 9 of pregnancy except for trypan blue which was injected on the days indicated. The doses were given sc except where indicated.

‡These doses were given orally.

§Control values are means ± SD: body weight is expressed in g; liver and kidney weights are expressed in g/kg body weight and adrenal and thyroid weights are expressed in mg/kg body weight.

Values marked with asterisks differed significantly (Student's *t* test) from the control value (*$P < 0·05$; **$P < 0·01$).

weight in pregnant and nonpregnant rats and sinigrin (sc) decreased the thyroid weight in nonpregnant rats alone. The following compounds had no effect on organ weights: allyl isothiocyanate, iberin nitrile, 3-indolylacetonitrile, indole-3-methanol and vitamin U.

Lethal effects

Because of the limited availability of some compounds, a systematic LD_{50} determination was not feasible; but only seven compounds in this series caused death at the doses tested (Table 4). *S*-1-Cyano-2-hydroxy-3-butene induced intermittent 'rolling seizures' and a loss of righting reflex preceding death (1–6 days). The rats treated with 1-cyano-3,4-epithiobutane, 3-indolylacetonitrile (iberin, allyl isothiocyanate) and indole-3-methanol were comatose before death. Lethal and near lethal doses of *p*-hydroxyphenylacetonitrile caused blinking of the eyelids and lachrymation within 10 min. In 30 min, clonic leg movements started, and the rats did not respond to painful stimuli and lost the righting reflex capability. When righted, the legs could not support the body, and the animals rested on the abdomen with legs spread and twitching. This clonic movement continued as long as an hour and was followed by a motionless period preceding death or recovery. Older male rats were more resistant to *p*-hydroxyphenylacetonitrile than young male rats (Table 4). The survivors did not show any adverse residual neurological effects. Young male rats were more sensitive than pregnant rats, which were older, to the lethal effects of allyl isothiocyanate and iberin.

Histology

Liver. The pregnant rats that had rolling seizures and died 2 to 6 days after sc injection of *S*-1-cyano-2-hydroxy-3-butene had liver necrosis in the subcapsular, or in certain midzonal areas spreading toward the centrilobular veins, which were intact. Some survivors had localized liver necrosis, replaced by scar tissue with disorganized proliferation of new biliary ducts and blood vessels when examined on the 12th day after receiving 200 mg/kg of this compound. Rats treated with the other compound in this series had liver histology similar to that of the controls, with occasional clusters of lymphocytes.

Kidney. A section of normal kidney from a control rat is shown in Fig. 1a for the purpose of comparison. The kidney sections of rats that died within 7 hr after treatment with 300 mg/kg of *S*-1-cyano-2-hydroxy-3-butene had swollen kidney tubular cells with swollen nuclei. Rats that died overnight after treatment had necrosis of the kidney tubular cells. The survivors of the group treated with 200 mg/kg of this compound did not show any kidney abnormality on the 12th day after treatment. The kidney of rats that died just 2–3 days after receiving 95 mg/kg, sc, of 1-cyano-3,4-epithiobutane had vacuolation and necrosis of the tubular cells (Fig. 1b,c). On the 12th day, some survivors of this group had kidneys with extremely dilated tubules, lined with flat epithelial cells, and had focal infiltration of lymphocytes in the interstitial space (Fig. 1d) of the kidney. Some kidneys that were extremely enlarged and pale had dilated tubules filled with a pink-staining material. Necrosis of kidney tubular cells was observed in rats that died 4 to 5 hr after 150 mg/kg, sc, of iberin.

Thyroid. The results of thyroid histological examination are summarized in Table 5, and representative photomicrographs are presented in Figs 2 & 3. There was considerable variation in the control thyroid with respect to the size of the follicles, height of the epithelial cells lining the follicles, and the absence or presence of vacuoles in the colloidal material filling the lumen. The thyroid gland of pregnant rats treated with the only compound in this series that increased the thyroid weight, *p*-hydroxyphenylacetonitrile, had enlarged follicles or a slightly hyperplastic appearance. The hyperplastic response to the test compounds seemed more intense in nonpregnant female rats than in the pregnant or male rats.

General observations

The following compounds caused lachrymation: *S*-1-cyano-2-hydroxy-3-butene, 1-cyano-3,4-epithiobutane, 3-indolylacetonitrile, *p*-hydroxyphenylacetonitrile, allyl isothiocyanate and iberin. These compounds also caused intense local oedema at the sc injection site. Indole-3-methanol and 3-indolylacetonitrile induced sedation, ataxia, loss of righting reflex and sleep. Treatment with 300 mg/kg, sc, of indole-3-methanol induced loss of righting reflex which lasted about 4 hr.

DISCUSSION

The previously demonstrated absence of teratogenicity of goitrin (Khera, 1977; Rakalska & Dzierzawski, 1972) and of allyl isothiocyanate (Ruddick, Newsome & Nash, 1976) was confirmed by using different doses, a different route of administration and different frequencies and times of administration (Table 2).

The occurrence of neurolathyrism and osteolathyrism due to the ingestion of certain leguminous plants (peas) has been known for centuries; but the identification of the aminonitriles responsible for those toxic signs is more recent, as described by Selye (1957), Gardner (1959) and others. We expected foetal malformations due to osteolathyrism which involves bones and cartilages, when we treated the pregnant rats with various nitrile derivatives of the glucosinolates. However, the nitriles did not cause a significant number of skeletal malformations. From nine pregnant rats treated with two doses of iberin nitrile (200 mg/kg, sc), only one foetus of 87 had defects involving the skeleton, namely, talipes and a curled tail.

The neurolathyrism caused by aminonitriles such as 2,2'-iminodipropionitrile in experimental animals is characterized by a lifelong hyperexcitability, choreiform and circling movements starting 3 to 4 days after treatment. The nitrile *S*-1-cyano-2-hydroxy-3-butene in lethal doses (200 mg/kg, sc) induced intermittent rolling seizures before death (1–6 days), but lower doses did not cause any visible permanent neurological effect in pregnant rats. Treatment with *p*-hydroxyphenylacetonitrile in high doses caused clonic leg movements, but the survivors were normal, without any residual neurologic effects.

By the action of the plant enzyme thioglucosidase (myrosinase), glucobrassicin forms 3-indolylaceto-

Table 4. *Lethal doses of compounds derived from glucosinolates (rats)*

Test chemical	$LD_{50} \pm SD$ (mg/kg body weight)	Sex	Dose (mg/kg body weight)	Death		Remarks
				Rate	Time (hr)	
3-Methylsulphinylpropyl isothiocyanate (iberin)	90 ± 2·89	F	150	6/6	4–6	Comatose, oedema at the injection site
		F	100	0/12		
		M	100	3/8	8–24	
		M(Y)	87·5	2/6	8–24	
Allyl isothiocyanate	92 ± 2·3	M(Y)	75	0/3		
		F	100 × 2	1/13	48	Comatose, oedema at the injection site
		M(Y)	100	3/3	7	
		M(Y)	90	2/6	5	
1-Cyano-3,4-epithiobutane	109 ± 4·8	M(Y)	75	0/3		
		F	132	6/6	4–8	Comatose
		F	95	1/13	27	Comatose
		M	95	1/7	72	Comatose
S-1-Cyano-2-hydroxy-3-butene	200 ± 12·5	F	88	1/6	96	Comatose
		M	250	3/5	1–6	Rolling seizures, liver necrosis
		F	200	9/18	24–144	Rolling seizures, liver necrosis
3-Indolylacetonitrile	255 ± 14	F	175	0/12		
		M(Y)	300	7/8	24	Comatose
		M(Y)	250	4/9	1·5–24	Comatose
Indole-3-methanol		M(Y)	225	0/3		
		M(Y)	500	3/4	1–3	Comatose
		M(Y)	300 × 2	0/3		
p-Hydroxyphenylacetonitrile		F	250	6/6	1·5	Clonic leg movements
		M(Y)	350	0/6		Clonic leg movements
		M(YY)	300	2/3	1–2	Clonic leg movements
		M(YY)	260	1/1	1·5	Clonic leg movements

F = female rats (pregnant + nonpregnant) weighing 250–280 g M = Male rats weighing 200–230 g
M(Y) = Young males, 100–150 g M(YY) = Younger male, 50–75 g

Table 5. *Thyroid histology of rats, 12 days after sc or oral administration of one or two doses of glucosinolates and related compounds*

Compounds*	Condition of rats....	Thyroid histology†		
		Pregnant	Nonpregnant	Male
Epiprogoitrin		Fig. 2b; normal	Normal; slightly hyperplastic	Normal; smaller follicles, columnar epithelial cells
S-1-Cyano-2-hydroxy-3-butene		Normal; hyperplastic, smaller follicles	Fig. 2c; very hyperplastic; smaller follicles; foamy, scanty colloid	Very hyperplastic; small follicles, columnar cells; vacuoles in the colloid
R-Goitrin		Fig. 2e; normal; smaller follicles; slightly hyperplastic	Normal; hyperplastic; smaller follicles; scanty colloid	Fig. 2d; small follicles; tall cells; scanty colloid or foamy colloid
1-Cyano-3,4-epithiobutane		Fig. 2f; normal; smaller follicles, cuboidal cells	Normal; smaller follicles	Normal
Sinigrin (sc administration)		Normal; smaller follicles; mitotic cells	Fig. 3b	Fig. 3a; large follicles columnar cells; papillary formations
Sinigrin (oral administration)		Normal; slightly hyperplastic	Normal; slightly hyperplastic vacuoles (lumen)	Normal
Allyl isothiocyanate		Fig. 3c	Hyperplastic areas; columnar cells	
Sinalbin hydrate		Normal; slightly hyperplastic; Fig. 3d	Normal; slightly hyperplastic; small follicles	
p-Hydroxyphenylacetonitrile		Normal; slightly hyperplastic		
Iberin nitrile		Normal; smaller follicles; columnar cells; scanty colloid	Fig. 3e	
Iberin		Normal	Normal	
3-Indolylacetonitrile		Normal; slightly hyperplastic	Fig. 3f; very hyperplastic; foamy or scanty colloid	
Indole-3-methanol		Normal; smaller follicles; columnar cells	Small follicles, columnar cells	
Vitamin U		Normal; smaller follicles;		

*All the compounds were administered sc except where indicated.
†Hyperplastic refers to an increase in the number of cells: cells are epithelial cells lining the thyroid follicles.

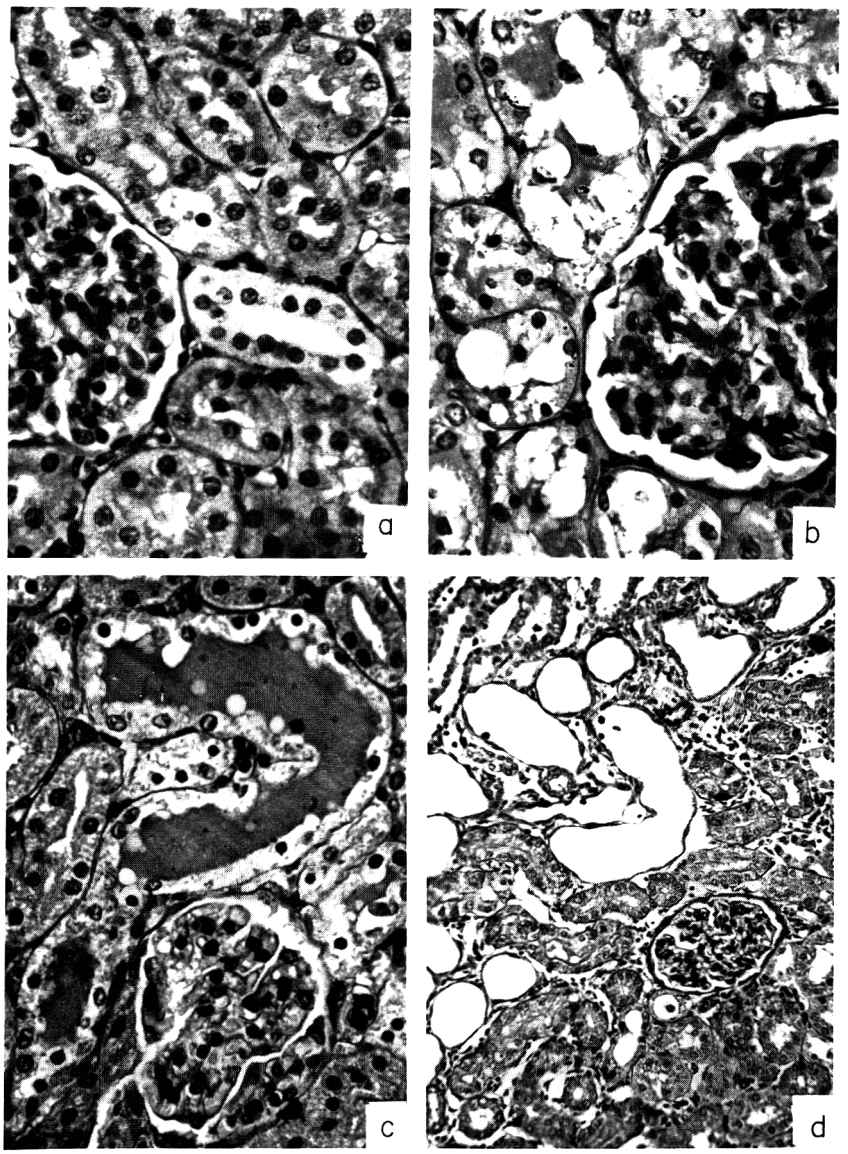


Fig. 1. Kidney sections of (a) a control rat; (b) a pregnant rat that died 27 hr after treatment sc with 95 mg 1-cyano-3,4-epithiobutane/kg showing vacuolated tubular cells; (c) a pregnant rat that died on the third day after treatment with 95 mg 1-cyano-3,4-epithiobutane/kg showing dilated tubules and necrosis of tubular cells; (d) a pregnant rat killed 12 days after treatment with 95 mg 1-cyano-3,4-epithiobutane/kg showing dilated tubules, lined with flat epithelial cells, and interstitial infiltration of lymphocytes. All the kidney sections were stained with periodic acid–Schiff and haematoxylin, a,b,c, × 400, d, × 160.

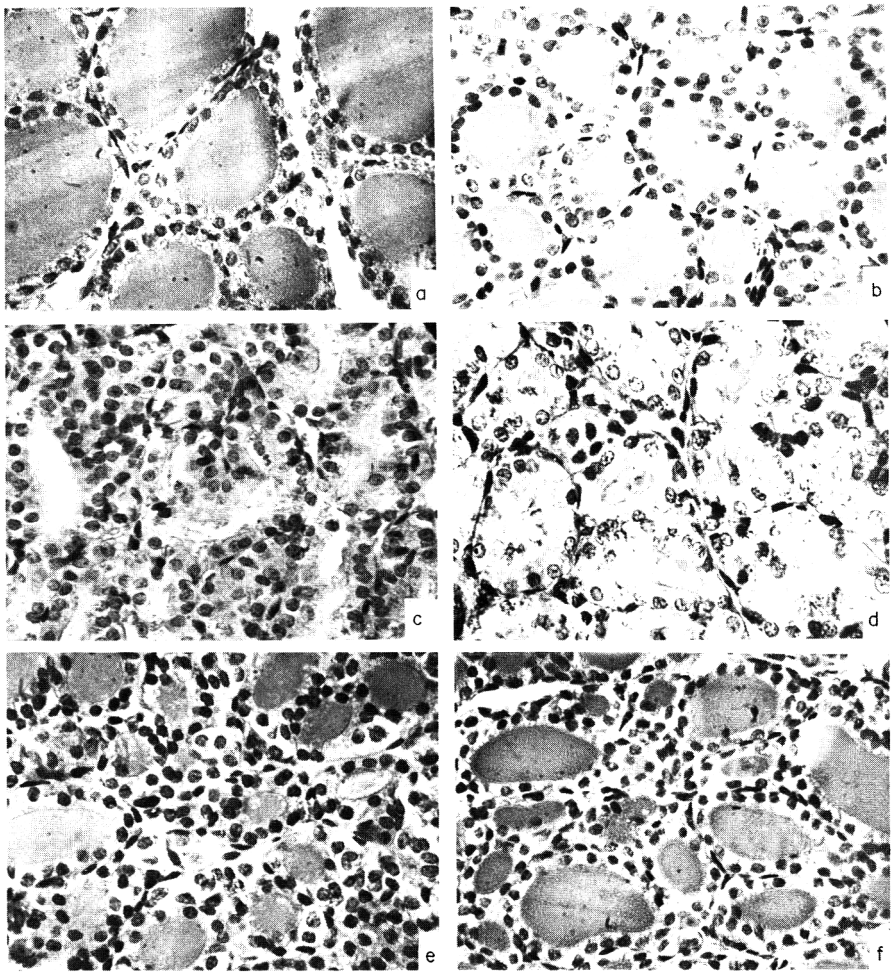


Fig. 2. Thyroid sections of (a) a control pregnant rat on day 20 of gestation; (b) a pregnant rat on day 20 of gestation, 12 days after two daily sc doses of 320 mg epiprogoitrin/kg showing an essentially normal thyroid; (c) a nonpregnant rat 12 days after an sc dose of 175 mg *S*-1-cyano-2-hydroxy-3-butene/kg showing columnar epithelial cells lining the follicles and lumens without colloid or with foamy colloid; (d) a male rat, 12 days after an sc dose of 200 mg *R*-goitrin/kg showing epithelial cell hyperplasia and scanty colloid; (e) a pregnant rat on day 20 of gestation, 12 days after an sc dose of 200 mg *R*-goitrin/kg showing smaller more cellular follicles than those of the control thyroid; (f) a pregnant rat on day 20 of gestation, 12 days after an sc dose of 95 mg 1-cyano-3,4-epithiobutane showing an essentially normal thyroid. All thyroid sections were stained with periodic acid–Schiff and haematoxylin × 400.

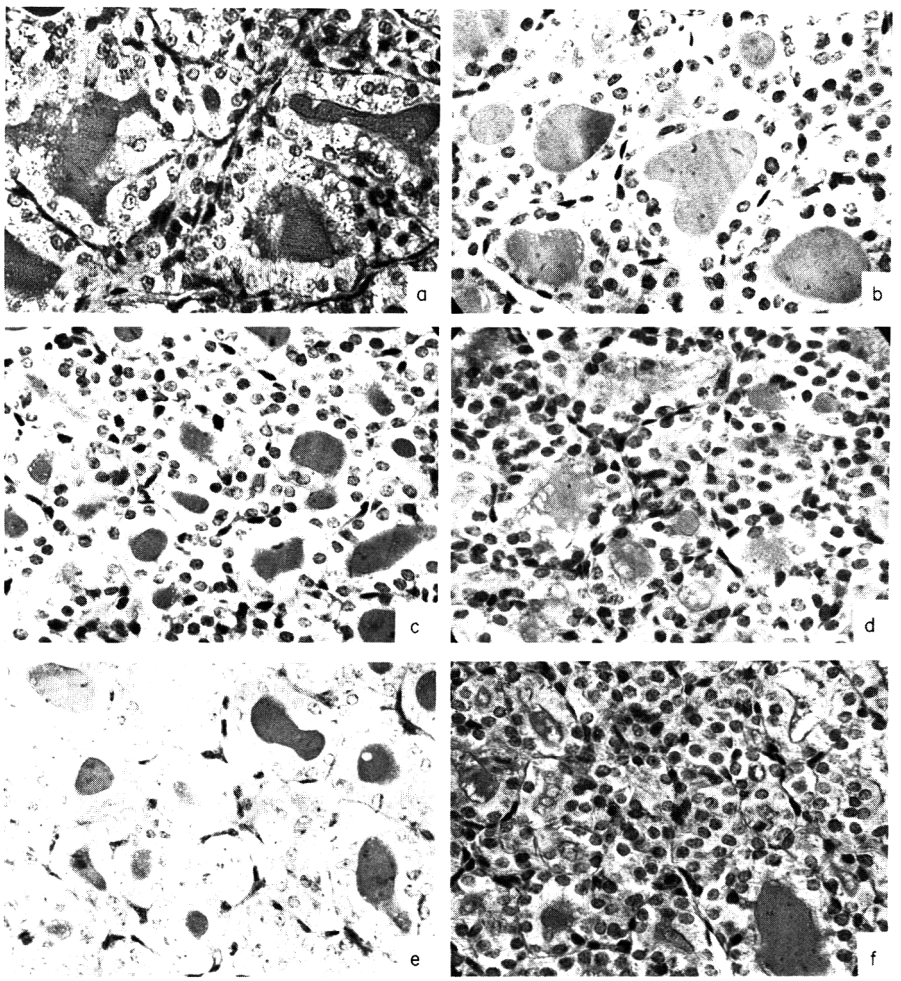


Fig. 3. Thyroid sections of (a) a male rat 12 days after two daily sc doses of 322 mg sinigrin/kg showing relatively large follicles lined with columnar epithelial cells; (b) a nonpregnant rat 12 days after two sc doses of 322 mg sinigrin/kg showing smaller follicles than the control lined with slightly taller epithelial cells; (c) a pregnant rat on day 20 of gestation, 12 days after two daily sc doses of 100 mg allyl thiocyanate/kg showing uniformly smaller follicles lined with tall epithelial cells; (d) a pregnant rat on day 20 of gestation, 12 days after two daily sc doses of 300 mg sinalbin hydrate/kg showing slightly smaller, more cellular follicles than the controls; (e) a nonpregnant rat, 12 days after two daily sc doses of 200 mg iberin nitrile/kg showing uniformly tall epithelial cells lining the follicles, scanty colloid, and some follicles with vacuoles; (f) a nonpregnant rat 12 days after an sc dose of 150 mg 3-indolylacetonitrile/kg showing extremely hyperplastic and cellular thyroid and scanty colloid. All thyroid sections were stained with periodic acid–Schiff and haematoxylin, × 400.

nitrile at pH 3-4 and indole-3-methanol and other compounds at pH 7 (Michajlovskij & Langer, 1967; Virtanen, 1965). 3-Indolylacetonitrile is a plant growth hormone (Jones, Henbest, Smith & Bentley, 1952), but in *in vitro* experiments it inhibited the growth of ascites sarcoma cells (Pilotti, Ancker, Arrhenius & Enzel, 1975). 3-Indolylacetonitrile is present in many plants, e.g. cabbage (Henbest, Jones & Smith, 1953), and in tobacco smoke (Pilotti *et al.* 1975). In rats, 3-indolylacetonitrile and indole-3-methanol induced sedation, ataxia, loss of righting reflex and sleep. These responses were not unexpected, because tryptophol (indole-3-ethanol), a homologue of indole-3-methanol, is known to cause sedation (Bosin, Campaigne, Dinner, Rogers & Maickel, 1976) and enhanced ethanol-induced sleep in mice (Blum, Calhoun, Merritt & Wallace, 1973). 3-Indolylacetonitrile caused marked hyperplasia in nonpregnant rat thyroid (Fig. 3f), but its effect on the thyroid of the pregnant rat was less pronounced. This diminished susceptibility of pregnant rats to the goitrogenic (hyperplastic) action of the nitriles and other compounds may have been due to the higher concentration of oestrogens during pregnancy, since Eskin & Bogdanove (1956) have shown that oestrogens reduced the hyperplastic response of the thyroid to propylthiouracil (goitrogen).

The goitrogenic effect of cabbage in rabbits was discovered by Chesney *et al.* (Chesney, Clawson & Webster, 1928; Webster & Chesney, 1930). The active compound L-5-vinyl-2-thiooxazolidone (goitrin) was identified by Astwood, Greer & Ettlinger (1949); and later, Greer isolated progoitrin, the precursor of goitrin (1956). Other goitrogenic compounds include allyl isothiocyanate and thiocyanate (Langer, 1966; VanEtten, 1969).

Whitelaw (1947) reported a case of human foetal anencephaly due to the maternal treatment of hyperthyroidism with thiouracil. Since thiouracil has goitrogenic and teratogenic properties, the possibility arose that the seasonal increases in human foetal anencephaly and spina bifida were due to the goitrogens present in Cruciferae and other plant-foods. However, the limited number of compounds we tested were not teratogenic, although they did cause varying degrees of thyroid hyperplasia. Further investigations are needed to resolve their possible role in birth defects.

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PATULIN MYCOTOXICOSIS IN THE SYRIAN HAMSTER

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Abstract—Patulin, a mycotoxin produced by species of the genera Penicillium and Aspergillus, was administered to Syrian golden hamsters by oral, sc and ip routes. The greatest number of deaths occurred 13–24 hr after administration. The LD_{50} values by the oral, sc and ip routes were respectively, 31·5, 23 and 10 mg/kg body weight. Gross alterations were few and consisted of gastric, duodenal and caecal hyperaemia in some hamsters. Histopathological alterations included ulceration and inflammation of the stomach and intestines, but these were not directly attributable to patulin. Patulin was also administered orally to hamsters daily or on alternate days for 2 wk at dosages of 50 or 75% of the oral LD_{50}. The mortality of the treated groups was greater than that of the controls, but was similar for all the treated groups. No evidence of cumulative toxicity was found and the gross and histopathological alterations were similar to those found in the LD_{50} studies. The cause of death and the enteric lesions were attributed to alterations of the gastro-intestinal flora by the antibiotic activity of patulin.

INTRODUCTION

Patulin, a mycotoxin produced by species of the fungal genera Penicillium and Aspergillus has been isolated from a wide variety of fruits and vegetables (Frank, 1977). The most consistent and significant source has been apple products contaminated with *P. expansum*, the common storage rot fungus of apples (Brian, Elson, & Lowe, 1956; Wilson & Nuovo, 1973).

The acute toxicity of patulin has been studied in several animals but the LD_{50} values and the lesions observed have varied. Oedema of the lungs and brain, visceral organ congestion and hepatic and renal necrosis have been reported in both rats and mice (Broom, Bülbring, Chapman, Hampton, Thomson, Ungar, Wien & Woolfe, 1944; Escoula, More & Baradat, 1977). In a toxicity study of patulin in rats, the only gross lesion was gaseous distention of the gastro-intestinal tract (Dailey, Brouwer, Blaschka, Reynaldo, Green, Monlux & Ruggles, 1977). Lovett (1972) reported that the 5-day LD_{50} for patulin in chickens was 170 mg/kg body weight and the principal gross lesion was extensive intestinal haemorrhage.

Dickens & Jones (1961) reported that patulin when given by the sc route produced sarcoma at injection sites. However, Osswald, Frank, Komitowski & Winter (1978) found that patulin did not display tumorigenic activity when administered orally to rats.

Because of the limited available morphological data and because observations of anatomical changes have varied greatly, studies were undertaken to define the mycotoxicosis produced by patulin in hamsters, mice and rats. This paper describes the mycotoxicosis produced in the Syrian hamster by patulin given by the oral, sc and ip routes.

EXPERIMENTAL

Animals. Weanling male Syrian golden hamsters (Ela:ENG (SYR)) weighing 51–60 g were used in these studies (Engle Laboratories, Farmersburg, IN). The hamsters were randomly assigned to clear plastic cages with aspen wood shavings for bedding. Feed and water were supplied *ad lib.* The ambient temperature was about 22°C and the relative humidity was 55 ± 5%. Hamsters were maintained under natural lighting with approximately 16 hr daylight and 8 hr darkness. They were transferred to clean cages with fresh bedding twice weekly and were acclimatized to their surroundings for 3 days before the dosing.

Materials. Patulin (lot number U-4847) obtained from the Upjohn Company, Kalamazoo, MI, was dissolved in 0·1 mM-citrate buffer, pH 5·0. It was mixed with the buffer at concentrations of 3 mg/ml for oral administration, at 1·2 mg/ml for ip administration and at 3·9 mg/ml for sc administration. For the multiple dose experiment patulin was given at a concentration of 3 mg/ml buffer; the solution was freshly-prepared every other day. The hamsters were weighed immediately before dosing. For the initial dosing, feed was withheld for 12 hr before administration of the toxin or control solvent. Oral dosing was by gastric intubation using an 18-gauge, 1·5-in., animal-feeding needle. A tuberculin syringe with a 25-gauge needle was used for the sc and ip injections.

Collection of tissues. Hamsters found dead during the experiment and survivors killed at termination by cervical dislocation were autopsied. Multiple tissues were fixed in neutral buffered 10% formalin. Fixed tissues were dehydrated, embedded in paraffin, sectioned and stained with haematoxylin and eosin for histopathological examination.

Design of experiments

Trial I. The single-dose toxicity of patulin was evaluated after oral, sc and ip administration. Control animals received similar amounts of the solvent. The LD_{50} values and 95% confidence intervals were calculated (Litchfield & Wilcoxon, 1949).

Trial II. Multiple doses of patulin of 50 and 75% of the oral LD_{50} (16 and 23 mg/kg respectively) were given either daily or on alternate days for one week.

Table 1. *Numbers and times of deaths after patulin administration (Trial I)*

Route of administration	Patulin dose (mg/kg body weight)	Time after dosing (hr)...	No. of deaths*		
			0–24	25–48	49–72
Oral	0		0	0	0
	25		2	0	0
	30		3	1	0
	35		7	0	0
Subcutaneous	0		0	0	0
	23		7	0	0
	25		4	0	0
	27		4	0	0
	30		6	2	1
Intraperitoneal	0		0	0	0
	8		1	2	1
	10		1	1	1
	12		5	2	0

*There were ten animals in each group.

The dosages during the second week remained the same or were increased from 16 to 23 mg/kg and the frequency of dosing remained the same or was increased from alternate days to daily dosing. Ten animals were randomly assigned to each group and three control groups received the solvent.

Trial III. Several authors have reported pulmonary oedema as a manifestation of patulin toxicosis in rats and mice, but we did not observe this lesion in our hamsters killed 72 hr after a single dose or in hamsters given multiple doses of the mycotoxin. To determine if pulmonary oedema was a transitory change in hamsters or if the method of tissue handling affected the occurrence of pulmonary oedema, two groups of 20 hamsters were given the oral LD_{50} doses of 31·5 mg/kg body weight. Ten animals served as controls for each group. One group was killed 6 hr after administration and the lungs of ten of the hamsters were fixed by immediate immersion. The lungs of the other ten animals were infused with fixative *in situ* and expanded to normal volume. The trachea was then tied with a string, and the thoracic viscera were removed *in toto* and placed in fixative. The second group was killed 12 hr after administration and the same procedures were used in handling the lungs. The lungs were prepared for histological examination as previously described.

RESULTS

Trial I

Clinical signs were similar regardless of the route of administration or the dose administered. Within a minute of administration, treated and control animals showed signs of agitation and irritation manifested by rapid movement around the cage. This activity decreased within 10–15 min and the hamsters assumed a grouped and huddled posture.

Beginning 60–90 min after administration, the hamsters again moved about the cage and dyspnoea was observed in most of the patulin-dosed hamsters. The dyspnoea became more severe with time in some hamsters. Arching of the back and a roughened coat were prominent signs. Within 10–24 hr after dosing the hamsters either recovered or the signs persisted and progressed to death.

Most of the treated hamsters that died did so within 24 hr of patulin administration. The route of administration did not greatly modify this pattern (Table 1) except that more deaths occurred beyond

Table 2. *Gross and histopathological alterations in hamsters given a single dose of patulin (Trial I)*

Route	Patulin dose (mg/kg body weight)	No. of animals with gross alterations*			No. of animals with microscopic alterations*		
		Gastric hyperaemia	Duodenal hyperaemia	Caecal hyperaemia	Gastritis	Enteritis	Caecitis
Oral	0	0	0	0	0	0	0
	25	2	1	0	2	0	0
	30	2	0	0	1	0	1
	35	2	0	1	5	3	1
Subcutaneous	0	0	0	0	0	0	0
	23	7	7	1	0	1	1
	25	3	3	1	5	2	0
	27	4	4	1	1	0	2
	30	7	0	1	1	1	0
Intraperitoneal	0	0	0	0	0	0	0
	8	0	0	0	1	0	0
	10	1	0	2	2	0	2
	12	7	4	0	2	1	1

*There were ten animals in each group.

Fig. 1. Ulceration of the glandular stomach with epithelial cell degeneration and necrosis in the hamster. Haematoxylin and eosin (H & E) × 88.

Fig. 2. Ulceration, haemorrhage, epithelial cell necrosis and desquamation of epithelial cells of the caecum of hamster. H & E × 88.

24 hr after administration in the group given patulin by the ip route than in other treated groups.

LD_{50} determination. The single-dose 72 hr-LD_{50} values and their 95% confidence intervals were as follows: oral, 31·5 (27·4–35·0); sc, 23·0 (19·5–27·1); ip, 10 (7·6–13·2) mg/kg body weight. The values were similar to those previously reported in other rodent species and confirm prior observations that the LD_{50} value was greatest for oral administration and least for the ip route.

Pathology. Gross lesions in hamsters were essentially limited to the gastro-intestinal tract (Table 2). Diffuse mucosal hyperaemia of the glandular stomach, duodenum and caecum was seen in all groups, but ulceration of the caecal mucosa was present only in hamsters given patulin by the oral route. Five animals in the ip dose group had haemorrhages in the peri-testicular fat and this was attributed to injection trauma.

The principal histopathological alterations were in the gastro-intestinal tract (Table 2). Erosion and ulceration of the mucosa of the glandular portion of the stomach was present in some hamsters of groups treated by the three routes of administration (Fig. 1). These lesions varied in severity and were characterized by epithelial cell degeneration and necrosis.

Duodenal lesions varied from a mild catarrhal to an ulcerative enteritis and were characterized by epithelial cell necrosis, cytoplasmic vacuolization, desquamation of epithelial cells and increased mucus production. The lamina propria contained increased numbers of neutrophils and lymphocytes and hyperaemia varied from mild to marked. The mucosa was ulcerated in two of the treated hamsters. The caecal alterations were similar to those observed in the duodenum (Fig. 2).

Incidental lesions included epididymitis and peritesticular steatitis in four hamsters treated by the ip route. Also, bronchiolitis, bronchopneumonia, pericholangitis and focal hepatic necrosis were found in treated hamsters and control animals with a similar prevalence and severity. A mild, chronic, diffuse tracheitis characterized by infiltration of the mucosa by mononuclear cells and mild dilation of submucosal glands was present in both treated and control animals.

Trial II

The clinical signs were similar to those observed in the single-dose study and no differences were found between the groups receiving 50% and those given 75% of the oral LD_{50}. Dyspnoea, piloerection, arching of the back and dispersion within the cage began 3–4 hr after administration. All surviving test hamsters had recovered by the fifth day of treatment. Some hamsters died without clinical signs of toxicity being observed. Loss of body weight was seen in all groups given patulin (Fig. 3) and was apparent within 48 hr of the first dose. The greatest number of deaths (71%) occurred 1 to 2 days after administration of patulin (Table 3).

Gross lesions were found principally in the stomach; duodenal distention was observed in three

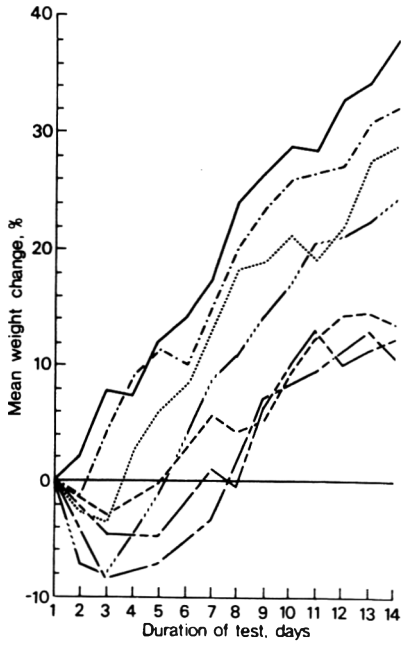

Fig. 3. Mean body-weight changes in hamsters given patulin daily (D) or on alternate days (AD) at doses of 50 and 75% of the oral LD_{50} (16 and 23 mg/kg body weight respectively) by gastric intubation. The groups were as follows: control (——), 16 mg/kg (D) on days 1–14 (–·–), 23 mg/kg (D) on days 1–14 (·····), 16 mg/kg (D) on days 1–7 then 23 mg/kg (D) on days 7–14 (–·····), 16 mg/kg (AD) on days 1–7 then 23 mg/kg (D) on days 7–14 (– – –), 23 mg/kg (AD) on days 1–7 then 23 mg/kg (D) on days 7–14 (————) and 23 mg/kg (AD) on days 1–14 (– –).

Table 3. *No. of deaths, and gross and histopathological alterations in hamsters given multiple doses of patulin (Trial II)*

Frequency and size of dose (mg/kg body weight)		Time after dosing (days)...	No. of deaths*			No. of animals with gastric hyperaemia*	No. of animals with microscopic alterations*	
Wk 1	Wk 2		1–2	3–7	8–14		Gastritis	Enteritis
0	0		0	0	0	0	0	0
D(16)	D(16)		3	0	0	1	1	2
D(23)	D(23)		3	0	1	1	1	2
D(16)	D(23)		3	0	1	0	3	2
AD(16)	D(23)		2	0	1	1	3	2
AD(23)	AD(23)		2	2	1	2	4	4
AD(23)	D(23)		4	0	1	4	3	3

D = Daily AD = On alternate days

*There were 30 control animals and ten animals in each treatment group.

treated hamsters (Table 3). The serosa of the stomach was grey and the glandular mucosa was red-black and mildly thickened. The duodenum was moderately distended with fluid.

The histopathological alterations related to compound administration were observed in the gastro-intestinal tract (Table 3). Gastric lesions, found in certain hamsters of all treated groups, consisted of haemorrhages within the glandular mucosa and ulceration of the lamina epithelialis of the mucosa with inflammatory cell infiltration of the lamina propria. Lesions occurred in all patulin-treated groups, but could not be correlated with dose or frequency of administration. Hyperaemia, catarrhal exudate, mucosal ulceration and inflammatory cell infiltrates within the lamina propria were present in the duodenum of certain test hamsters and were not found in controls.

Pericholangitis characterized by a mononuclear cell infiltration, principally by lymphocytes in the portal areas, and bronchiolitis and bronchopneumonia were observed in both treated and control hamsters with similar prevalence and severity.

Sections of brain, heart, thyroid, thymus, oesophagus, bladder, spleen, testicle, adrenal, pancreas, bone marrow, eye and kidney were not histologically remarkable.

Trial III

No pulmonary oedema was observed in patulin-treated hamsters killed at 6 or 12 hr after treatment in lungs fixed either by immersion or by intratracheal perfusion.

DISCUSSION

Patulin was most toxic to the hamster when given by the ip route and was least toxic by the oral route. The toxicity by various routes of administration was in general similar for the hamster, the mouse and the rat, but the LD_{50} values of individual reports have varied. The oral LD_{50} value of 31·5 mg/kg obtained for the hamster in this study was similar to that reported for the rat (Dailey *et al.* 1977a; Escoula *et al.* 1977) and the mouse (Broom *et al.* 1944), but was less than that reported by Escoula *et al.* (1977) for the female mouse (46·3 mg/kg). The sc LD_{50} of 23 mg/kg and the ip LD_{50} of 10 mg/kg obtained for patulin in the hamster were above some values for these routes reported for the rat and the mouse (Broom *et al.* 1944; Ciegler, Beckwith & Jackson, 1976; Katzman, Hays, Cain, van Wyk, Reithel, Thayer, Doisy, Wade, Gaby, Carroll, Muir & Jones, 1944; Escoula *et al.* 1977) and below other reported values for these species (Andraund, Tronche & Couquelet, 1964; Broom *et al.* 1944; Dailey *et al.* 1977a; Hofmann, Mintzlaff, Alperden & Leister, 1974; Katzman *et al.* 1944; Raistrick, Birkinshaw, Bracken, Michael, Hopkins & Gye, 1943).

Some hamsters given a single dose of patulin died, and usually within 24 hr of administration. The number of deaths was dose-dependent by all routes, but this was less evident in the group given patulin by the sc route. Several deaths, occurring more than 24-hr after administration, as observed in our hamsters given patulin by the ip route, were also observed in the mouse (Escoula *et al.* 1977). Most of the deaths in the multiple-dose study occurred within 1 to 2 days of patulin administration.

The clinical signs of toxicity included listlessness, piloerection, reduced spontaneous activity and isolation and separation from other hamsters within the same cage. These cannot be considered specific for patulin as they are general signs of toxicosis in rodents. These signs were not seen in all the hamsters as some hamsters were found dead without signs of toxicity being observed. Escoula *et al.* (1977), Broom *et al.* (1944) and Raistrick *et al.* (1943) reported that mice treated with patulin had convulsions prior to death, but convulsions were not observed in our treated hamsters.

Data from the 14-day multiple-dose study indicated no cumulative toxicity, and susceptibility was similar regardless of the frequency of administration. Hamsters not dying early in the dosing period appeared to develop a tolerance, because few deaths occurred during the second week of treatment. These results are similar to those reported by Freerksen & Bönicke (1951) who found no cumulative toxicity in mice after 8 days of treatment, but differ from the report of Broom *et al.* (1944) who described cumulative toxicity after 2 wk of patulin treatment. An initial loss in body weight was attributed to dehydration. This weight-loss was followed by recovery of body weight by day 3 for most groups since most of the 'susceptible' hamsters had died.

The gross lesions in patulin-treated hamsters were hyperaemia of the stomach and intestinal tract. Hyperaemia of the gastro-intestinal tract was previously reported for the rat and the mouse (Broom *et al.* 1944; Escoula *et al.* 1977; Katzman *et al.* 1944) and in chickens (Lovett, 1972). The histopathological alterations were those of gastroenteritis, but such lesions were not found in all treated hamsters and a dose-effect relationship was not evident. Escoula *et al.* (1977) reported ulceration and haemorrhages in the stomach and jejunum of rats and jejunal ulcerations in mice given patulin. We could not substantiate pulmonary oedema as a feature of patulin toxicosis in the hamster, although this lesion has been described in the mouse (Escoula *et al.* 1977) and the rat (Broom *et al.* 1944).

The exact mechanism of toxic action by patulin is undetermined. Patulin is rapidly metabolized and eliminated via the faeces and in the urine; 70% was eliminated within 24 hr and 79% within 72 hr, the major retention site for ^{14}C activity being the erythrocyte (Daily, Blaschka & Brouwer, 1977). The early deaths of patulin-treated hamsters were probably due to unmetabolized patulin.

Mortality and the gastro-intestinal lesions can be attributed to patulin's antibiotic activity resulting in changes in the bacterial flora of the intestinal tract. Patulin, as an antibiotic, inhibits gram-positive organisms (Singh, 1967). Such antibiotics as lincomycin hydrochloride and clindomycin when administered to hamsters caused a marked overgrowth of enteric gram-negative organisms and histopathological alterations in the gastro-intestinal tract similar to those seen in our hamsters (Lusk, Fekety, Silva, Browne, Ringler & Abrams, 1978; Small, 1968). These investigators speculated that death of antibiotic-treated hamsters was due to an enterotoxaemia produced by

the overgrowth of gram-negative organisms. Such a mechanism appears to be the most likely one for the toxicity of patulin observed in the hamsters of this study.

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PATULIN MYCOTOXICOSIS IN SWISS ICR MICE

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Abstract—Patulin is a toxic metabolite of several species of the genera Aspergillus and Penicillium. When administered to 10–15-g Swiss ICR mice by the oral, sc and ip routes, the 72-hr LD_{50} values were: oral 48·0, sc 10·0 and ip 7·5 mg/kg body weight. Gross lesions consisted of gastric and duodenal hyperaemia and intestinal distention. Histopathological lesions included ulceration and inflammation of the stomach and intestines. Mortality was greatest 0–24 hr post-administration when patulin was administered by oral and sc routes, but most deaths occurred after 49–72 hr in the ip-dosed group. When patulin was administered orally to mice daily or on alternate days for 2 wk at doses of 50 or 75% of the oral LD_{50} mortality was dose-dependent and no cumulative toxicity was indicated. The cause of death and the lesions were attributed to enterotoxaemia from alteration of the gastro-intestinal flora by patulin's antibiotic activity.

INTRODUCTION

Patulin is a metabolite of several species of the genera Penicillium and Aspergillus (Abraham & Florey, 1949) which are natural fungal contaminants of various foods and grains. Isolation of patulin has been most frequent from apples rotted with *P. expansum* (Brian, Elson & Lowe, 1956; Harwig, Chen, Kennedy & Scott, 1973; Wilson & Nouvo, 1973), but other patulin-contaminated foods include pears, peaches, apricots, bananas, pineapples, grapes, plums and sweet cherries (Buchanan, Sommer, Fortlage, Maxie, Mitchell & Hsieh, 1974; Frank, 1971).

The initial interest in patulin as an antibiotic has given way to a concern for its role as a mycotoxin as its presence in the environment has become known. In addition to the concern for its acute toxicity, patulin was reported to produce sarcomas at injection sites in rats (Dickens & Jones, 1961). However, patulin was not found to be a carcinogen in rats when administered orally for 64 wk (Osswald, Frank, Komitowski & Winter, 1978).

The acute toxicity of patulin for mice has been studied by several authors, but the results have varied among investigators. Lesions ranging from diffuse congestion and pulmonary oedema (Broom, Bülbring, Chapman, Hampton, Thomson, Ungar, Wien & Woolfe, 1944; Katzman, Hays, Cain, Van Wyk, Reithel, Thayer, Doisey, Wade, Gaby, Carroll, Muir & Jones, 1944) to abscesses in different organs (Bollag, 1949) have been reported. Escoula, More & Baradat (1977) reported necrosis of renal tubules and jejunal mucosal necrosis as well as diffuse congestion and haemorrhages of the kidney, liver, lung and jejenum in mice given patulin.

The results of subacute studies have also been contradictory. Broom *et al.* (1944) noted evidence of cumulative toxic action in mice given 25 mg/kg orally daily for 2 wk. However, Freerksen & Bönick (1951) found that mice tolerated 0·6 mg per animal (c. 30 mg/kg for a 20-g mouse) given orally every 3 hr for 8 days and no deaths occurred. De Rosnay, Martin-Dupont & Jensen (1952) injected patulin sc at a dose of 0·1 mg/mouse daily for 26 wk and observed hepatic necrosis and renal and pulmonary congestion.

These studies were undertaken to define the acute mycotoxicosis produced in mice by patulin given by oral, sc and ip routes and to determine the effects of multiple oral doses of patulin.

EXPERIMENTAL

Animals. Weanling male Swiss mice of the ICR strain weighing 10–15 g were used (Harlen Industries, Cumberland, IN). Mice were randomly assigned to clear plastic cages with ground corn cobs (Bed-o-Cobs, Anderson Cob Mills, Anderson, IN) as bedding material. Water and feed (Wayne Laboratory Animal Diet, Allied Mills, Castleton, IN) were supplied *ad lib.* The ambient temperature was about 22°C and the relative humidity was 55 ± 5%. Mice were maintained under natural lighting with approximately 16 hr daylight and 8 hr darkness. Mice were transferred to clean cages with fresh bedding twice weekly and were acclimatized to their surroundings for 3 days before dosing.

Materials. Purified patulin (lot number U-4847, Upjohn Company, Kalamazoo, MI) was dissolved in 0·1 mM-citrate buffer, pH 5·0. It was mixed at concentrations of 2 mg/ml (oral), 0·55 mg/ml (ip) and 0·55 mg/ml (sc). For the multiple-dose experiment, patulin was mixed at a concentration of 1·34 mg/ml every other day. The mice were weighed immediately before dosing. For the initial dosing, feed was withheld for 12 hr before toxin or control solvent administration. Oral dosing was by gastric intubation using an 18-gauge, 1·5-in. animal-dosing needle. A tuberculin syringe with a 25-gauge needle was used for the sc and ip injections.

Collection of tissues. Mice found dead during the experiment and survivors killed at termination by cervical dislocation were autopsied. Multiple tissues were fixed in neutral buffered 10% formalin. Fixed tissues were dehydrated, embedded in paraffin, sectioned and

stained with haematoxylin and eosin for histopathological examination.

Design of experiments

Trial I. The single-dose toxicity of patulin was evaluated after oral, sc and ip administration. Control animals received similar amounts of the solvent. The LD_{50} values and 95% confidence intervals were calculated (Litchfield & Wilcoxon, 1949).

Trial II. Multiple doses of patulin of 50 and 75% of the oral LD_{50} (24 and 36 mg/kg respectively) were given either daily or on alternate days for 1 wk. The doses for the second week remained the same or were increased from 24 to 36 mg/kg and the frequency of dosing remained the same or was increased to daily administration. Ten animals were randomly assigned to each group and one control group received the solvent at a volume equivalent to the greatest quantity used for trial test groups.

RESULTS

Trial I

Clinical signs were similar regardless of the route of administration, but were more severe with more immediate onset in groups given the larger doses of patulin. Beginning several minutes after administration, control and treated animals exhibited signs of agitation and irritation manifested by rapid movement around the cage and by salivation in the mice dosed orally with patulin. This activity decreased within 5–10 min and the mice assumed a huddled posture and were grouped together.

Beginning 45–60 min after patulin administration, the treated mice again moved about the cage and some were hyperactive. Respiration rates increased and progressed to dyspnoea in some mice. The mice became dispersed within the cage and some became listless, developed arching of the back, anorexia, a roughened pelage and distended flaccid abdomens. Within 6–24 hr post-dosing, affected mice either recovered or the signs persisted and progressed to death.

Most of the mice that were given patulin by the oral and sc routes and that died did so within 24 hr of patulin administration (Table 1). Death was delayed in mice given patulin by the ip route as 39% of the deaths were within 25–48 hr of the patulin administration and 48% of the deaths occurred 49–72 hr after administration.

LD_{50} determination. The single-dose 72-hr LD_{50} values and their confidence intervals were as follows: oral: 48·0 (35·8–64·3); sc 10·0 (7·7–13·0); ip 7·5 (5·4–10·5) mg/kg body weight. These values confirm reports by other authors that patulin is most toxic by the ip route and least toxic by the oral route.

Pathology. The principal gross alterations were in the gastro-intestinal tract (Table 2). Hyperaemia of the intestinal mucosa, principally in the duodenum, was found in some mice of all treated groups. In mice killed or dying immediately before autopsy, gaseous distention of the intestinal tract was present in some mice of all treated groups administered patulin by the oral and ip routes and in mice of three of the five groups given patulin by the sc route. Gastric hyperaemia was observed only in the two highest dosage groups of mice dosed ip. Cutaneous and subcutaneous necrosis and oedema were present at the injection sites in some mice of all ip groups. Two animals in the ip-dosed groups had peritonitis and this lesion was attributed to injection trauma.

The principal histopathological alterations were in the gastro-intestinal tract (Table 2). Erosion and ulceration of the mucosa of the glandular portion of the stomach and submucosal oedema were present only in mice dosed orally with patulin (Fig. 1). These lesions were not dose-related and were characterized by epithelial cell degeneration and necrosis.

Duodenal lesions were mild and varied from a non-suppurative to a catarrhal enteritis (Fig. 2). They were characterized by increased numbers of neutrophils, lymphocytes and macrophages in the lamina propria, congestion of proprial blood vessels, epithelial cell necrosis and desquamation, cytoplasmic vacuolization and increased mucus production. Some leucocytes within the lamina propria were necrotic as evi-

Table 1. *Number of mice dying and time to death after patulin administration (Trial I)*

Route of administration	Patulin dosage group* (mg/kg body weight)	Time after dosing (hr)...	No. of deaths* 0–24	25–48	49–72
Oral	0		0	0	0
	35		2	1	0
	40		3	0	0
	45		6	0	0
Subcutaneous	0		0	0	0
	6		0	0	0
	7		4	0	0
	8		5	0	0
	9		4	0	0
	10		6	0	0
Intraperitoneal	0		0	0	0
	5		0	1	1
	7		3	1	0
	10		0	5	5
	15		0	2	5

*There were ten animals in each group.

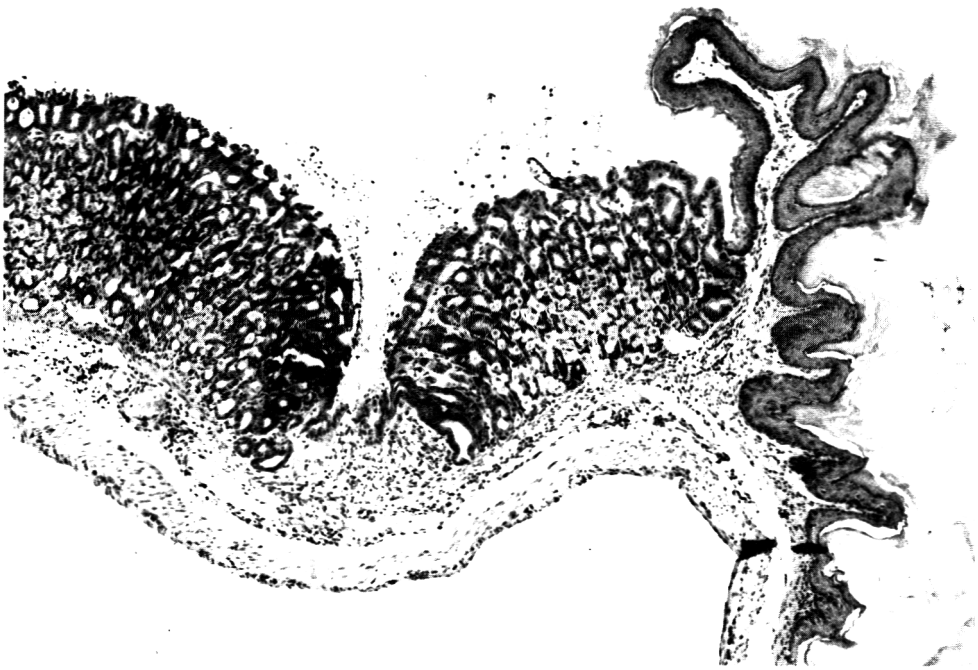

Fig. 1. Ulceration, necrosis and neutrophilic infiltration in the glandular mucosa of the stomach of a mouse. Haematoxylin and eosin (H & E) × 56.

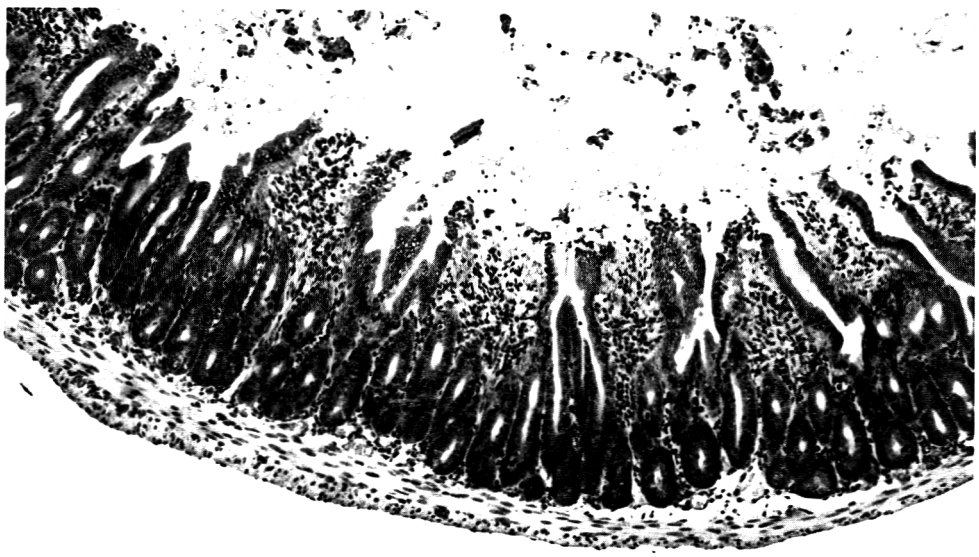

Fig. 2. Vascular congestion, prominent acute inflammatory cell infiltration of the lamina propria, disruption and desquamation of surface epithelial cells of the duodenum. H & E × 88.

Table 2. *Gross and histopathological alterations in mice given a single dose of patulin (Trial I)*

Route	Patulin dose (mg/kg body weight)	No. of animals with gross alterations*			No. of animals with microscopic alterations*	
		Gastric hyperaemia	Intestinal hyperaemia	Intestinal distention	Enteritis	Gastritis
Oral	0	0	0	0	0	0
	35	0	1	2	1	3
	40	0	1	1	3	2
	45	0	1	1	5	1
Subcutaneous	0	0	0	0	0	0
	6	0	1	0	3	0
	7	0	3	3	5	0
	8	0	3	2	1	0
	9	0	2	0	2	0
	10	0	2	1	3	0
Intraperitoneal	0	0	0	0	0	0
	5	0	2	2	1	0
	7	0	1	2	3	0
	10	1	3	2	3	0
	15	1	3	2	3	0

*There were ten animals in each group.

denced by nuclear pyknosis, karyorrhexis and karyolysis.

Peritonitis characterized by fibrin strands and degenerating leucocytes on the intestinal serosa was observed in three ip-dosed mice and was attributed to injection trauma. Other incidental lesions included a bronchopneumonia and bronchiolitis and these were observed in treated and control mice with a similar severity and prevalence.

Trial II

The clinical signs were similar to those observed in the single-dose study, although the signs were less severe and the time of onset was delayed. Roughened pelage, increased respiratory rates, arched back, anorexia, lethargy, distended abdomens and dispersion within the cage began 1–2 days after the start of administration. Clinical signs decreased in severity until day 6 or progressed to death. Some mice died without showing clinical signs of toxicity. An initial loss of body weight was observed in mice given daily doses of patulin and the weight-loss was observed within 24 hr in the group given 36 mg/kg body weight and within 48 hr in the group given 24 mg/kg body weight (Fig. 3). The greatest number of deaths (64%) occurred during the period from day 3 to 7 after the start of administration (Table 3).

Gross lesions were principally of the stomach and small intestine. The mucosa of the glandular stomach was hyperaemic and was either thin-walled or mildly thickened. The duodenum and jejunum were hyperaemic and distended with fluid or gas.

Histopathological alterations were observed in the stomach and duodenum. In the gastric mucosa of some mice of all treated groups, the lesions were epithelial cell degeneration, haemorrhages, and ulceration or erosion of the mucosal epithelium with neutrophilic and mononuclear cell infiltration of the lamina propria. The prevalence and severity of the lesions could not be correlated with patulin dose or frequency of administration. Duodenal lesions were catarrhal exudate, epithelial cell desquamation and inflammatory cell infiltrates within the lamina propria.

Sections of brain, heart, liver, kidney, trachea, thyroid, thymus, oesophagus, bladder, spleen, testicle,

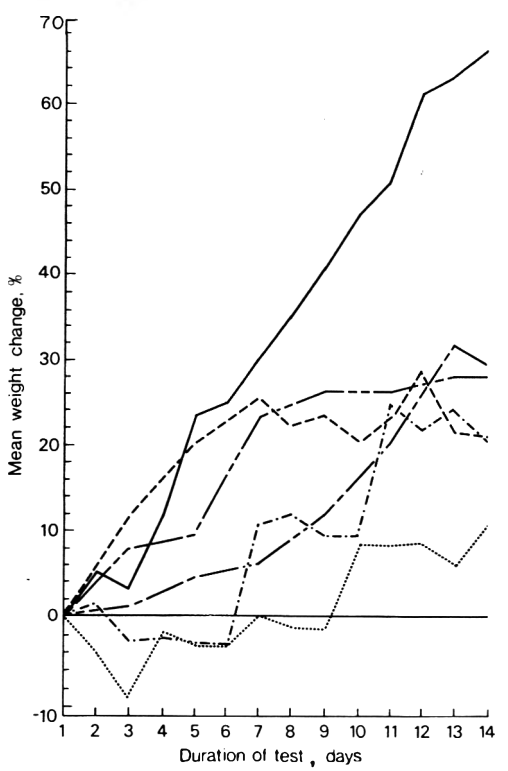

Fig. 3. Mean body-weight changes in mice given patulin daily (D) or on alternate days (AD) at doses of 50 and 75% of the oral LD_{50} (24 and 36 mg/kg body weight respectively) by gastric intubation for 14 days. The groups were as follows: control (——), 24 mg/kg (D) on days 1–14 (–·–), 36 mg/kg (D) on days 1–14 (···), 24 mg/kg (AD) on days 1–7 and 36 mg/kg (D) on days 7–14 (– – –), 24 mg/kg (AD) on days 1–14 (— — —) and 36 mg/kg (AD) on days 1–14 (— – —).

Table 3. *No. of deaths and gross and histopathological alterations in mice given multiple oral doses of patulin (Trial II)*

Frequency and size (mg/kg body weight) of dose		Time after administration (days)...	No. of deaths*			No. of animals with gross alterations*			No. of animals with microscopic alterations*	
Wk 1	Wk 2		1–2	3–7	8–14	Gastric hyperaemia	Intestinal hyperaemia	Intestinal dilation	Gastritis	Enteritis
0	0		0	0	0	0	0	0	0	0
D(24)	D(24)		0	5	1	1	2	2	1	2
D(36)	D(36)		0	6	1	3	3	2	3	3
AD(24)	AD(24)		0	0	2	1	1	0	1	2
AD(36)	AD(36)		0	2	2	2	2	0	3	4
AD(24)	AD(36)		1	1	1	1	2	1	3	4

D = Daily AD = On alternate days

*There were ten animals in each group.

adrenal and pancreas were histologically not remarkable.

DISCUSSION

Patulin was most toxic for the mouse when administered by the ip route and was least toxic by the oral route. The relative toxicity of patulin in mice according to route of administration was in general similar to that reported by other investigators (Broom *et al.* 1944; Escoula *et al.* 1977; Katzman *et al.* 1944). The oral LD_{50} value of 48 mg/kg obtained in this study for the mouse was higher than that reported by other authors, but the values previously reported generally fall within our confidence intervals (Broom *et al.* 1944; Escoula *et al.* 1977; Katzman *et al.* 1944). The sc LD_{50} of 10 mg/kg and the ip LD_{50} of 7·5 mg/kg were above those reported by Katzman *et al.* (1944). Ciegler, Beckwith & Jackson (1976) and Lochhead, Chase & Landerkin (1946), and below other reported values for the mouse (Broom *et al.* 1944; Escoula *et al.* 1977; Hofmann, Mintzlaff, Alperden & Leistner, 1971; Raistrick, Birkinshaw, Bracken, Michael, Hopkins & Gye, 1943).

Mortality was dose-dependent in groups treated by all routes, but this relationship was less evident in the groups treated ip. A longer survival time after ip-dosing of patulin was observed in mice (Escoula *et al.* 1977) and in hamsters (McKinley & Carlton, 1980).

The data from the 14-day multiple-dose study indicated no cumulative toxicity. The response to patulin administration was similar at doses that were either 50 or 75% of the oral LD_{50}, but the number of deaths was greater during the first week for rats dosed daily than for those dosed on alternate days. The mice dosed daily that did not die during the first week appeared to develop a tolerance; few deaths occurred during the second week of treatment.

The initial loss of body weight observed in groups administered patulin daily (Fig. 3) was attributed to dehydration and some weight was regained after 7–10 days. The loss in body weight and time required for recovery was similar to that reported (Escoula *et al.* 1977) after a single oral dose of patulin (minimum lethal dose).

Clinical signs of toxicity included dyspnoea, anorexia, lethargy, arching of the back, roughened pelage and listlessness and as general signs of toxicoses in rodents cannot be considered specific for patulin mycotoxicosis Escoula *et al.* (1977) reported neuromuscular difficulties in the rear legs and cyanosis of the feet, tail and ears in patulin-treated mice, but these signs were not observed in our treated mice.

Gross lesions as seen in the intestinal tract of our patulin-treated mice have been reported previously in the mouse (Escoula *et al.* 1977; Katzman *et al.* 1944), the rat (Escoula *et al.* 1977), the chicken (Lovett, 1972) and the hamster (McKinley & Carlton, 1980). Such gross lesions as ascites, hydrothorax and pulmonary haemorrhage were reported by others (Broom *et al.* 1944; Escoula *et al.* 1977) but were not observed in our mice.

Histopathological alterations included enteritis in some mice of all patulin-treated groups and gastritis in those orally dosed, but a dose-effect relationship was not evident. The gastritis, as it was limited to

those orally-dosed, could have been due to the irritative effect of patulin on the gastric mucosa, as others (Escoula *et al.* 1977) have reported ulceration and necrosis of the jejunum in mice and gastro-intestinal ulceration in the rat after patulin administration. Haemorrhages and necrosis of the lung, liver and kidney of the mouse and rat as reported by others (Escoula *et al.* 1977) were not observed by us.

The biochemical mechanism of patulin toxicity is unknown. Patulin reacts with sulphydryl compounds (Atkinson & Stanley, 1943; Du Rosnay *et al.* 1952; Geiger & Conn, 1945) and may do so with sulphydryl groups of critical enzymes (Cavallito & Bailey, 1944; Geiger & Conn, 1945). The toxicity of patulin may be related to its antibiotic activity and the change in the intestinal flora because patulin inhibits a wide variety of bacterial organisms (Singh, 1967). Other antibiotics such as lincomycin hydrochloride and clindamycin caused a marked overgrowth of enteric gram-negative organisms in hamsters and the histopathological alterations in the gastro-intestinal tract were similar to those seen in our mice (Lusk, Fekety, Silva, Browne, Ringlar & Abrams, 1978; Small, 1968). An enterotoxaemia produced by overgrowth of gram-negative organisms after antibiotic or patulin administration could be responsible for their toxicity. Such a mechanism was proposed for patulin-treated hamsters and could account for the toxicity of patulin to mice in this study. Mice appear less susceptible to the toxicity of penicillin than do hamsters and guinea-pigs (Schneierson & Perlman, 1956). We found mice less susceptible to patulin than were hamsters (McKinley & Carlton, 1980).

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TERATOGENICITY STUDY OF DICETYLDIMETHYLAMMONIUM CHLORIDE IN MICE

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Abstract—Pregnant JCL-ICR mice were treated with single sc doses of 50 or 200 mg dicetyldimethylammonium chloride/kg body weight on day 7, 9, 11, 13 or 15 of pregnancy. Maternal responses included local reaction at the injection site at both dose levels. Litter parameters were unaffected at the doses tested. There was no significant increase in the incidence of malformations. However there was generally an increased incidence of foetuses with split or bifurcated cervical vertebral arches at all doses and a lower overall incidence of foetuses with extra sternebrae in all treated groups.

INTRODUCTION

Dicetyldimethylammonium chloride, a quaternary ammonium surfactant, has been widely used as an active ingredient in domestic fabric softeners. However, to our knowledge few investigations of the toxicity of this surfactant have been reported. It has been demonstrated that the administration ip of cetyltrimethylammonium bromide (CTAB), a structurally related compound of dicetyldimethylammonium chloride, to pregnant mice at a dose corresponding to 10 or 33% of the LD_{50} (10·5 or 35·0 mg/kg) increased the incidence of malformations, principally cleft palate and minor skeletal defects of the skull and sternum, and at the higher dose CTAB increased foetal mortality (Isomaa & Ekman, 1975). The present experiment was performed in order to examine the effects (embryotoxicity and teratogenicity) of a single sc dose of a dialkyldimethylammonium surfactant given to pregnant mice.

EXPERIMENTAL

Chemicals. Dicetyldimethylammonium chloride (C_{16}, 100%) was supplied by Mr. Y. Minegishi of our Tokyo research laboratories and was shown to be 97·5% pure.

Animals. Six-week-old virgin JCL-ICR mice, supplied by Nippon CLEA Co., Ltd., Tokyo, were maintained on a commercial diet (Nippon CLEA, CA-1) and tap water under controlled conditions of temperature (22 ± 1°C) and humidity (55 ± 5%) until they reached 10 wk. Female mice of 10 wk or older, weighing 25–30 g, were used for embryotoxicity and teratogenicity studies.

Embryotoxicity and teratogenicity studies. Female mice were caged with potent males of the same strain in pairs overnight, and the next morning females with vaginal plugs were regarded as at day 0 of pregnancy. Dicetyldimethylammonium chloride, dispersed in water, was administered sc to the pregnant mice in a single dose of 50 or 200 mg/kg body weight (based on the weight of the animal on conception day) on day 7, 9, 11, 13 or 15 of pregnancy. These doses were chosen on the basis of preliminary studies. The volume administered was 10 ml/kg body weight and control animals received an equal volume of water. The animals were weighed at regular intervals and killed on day 18 of pregnancy. Foetuses were removed from the uterine horn, weighed and inspected for external malformations. About half of the living foetuses from each litter were preserved in alcohol and subsequently stained with alizarin red S for skeletal examination. The remainder were fixed in Bouin's fluid and examined for visceral abnormalities by a modified razor-blade sectioning method (Wilson, 1965).

RESULTS AND DISCUSSION

Maternal toxicity

Local tissue reactions consisting of dermal thickening and hardening, subcutaneous swelling and occasional scab formation were observed in all animals at the 200 mg/kg dose level. At 50 mg/kg all animals showed slight thickening and hardening of the skin or subcutaneous swelling at the injection site. No other systemic signs of reaction were observed and no deaths occurred. Weight changes of treated groups were essentially comparable with those of controls. Terminal examination of internal organs and viscera of maternal animals did not reveal any gross pathological changes that could be attributed to treatment.

Embryotoxicity and teratogenicity

The effects on the foetuses are summarized in Table 1. Within the control group, no significant differences were observed between foetuses from mothers injected on different days of gestation. The controls were therefore treated as a single group in the statistical evaluations. No significant differences were observed between the parameters revealed in the different experimental groups, i.e. implantations, viable foetuses, foetal weight and sex ratio. The number of dead or resorbed foetuses in treated groups was similar to, or compared favourably with, that of the control groups. The group treated on day 9 (200 mg/kg) had the lowest incidence of embryonic death (1·4%) among all groups and was significantly different from that of the controls. Foetuses with external malformations and other defects (petechiae)

Table 1. *Effects of dicetyldimethylammonium chloride on mouse foetuses*

	Effects of a dose (mg kg) of										
	0 on day	50 on day					200 on day				
Parameter	7–15†	7	9	11	13	15	7	9	11	13	15
No. of dams	30	7	11	11	10	10	9	11	11	11	10
No. of implantations/dam‡	12·4 ± 2·65	13·4 ± 2·22	12·6 ± 2·16	13·4 ± 2·06	12·7 ± 1·49	12·2 ± 1·93	12·0 ± 1·41	12·9 ± 1·64	11·2 ± 3·71	13·0 ± 1·84	13·2 ± 2·39
No. of viable foetuses/dam‡	11·4 ± 2·44	12·4 ± 1·99	12·1 ± 2·17	12·5 ± 2·16	12·2 ± 1·48	11·6 ± 1·71	11·0 ± 1·22	12·7 ± 1·62	10·5 ± 3·72	12·0 ± 2·28	12·6 ± 2·32
Percentage of foetuses dead or resorbed	8·3	7·5	3·6	6·8	3·9	4·9	8·3	1·4**	6·5	7·7	4·6
Foetal weight‡ (g)	1·33 ± 0·089	1·30 ± 0·107	1·35 ± 0·087	1·37 ± 0·106	1·28 ± 0·107	1·29 ± 0·070	1·35 ± 0·080	1·29 ± 0·073	1·35 ± 0·053	1·32 ± 0·101	1·28 ± 0·96
Foetal sex ratio (M:F)	164:178	44:43	72:61	65:72	57:65	66:50	52:47	75:65	56:59	63:69	66:60
Type and no. of external malformations	OE:12, CF:2		OE:1, KT:1	OE:1	OE:2	CP:1	OE:1	OE:1, CF:3	OE:2, CP:1	OE:2	OE:3
Percentage of foetuses with external malformations	4·1	0	1·5	0·7	1·6	0·9	1·0	2·9	2·6	1·5	2·4
Percentage of foetuses with other defects§	3·8	2·3	2·3	1·5	1·6	7·8	5·1	0·7	0	1·5	0
No. of foetuses examined for visceral anomalies‖	94	24	35	35	32	34	28	36	32	35	37

OE = Open eyelids CF = Club foot KT = Kinky tail CP = Cleft palate

†Controls injected on day 7, 9, 11, 13 or 15 are all treated as a single group.
‡Means ± SD.
§Petechiae.
‖None of the foetuses examined showed any visceral anomalies.
The value marked with asterisks differs significantly from the control value (**$P < 0·01$)

Table 2. *Effects of dicetyldimethylammonium chloride on skeletal development in mouse foetuses*

Parameter	Effects of a dose (mg/kg) of										
	0 on day	50 on day					200 on day				
	7 15†	7	9	11	13	15	7	9	11	13	15
No. of foetuses examined	171	41	70	72	63	53	46	74	61	68	60
No. of ossified caudal vertebrae/foetus‡	9·38 ± 1·80	8·71 ± 1·79	9·74 ± 1·39	9·95 ± 1·53	9·19 ± 1·67	8·45 ± 1·18	9·51 ± 1·43	9·51 ± 1·23	10·10 ± 1·14	9·38 ± 1·34	8·10 ± 1·06*
No. of ossified middle phalanges in forelimbs§	2·81 ± 0·52	2·71 ± 0·58	2·95 ± 0·57	2·99 ± 0·44	2·51 ± 0·62	2·77 ± 0·36	2·92 ± 0·44	2·64 ± 0·83	3·02 ± 0·47	2·86 ± 0·45	2·75 ± 0·32
No. of ossified middle phalanges in hindlimbs§	1·08 ± 1·06	1·12 ± 1·09	1·47 ± 1·02	1·47 ± 1·30	0·91 ± 1·03	0·71 ± 0·75	1·34 ± 1·02	1·14 ± 1·05	1·52 ± 1·09	1·08 ± 0·83	0·82 ± 0·94
					Skeletal anomalies						
Percentage of foetuses with:											
Split or bifurcation of 1st or 2nd cervical vertebral arch	17·5	43·9**	18·6	23·6	36·5**	18·9	43·5**	31·1**	29·5	20·6	10·0
Cervical ribs	7·6	7·3	5·7	1·4	1·6	5·7	10·9	4·1	6·6	7·4	0
Irregularity of sternebrae	1·2	9·8*	4·3	5·6	1·6	1·9	4·3	1·4	3·3	0	0
Extra sternebrae	21·6	7·3	18·6	8·3*	11·1	15·1	13·0	14·9	21·3	19·1	8·3*
Lumbar ribs	49·7	78·0**	51·4	43·1	52·4	62·3	58·7	43·2	41·0	50·0	63·3

†Controls injected on day 7, 9, 11, 13 or 15 are all treated as a single group.
‡Mean ± SD.
§Values are means ± SD for both right and left limbs.
Values marked with asterisks differ significantly from the control value (*$P < 0.05$; **$P < 0.01$).

were observed in all groups including the controls. The malformations manifested were open eyelids, club foot, cleft palate and kinky tail. All the malformations that occurred in this study are among the most common spontaneous abnormalities in the JCL-ICR strain used and they have previously occurred spontaneously in controls in our laboratory. The incidence of external malformations amongst test groups was generally lower than that of the control group and there was no evidence of treatment-related effects. None of the intergroup differences in the incidence of foetuses with petechiae between groups dosed on different days of gestation were statistically significant. There was no indication that the incidences of external malformations and petechiae were dose-related. No visceral anomalies of foetuses examined were observed in the control or treated groups.

Observations of skeletal abnormalities are shown in Table 2. No skeletal malformations were observed with the exception of one foetus with fused sternebrae at 200 mg/kg in the group treated on day 13. Skeletal variations such as split or bifurcation of 1st or 2nd cervical vertebral arch, cervical ribs, irregularity of sternebrae, extra sternebrae and lumbar ribs were found in the control and treated groups. A generally higher incidence of foetuses with variants of the cervical vertebral arches was observed amongst the treated groups and this was significant at the 50 mg/kg level on day 7 or 13 and at the 200 mg/kg level on day 7 or 9. A significant increase in the number of foetuses with lumbar ribs and irregularity of sternebrae was found at the 50 mg/kg level in the group treated on day 7 but there was no obvious relationship to dose. The incidence of foetuses with extra sternebrae amongst test groups was generally lower than that of the controls and significantly lower values for the incidence were observed at the 50 mg/kg level on day 11 and at 200 mg/kg on day 15. At the 50 mg/kg dose level on day 7 the value of the incidence was low but not statistically significant. The variations in the incidences of these skeletal variants showed no consistent dose-related trends. The effects of dicetyldimethylammonium chloride on skeletal variation were puzzling. The values for all groups, including controls, show that this batch of mice produced a higher than usual proportion of foetuses with variants of cervical vertebral arches or extra sternebrae, and the distribution of these foetuses showed a strong litter bias.

No delayed ossification was observed at either dose tested on any day of pregnancy except for a significant decrease in the number of ossified caudal vertebrae at the 200 mg/kg level injected on day 15 which was probably related to the slightly lower mean foetal weight at this dosage.

When administered sc, dicetyldimethylammonium chloride showed no conclusive evidence of specific teratogenic and embryotoxic activity at the dosages tested. However, there was generally an increase in the incidence of variations of cervical vertebral arches and a lower overall incidence of extra sternebrae in all the treated groups.

REFERENCES

Isomaa, B. & Ekman, K. (1975). Embryotoxic and teratogenic effects of CTAB, a cationic surfactant, in the mouse. *Fd Cosmet. Toxicol.* **13**, 331.

Wilson, J. G. (1965). Methods for administering agents and detecting malformations in experimental animals. In *Teratology: Principles and Techniques*. Edited by J. G. Wilson and J. Warkany. p. 262. University of Chicago Press, Chicago.

Fd Cosmet. Toxicol. Vol. 18, pp. 193 to 195
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Review Section

REVIEWS OF RECENT PUBLICATIONS

Advances in Modern Toxicology. Vol. 1. New Concepts in Safety Evaluation. Edited by M. A. Mehlman, R. E. Shapiro and H. Blumenthal. John Wiley & Sons Ltd., Chichester. Part 1 (1976): pp. xix + 455; £18.30. Part 2 (1979): pp. xiii + 191; £16.30.

The first volume in the series *Advances in Modern Toxicology* has been published in two parts, separated by a 3-year interval. Dedicated to the late Dr. Leo Friedman, the volume as a whole reviews some of the concepts that are shaping the science of toxicology in the late 1970s.

Part 1 introduces such diverse themes as interactions between environmental chemicals, the role of nutrition and diet in toxicity assessments, the use of the Syrian hamster in toxicology, methods used in teratology, pharmacokinetic studies, radioautography, aspects of drug and pesticide metabolism and the transplacental toxicity of diethylstilboestrol.

A chapter by B. P. McNamara on systemic toxicity studies is perhaps the most controversial and thought-provoking, in its attempt to streamline conventional toxicity testing procedures. The author propounds the argument that the number of chemicals evaluated for safety can be increased greatly if evaluation is based on the absence of toxic signs rather than on their demonstration. On the basis of an evaluation of toxicity data for over 150 chemicals, he claims that there is 'good evidence' that satisfactory long-term no-effect doses can be established by toxicity tests of about 4 months' duration. This statement obviously excludes the possibility of carcinogenesis and mutagenesis, for which a combination of screening tests is suggested. Another probable exception to the no-effect approach is the evaluation of new drugs, for which the nature of any toxic effects can be an important guide to members of the medical profession and others. Of particular interest in this chapter are the tables in which the author has compared the short- and long-term no-effect doses for many pesticides, food additives and other types of compound.

In Part 2, consideration is given to the controversial issue of environmental carcinogenesis, with an authoritative chapter from J. Higginson of the International Agency for Research on Cancer. This author recommends a sequential approach to the demonstration and control of carcinogenic hazard for an environmental chemical. This would involve developing epidemiological surveillance mechanisms embracing not only exposure risk but also cancer frequencies to supplement animal data. More technical contributions to Part 2 analyse mathematical approaches to the extrapolation of animal data for carcinogenic-risk assessment and to the interpretation of the linear non-threshold dose-response model for mouse-skin carcinogenesis. The concluding chapters discuss the interactions of metals and tissues, the toxicity assessment of biomedical devices and some current views on the health status of nitrites, nitrates and nitrosamines.

The contribution from J. L. Radomski on the identification and evaluation of environmental carcinogens is particularly recommended. The problem is elegantly summarized: "It is indeed a strange situation where two good scientists reviewing the same body of information can come to diametrically opposed conclusions with essentially equal justification: (1) that chemicals in the environment have no significant role in producing cancer in humans, and (2) that 90% of present human cancer is caused by environmental chemicals". The author's plea for a planned and coordinated approach to the investigation of any single environmental chemical suspected of carcinogenicity, or to the establishment of a programme for solving a particular cancer problem, is developed with conviction.

It is clear that the variety of events occurring as a result of the exposure of the animal organism to foreign chemicals provides an endless source of problems for the toxicologist. The elucidation of these events, as portrayed in these books, is not only of practical importance but also inspires confidence in the discipline of toxicology.

Residue Reviews: Residues of Pesticides and Other Contaminants in the Total Environment. Vol. 67. Edited by F. A. Gunther. Springer-Verlag, New York, 1977. pp. x + 139. $16.80.
Residue Reviews: Residues of Pesticides and Other Contaminants in the Total Environment. Vol. 68. Edited by F. A. Gunther. Springer-Verlag, New York, 1977. pp. viii + 154. $19.80.

It is perhaps a reflection of their severity that the hazards facing those who have to work with crops previously treated with pesticides have come to be known simply as 'the re-entry problem'. Another indication of the magnitude of this problem—and its complexity—is the appearance of a volume of *Residue Reviews* concerned solely with this subject.

Associated particularly with the cholinesterase-inhibiting organophosphorus compounds, re-entry problems have arisen with many fruit and vegetable crops, and while studies have centred particularly on citrus groves treated with parathion, much of the information in this detailed monograph is widely applicable. Thus the procedures outlined for acquiring the data necessary for estimating re-entry hazard and establishing safe re-entry intervals for citrus-treated crops include steps likely to be applicable to other crops. On the other hand, for any given crop and treatment, the degree of hazard is greatly affected by local factors such as the rainfall pattern and soil type. (It is the lack of rainfall in California between March and November that gives that area a citrus

re-entry problem from which Florida escapes.) This comprehensive report is backed by numerous graphs and tables and an impressive list of references.

The next volume of *Residue Reviews* is one of the more conventional collections of unrelated papers. Two of these continue the organophosphate theme. The first presents a considerable amount of information on the toxicity of fenitrothion, as part of an assessment of the ecological hazards of spray programmes aimed against the spruce budworm in Canada and parts of the USA. A later chapter reviews some aspects of the persistence of parathion. While organophosphorus pesticides are generally accepted as being less persistent than organochlorine compounds and are rapidly metabolized in plant and animal tissues, relatively little has been known about their fate in soils and water. Now, however, it appears that the widely-used parathion is stable enough for some accumulation to be possible in soil and water in areas of intensive cultivation. Microbial degradation is more effective than chemical detoxication in removing parathion from the environment, and flooding is apparently another, if drastic, means of speeding its breakdown.

A review of the literature on lindane metabolism concentrates on the identification of its metabolites and on the distribution of radioactivity from labelled lindane in mammals, mainly the rat. In the light of this information, possible degradation pathways are discussed with the aid of numerous diagrams.

The remaining contributions deal with two widespread contaminants of food—fluorine and arsenic. In the latter case, the approach is analytical, and the author deals methodically with the determination of arsenic in foods and other biological materials, moving from the principles of representative sampling, through sample preparation to the identification and estimation of specific arsenic compounds. In contrast, the fluorine review considers in some detail the levels of fluorine found in different types of food and the environmental and social factors liable to affect the fluorine intake of individuals. The general impression is of a complex and variable situation, but one that needs to be taken into account in any locality where water fluoridation is under discussion.

Toxic Substances Control Act Chemical Substance Inventory. Initial Inventory. Vol. I. US Environmental Protection Agency. Published with accompanying Substance Name Index (Vols II and III), Molecular Formula and UVCB Indices (Vol. IV) and Trademark and Product Name List. EPA, Washington, DC, May 1979.

The initial inventory of chemical substances required by the Toxic Substances Control Act (TSCA) and based on information requested in 1977 (*Federal Register* 1977, **42**, 64572) has been published by the EPA. The inventory, which with its subsidiary parts occupies six large volumes, lists more than 44,000 materials covered by the TSCA definition of a chemical substance. While 'naturally occurring substances', again as defined by the TSCA, have been automatically included, all other substances have been identified from reports submitted to the EPA by manufacturers or importers. All the substances in the inventory have been manufactured, imported or processed for commercial purposes in the USA since 1 January 1975. The list is in no way related to the possible toxicity of any of the chemicals, but merely indicates their commercial manufacture or use.

The chemicals are identified in the 'Initial Inventory' by the Chemical Abstracts Service (CAS) Registry Number and by a preferred name, which identifies the composition of the substance or category of substance. They are listed in order of CAS number. This volume also includes two appendices, the first providing further definitions to assist in the identification of some of the substances in the main list and the second giving generic names for substances requiring confidential treatment. Three of the supporting volumes are designed to facilitate use of the Initial Inventory: the Substance Name Index (Volumes II and III) provides an alphabetical list of the preferred names of all substances in the inventory of their synonyms, while in Volume IV the substances appear again, either in the Molecular Formula Index or in the 'UVCB Index'. The latter covers substances of unknown or variable composition, complex reaction products and biological materials, a heterogeneous group which accounts for about one third of the substances included in the Initial Inventory.

Chemical substances that are not in the Initial Inventory but have been processed, or imported as part of mixtures or articles, since 1 January 1975 may be added during a 210-day reporting period that began in May 1979. These, together with some substances reported too late for the current publication will be included in a Revised TSCA Chemical Substance Inventory, likely to be published during 1980. Subsequently, supplements will be published at intervals to incorporate substances for which premanufacture review has been completed and commercial handling is beginning. Two further volumes have been issued with the Initial Inventory to assist those who have to determine whether to report substances for the Revised Inventory. Together these volumes form the Trademark and Product Name List, which is designed to function solely in connection with the current 210-day reporting period and therefore will not be updated. The first part of this list presents a list of reporting companies together with their trademarks or product names, while in the second, the reporting company is identified for each trademark or product name in an alphabetically arranged list.

GLC and HPLC Determination of Therapeutic Agents. Part 1. Edited by K. Tsuji and W. Morozowich. pp. xiv + 415. Sw.fr. 98.00. Part 2. Edited by K. Tsuji. pp. xiv + 519. Sw.fr. 95.00. Marcel Dekker, Inc., New York, 1978.

The first two volumes of this three-volume work are advertised as comprehensive with respect to both the theory and methodology of gas and high-performance liquid chromatography. They are nothing less. Both volumes provide an extremely well-written account of these two related and axial fields of modern chromatographic science; the illustrations are

numerous, informative and superbly presented and the references cited are relevant and up-to-date.

Part 1 covers the theory of chromatography and the instrumentation used for the separation and detection of substances, column selection, sample derivatization and isolation, mass spectrometer combination (both with gas–liquid chromatography and with high-performance liquid chromatography), quality control, and computer interfacing and data handling. Part 2 serves as a detailed laboratory handbook and reference guide to the chromatographic analysis of a variety of drugs in pharmaceutical formulations or biological samples. Although Part 2 refers to specific drug analyses, the wealth of experimental detail will provide useful comparative information for analysts developing methods for a wide variety of compounds.

It is unlikely that any serious chromatographer will wish to be without at least part 1 of this series.

BOOKS RECEIVED FOR REVIEW

Mass Spectrometry. Vol. 5. **A Review of the Recent Literature Published between July 1976 and June 1978.** Senior Reporter R. A. W. Johnstone. The Chemical Society, London, 1979. pp. xii + 450. £25.00.

DDT and its Derivatives. Environmental Health Criteria 9. Published under the joint sponsorship of the United Nations Environment Programme and the World Health Organization. WHO, Geneva, 1979. pp. 194. Sw.fr. 16.00 (available in the UK through HMSO).

Residue Reviews. Residues of Pesticides and Other Contaminants in the Total Environment. Edited by F. A. Gunther. Springer-Verlag, New York, 1979. pp. viii + 144. DM 39.50.

New Concepts in Safety Evaluation. Part 2. Advances in Modern Toxicology. Vol. 1. Edited by M. A. Mehlman, R. E. Shapiro and H. Blumenthal. John Wiley & Sons, Chichester, 1979. pp. xiii + 191. £16.30.

Progress in Drug Metabolism. Vol. 3. Edited by J. W. Bridges and L. F. Chasseaud. John Wiley & Sons, Chichester, 1979. pp. ix + 372. £19.25.

Chlorine Dioxide. Chemistry and Environmental Impact of Oxychlorine Compounds. By W. J. Masschelein. Ann Arbor Science Publishers Inc., Ann Arbor, 1979. pp. ix + 190. £14.85.

The Biochemistry of Atherosclerosis. Edited by A. M. Scanu. Marcel Dekker, Inc., New York, 1979. pp. xx + 548. Sw.fr. 112.00.

The Alkaloids: The Fundamental Chemistry—A Biogenetic Approach. By D. R. Dalton. Marcel Dekker, Inc., New York, 1979. pp. x + 789. Sw.fr. 110.00.

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

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Information Section

ARTICLES OF GENERAL INTEREST

ERUCIC ACID AGAIN

Rapeseed oil contains variable amounts of the mono-unsaturated fatty acid *cis*-13-docosenoic acid, commonly known as erucic acid (EA; C22:1, n-9). This fatty acid has been held responsible for the fatty deposits in heart muscle and associated myocardial lesions found in experimental animals fed rapeseed oil (*Cited in F.C.T.* 1975, **13,** 130). Many experiments have been carried out to follow triglyceride and free fatty acid accumulation in cardiac lipids and to determine the role of EA in the lesion response, and there is considerable evidence that EA is not solely responsible for these toxicological effects (*ibid* 1978, **16,** 619). For instance there have been many suggestions that linolenic acid plays an important part. In addition, it has been shown that the effects of EA vary, not only between different species of experimental animal but also between different sexes and strains of the same test species (*ibid* 1978, **16,** 620).

Although there is relatively little information on the effects of EA in humans, several groups of workers are studying the effects of this fatty acid in non-human primates. The demonstration of toxic effects of EA in experimental animals has led to the restriction of levels of EA in edible fats and oils (*ibid* 1977, **15,** 352) and plant breeders have made considerable progress in the production of low-EA varieties of rapeseed. Oil from some of the new varieties contains as little as 0·2% EA. Toxicologists have turned their attention to these new varieties, while continuing their efforts to determine the mechanisms behind the effects of EA and the high-EA rapeseed oils.

A study carried out by Kramer & Hulan (*Lipids* 1978, **13,** 438) produced further evidence that the long-term development of myocardial lesions in rats fed diets containing EA could not be attributed to the early accumulation of either triglycerides or free fatty acids. Using an extraction procedure that minimized the autolysis of cardiac lipids, they determined quantitative and qualitative changes in the fatty acid composition of cardiac triglycerides and free fatty acids during the first week of feeding and after 16 wk of feeding oils containing up to 22·3% EA to male Sprague-Dawley rats. The results were similar to those in earlier studies; there was transient myocardial lipidosis during the first week of feeding, but this had decreased considerably after 16 wk. However, the free fatty acid levels determined were about 5% of those that had been reported previously, a reflection of the modification of the extraction procedure. It was interesting to note, though, that although by this method the cardiac free fatty acid levels were lower, the proportion of EA in the free fatty acids was markedly higher than that reported in previous studies. Indeed the temporary accumulation of free fatty acids in the heart tissue of rats fed high-EA rapeseed oil could be almost entirely explained by the accumulation of EA and eiconenoic acid. After 16 wk the levels of these acids in cardiac free fatty acid and triglyceride had decreased significantly.

Other workers have continued the search for factors that may be involved in the cardiopathogenic effects of high-EA rapeseed oil, and there have been further studies on the role of linolenic acid. In one such study, both Sprague-Dawley and Wistar rats were fed Span (4·6% EA) and Tower (1·4% EA) rapeseed oils which had been partially hydrogenated to iodine values (IV) of 78·4 and 76·6 respectively (Beare-Rogers & Nera, *ibid* 1977, **12,** 769). The rats fed the partially hydrogenated rapeseed oil developed fewer and less severe lesions than rats given untreated oil or oil from which polar compounds had been removed or which had been treated with nitrogen under the conditions used for hydrogenation. Although hydrogenation considerably reduced the levels of both linolenic and linoleic acids, the improved nutritional quality of the hydrogenated oil did not seem to be related to the decreased levels of linolenic acid, because feeding linseed oil high in linolenic acid, with or without added EA, did not increase the incidence of lesions.

A better indicator of cardiac pathology appeared to be the level of docosahexaenoic acid in the cardiac fatty acids. There were relatively high concentrations of this acid in the cardiac fatty acids of rats fed the unhydrogenated Tower oil or a Tower oil hydrogenated to IV 97·1, but rats fed Tower oil of IV 76·6 had docosahexaenoic acid levels similar to those of rats given a mixture of lard and corn oil. Roquelin (*Nutr. Metabol.* 1979, **23,** 98) found that in weanling male Wistar rats, the administration of linseed oil containing a high proportion of linolenic acid (8% of the calories in the diet) did not affect the EA-induced accumulation of cardiac triglycerides but did increase the hepatic conversion of EA into oleic acid and other shorter monoenes. He gave the rats diets containing 15% (w/w) of four lipid mixtures—trierucin + groundnut oil (1:1), trierucin + linseed oil (1:1), triolein + groundnut oil (1:1) and triolein + linseed oil (1:1). The relative proportion of (n-3):(n-6) polyunsaturated fatty acids in liver and heart phospholipids was considerably increased when linseed oil was fed, but EA had little effect on the (n-3):(n-6) ratio.

Dewailly *et al.* (*Lipids* 1978, **13,** 301) also implicated linolenic acid in the pathological effects of feeding high-EA rapeseed oil. They fed rapeseed oils containing high (46·2%) and low (3·7%) levels of EA to male Wistar rats for 20 wk and studied changes in the fatty acid composition of the cardiac mitochondrial phospholipids. Cardiolipin isolated from the heart mitochondria of rats fed the high-EA rapeseed oil con-

tained about 5·6% EA. Linolenic acid was present in cardiolipid isolated from the mitochondria of animals given high- and low-EA rapeseed oil at levels of 1·8 and 1·1% respectively. In addition, with both oils there was a highly significant increase in the linoleate:arachidonate molar ratio in the total fatty acids of phosphatidylethanolamine and phosphatidylcholine. In these phospholipids the linoleate:arachidonate ratio was also significantly increased in fatty acids esterified at the β-position. The incorporation of EA and linolenic acid into mitochondrial phospholipid may have an inhibitory effect on mitochondrial function. Linolenic acid inhibits chain elongation of linoleic acid, and may act as an inhibitor of heart microsomal enzymes involved in linoleate–arachidonate conversion.

Vasdev & Kako (*Am. J. Physiol.* 1978, **235**, E509) fed male Sprague-Dawley rats diets containing 20% of either mustard-seed oil (37% EA) or corn oil (62% linoleic acid). EA did not accumulate in the heart, liver or adipose tissues. Instead, during 6 wk of treatment EA levels in these tissues decreased, although hyperlipaemia persisted. Chylomicrons from rats fed mustard-seed oil for 6 wk contained relatively less EA than those isolated from rats given the EA-containing diet for 3 wk. After overnight fasting, EA was reduced to very low levels in the plasma triglycerides and free fatty acids. The composition of the diet had hardly any effect on the fatty acid content of heart, liver and plasma phospholipids. The results indicated that feeding an EA-enriched diet induces a metabolic adaptation in rat tissues so that over a period of 6 wk the rate of EA metabolism increases.

Other workers have also investigated the effects of starvation on EA incorporation in the serum lipids and lipoproteins of rats fed rapeseed oil (Szlam & Sgoutas, *Lipids* 1978, **13**, 121). In male Sprague-Dawley rats fed 20% rapeseed oil (46·5% EA) in the diet for 7 days, only very small proportions of the EA and eicosenoic acid administered in the diet were incorporated into the blood lipids. In rats adequately fed on the rapeseed-oil diet, EA and eicosenoic acid were incorporated into all the lipoproteins (very low-density lipoprotein, VLDL: low-density lipoproteins, LDL; high-density lipoprotein, HDL) and their lipid sub-classes (cholesteryl esters; triacylglycerols; phospholipids) with the exception of EA which was not incorporated into the cholesteryl esters and phospholipids of HDL. However, after 16 hr starvation of these rapeseed-oil fed rats and EA and eicosenoic acid contents of VLDL were greatly depleted; only in HDL triacylglycerols were levels similar to those before starvation. It seems that the rapeseed-oil diet caused an increased rate of clearance of chylomicrons and VLDL containing EA and eicosenoic acid, but the rate of breakdown of HDL triglyceride containing these fatty acids remained slow, in spite of starvation.

Much of the work on the effects of rapeseed oil continues to be carried out on the rat. We have discussed before how the sex or strain of an experimental animal may play a part in the cardiopathogenic effects of EA (*Cited in F.C.T.* 1978, **16**, 620) and have commented on the need for more information from studies on non-human primate species (*ibid* 1978, **16**, 622). Recent studies have confirmed the susceptibility of the Sprague-Dawley strain of rats to myocardial lesions (Beare-Rogers & Nera, *loc. cit.*: Vles, Paper presented at an EEC Symposium on New Varieties of Rapeseed, held in Brussels, 11–12 April 1978).

Several investigations have been carried out into the effects of rapeseed oil and EA in primates. Cynomolgus monkeys (*Macaca fascicularis*) were fed diets containing either 25% rapeseed oil (containing 24·6% EA), partially hydrogenated herring oil (PHHO; containing 24·4% C22:1, mainly as cetoleic acid, C22:1, n-11), or a 3:1 mixture of lard and corn oil (containing no C22:1) for 4 months (Loew *et al. Nutr. Metabol.* 1978, **22**, 201; Schiefer *et al. Am. J. Path.* 1978, **90**, 551). Monkeys fed PHHO or rapeseed oil developed myocardial lipidosis and analysis showed an accumulation of C22:1 in the heart lipids. Infrequent foci of mild mononuclear cell infiltration occurred in all groups, although the infrequency may have been related to the sectioning techniques used. There were no significant differences between the monkeys in the three dietary groups in terms of growth rates or electrocardiograms, and the only significant inter-group differences in biochemical or haematological measurements were as follows: serum cholesterol concentrations increased in the group fed rapeseed oil after 30 and 120 days of treatment; glutamic–oxalacetic transaminase activity increased both in the rapeseed-oil group and in the PHHO group after 60 and 120 days of treatment. Electronmicrographs of heart tissue of monkeys from the rapeseed oil- and PHHO-treated groups showed mild mitochondrial degeneration. In the rapeseed-oil group the P:O ratio was decreased and in the PHHO-treated group the state III respiratory rate (μmol O_2/min/mg protein) was decreased.

In another study in the same species, Kramer *et al.* (*Can. J. Anim. Sci.* 1978, **58**, 245 & 257) examined the effects of feeding low-EA (0·2%) rapeseed oil or soyabean oil at levels of 20% in the diet for 24 wk. Feeding the low-EA rapeseed oil resulted in increased adrenal weights in the female monkeys and decreased kidney weights in the males, but in other respects growth was normal. Electrocardiograms and studies of haematology and clinical biochemistry revealed no significant differences between the two dietary groups. Long-chain monoenes (C18:1, C20:1 and EA) accumulated in the cardiac lipids of both sexes fed the rapeseed oil, but histological studies showed no myocardial damage associated with the feeding of either diet. The slow accumulation of EA in the monkeys on the rapeseed-oil diet contrasts with the effects of rapeseed oil on the rat, in which species there is a rapid initial increase in EA in heart lipids, followed by a decrease (Kramer & Hulan, *loc. cit.*). The concentration of cardiac EA relative to dietary EA is much greater in monkeys than in pigs, rats and chickens fed low-EA rapeseed oil. Monkeys, pigs and chickens differ from the rat in cardiac levels of docosahexaenoic acid, considerably larger amounts occurring in rats than in the other three species.

As in other studies (*Cited in F.C.T.* 1978, **16**, 621) these results indicate differences in response to the feeding of EA between monkeys and rats. Further work by Vogtmann *et al.* (*Int. J. Vit. Nutr. Res.* 1978, **48**, 90) confirmed the differences in the effects of EA in chickens and rats (*Cited in F.C.T.* 1977, **15**, 351). There was no peak in the lipid content of the hearts of

chicks fed rations containing 15% rapeseed oil (22% EA) from days 1 to 10 of age.

The search continues for the biochemical basis of the effects of EA in rats and other species. Many studies continue to focus on the effects of EA on mitochondrial structure and function. Others have been carried out on the chain-shortening of EA, attempting to identify the cellular location of the chain-shortening mechanisms. We shall consider some of these biochemical studies in a later issue.

[M. E. Morris—BIBRA]

DIFFERENCES OF OPINION OVER TDI

Debate over a safe level of toluene diisocyanate (TDI) has continued. In particular, the claim of Wegman *et al.* (*Br. J. ind. Med.* 1977, **34**, 196; *cited in F.C.T.* 1979, **17**, 89), that chronic occupational exposure to TDI levels in excess of 0·003 ppm resulted in both an acute and chronic decrease in respiratory performance, has not been received in silence.

Adams *et al.* (*Lancet* 1978. **I**, 1308) considered that although a small number of atopic individuals became sensitized at very low TDI levels (even below 0·003 ppm), the TLV of 0·02 ppm was sufficient to protect the vast majority of workers. This view was based both on an earlier ICI study (*Cited in F.C.T.* 1976, **14**, 218), in which no association was observed between TDI exposures below 0·02 ppm and the forced expiratory volume (FEV) of workers, and on the negative findings of Butcher *et al.* (*Am. Rev. resp. Dis.* 1977, **116**, 411; *Cited in F.C.T.* 1979, **17**, 90). The latter study did not demonstrate a decline in the pulmonary function of employees over a 3·5 yr period of TDI exposure and was particularly important in the opinion of Adams *et al.* (*loc. cit.*) because it involved men employed on a new plant and true baseline data on lung function was therefore available. However, Peters & Wegman (*Lancet* 1978, **II**, 472) were not impressed with either report. They expressed doubts over the accuracy of the pulmonary-performance measurements of Butcher *et al.* (*loc. cit.*) and considered that the negative ICI result (*Cited in F.C.T.* 1976, **14**, 218) may have been due simply to the selection of workers for examination, the removal of a sizeable minority of individuals with respiratory symptoms from the TDI plant (and therefore from the study population) having led to an artificial rise in the health of the remaining cohort.

In their own paper, Wegman *et al.* (*loc. cit.*) concluded that the FEV losses observed in the workforce were a consequence of TDI exposure and were not related to lung size. However, analysing the published summary of data from this study, Adams *et al.* (*loc. cit.*) deduced widely differing lung performances (FEV) for the three exposure groups at the beginning of the study. The inter-group differences of up to 666 ml were much higher than the average reductions in lung capacity of 12, 85 and 205 ml seen in the groups on low, medium and high levels of TDI exposure, respectively, over the 2-yr monitored period. Although Peters & Wegman (*loc. cit.*) did not consider that this invalidated their conclusion, and emphasized that it was not reasonable for occupational exposure to result in a loss of over 100 ml of FEV per year (as was observed at the TLV exposure of 0·02 ppm), they did not respond specifically to their critics' suggestion that the full data should be published so that doubts about their original analysis could be resolved.

In the study of Wegman *et al.* (*loc. cit.*), the lung function of TDI workers was measured immediately before the start of the first shift of the week, in order, it was said, to avoid the complicating acute effects of TDI. Adams *et al.* (*loc. cit.*) expressed reservations about this procedure. They favoured measurements taken in the afternoon of a workshift, for at this time non-reaginic late (asthmatic) reactions, which do not occur until 4–6 hr after exposure to TDI, would also be detectable. However, Peters & Wegman (*loc. cit*). maintained their view that a baseline measurement of pulmonary function was possible only on a 'Monday morning', when the acute symptom-free falls, which were seen over a workshift and were clearly different from the sensitization phenomenon mentioned by Adams *et al.* (*loc. cit.*), were avoided.

Burge *et al.* (*Lancet* 1978, **II**, 96) also drew attention to the difference between sensitization and the other effects of TDI and emphasized that even if unequivocal data were eventually forthcoming to show that TDI exposure at 0·02 ppm did not produce a decline in FEV with age in asymptomatic workers, there would still remain the quite distinct problem of bronchial asthma. In a study conducted at the Brompton Hospital on 37 individuals with TDI-induced occupational asthma, asthmatic reactions were usually produced by 0·005 ppm TDI and often by 0·001 ppm. Burge *et al.* (*loc. cit.*) noted that it was not yet possible to define the concentration required to sensitize a worker to TDI; in the population covered by the original ICI study, 61 of the 241 exposed workers had left employment with respiratory disease, and at least 29 of the 146 exposed workers examined by Butcher *et al.* (*loc. cit.*) were shown to be sensitized to TDI.

In 1973, NIOSH recommended a time-weighted average of 0·005 ppm for occupational exposure to TDI, with a ceiling value of 0·02 ppm. The same recommended limits appeared in the diisocyanate criteria document published in September 1978 (*Criteria for a Recommended Standard..... Occupational Exposure to Diisocyanates*; NIOSH, Cincinnati, OH, 1978). A consideration of further research requirements specified epidemiology studies of respiratory symptoms and lung function, dose-response studies of sensitization in animals, the development of screening tests for identifying sensitized individuals and the mutagenicity (carcinogenicity) screening of TDI.

[J. Hopkins—BIBRA]

VINYL CHLORIDE—PART 5: MUTAGENICITY IN MAN

Since it is widely accepted that somatic mutation is frequently a step in tumour formation, the demonstrable carcinogenicity of vinyl chloride (VC) indicates that *in vivo* mutagenicity does occur in man. Cytogenetic examination is one means of evaluating this aspect of the monomer's biological profile.

Until recently, studies of this type have given equivocal results, the reports identifying a VC-associated increase in chromosomal aberrations being balanced by those showing no effect. Several of the positive studies are of limited value, involving only small and therefore possibly non-representative groups of workers. Ducatman *et al.* (*Mutation Res.* 1975, **31**, 163), for example, examined lymphocyte cultures from 11 VC-exposed workers. Kučerová *et al.* (*ibid* 1979, **67**, 97) from nine and Funes-Cravioto *et al.* (*Lancet* 1975, **I**, 459) from seven.

The experiment of Léonard *et al.* (*J. Toxicol. envir. Hlth* 1977, **2**, 1135), in which lymphocyte cultures were taken from ten "control" individuals, seven men who had worked in the laboratory of a VC plant and who were subjected to only limited VC exposure and 11 workers in a VC polymerization plant, is open to the same criticism. While these authors found that the incidence of chromosomal aberrations, such as chromatid gaps and breaks and chromosome gaps, were comparable in all groups, more severe abnormalities, such as dicentrics, fragments, translocations and rings, were observed in ten of the 11 workers from the polymerization plant but in none of the controls. Levels of VC in the plant were less than 10 ppm at the time of the study, but had been as high as 500 ppm a few years earlier. The authors did not consider that these findings incriminated VC as the causative factor. Many of the workers in the polymerization plant had evidently been given several X-rays of the hands and feet (in order to check on possible acro-osteolysis), while the control group had no similar exposure to radiation, and Léonard *et al.* (*loc. cit.*) considered it likely that the observed cytogenetic changes were attributable to X-rays rather than to VC exposure. They thought that a suitably representative sample of VC workers would be difficult to identify because of the practice in the industry of monitoring the workforce regularly by various scanning techniques. Nevertheless, this is a fairly recent development and in at least one of the earlier studies, that of Purchase *et al.* in 1975 (*Lancet* 1975, **II**, 410), the test and control groups were said to be comparable in their exposure to radiation. In that study, lymphocyte cultures from 56 workers exposed to VC and from 24 controls were examined and it was found that VC-exposure was associated with an increased incidence of chromosomal aberrations including major chromosomal abnormalities.

On further analysis of essentially the same data (the exposed group had been increased to 57 men), Purchase *et al.* (*Mutation Res.* 1978, **57**, 325) found an association between chromosomal aberration and four factors: job category, length of employment, experience of exposure to short-term excursion levels of VC, and smoking habits (as ascertained in an interview 18 months after lymphocyte sampling). It was not possible to estimate which of these parameters was the most important. With respect to job category, the greatest statistically significant increase in both minor and major chromosome abnormalities occurred in autoclave operators/cleaners (17 men in all). Smaller increases in one or more of the types of aberration were commonly seen with other job categories. The autoclave operators were subject to average VC levels of 300–400 ppm for the decade up to 1970, but the levels had been reduced to 15 ppm by mid-1973 and to 5 ppm by 1975. Blood samples for chromosomal analysis were taken in mid-1974.

Fleig & Thiess (*J. occup. Med.* 1978, **20**, 557) compared lymphocyte cultures from ten workers exposed to VC (but showing no symptoms of VC disease) with those from 20 matched unexposed controls. There were no statistically significant differences between the two groups. However a significantly higher incidence of aberrations was found in a group of 20 workers suffering from various symptoms of VC disease than in the control group of 20 individuals, the control incidence of aberrations of 5·5% including gaps and 2·1% excluding gaps being raised to 11·2 and 5·2%, respectively. Furthermore, a 16·6% incidence of chromosomal aberrations (7·3% excluding gaps) was measured in a 43-yr-old patient, who had developed an angiosarcoma after working in the PVC industry for 13 yr. His exposure to VC in the past few years, resulting from the cleaning of autoclaves, was estimated at between 35 and 150 ppm for about 20–30 hr a month. Surprisingly, there is no specific mention of the X-ray experience of the workers suffering from VC disease and these individuals are likely to have been subjected to X-ray examinations. As for the cancer victim, the authors noted that cancer chemotherapy had been received 9 months before the lymphocyte examination.

A study of a large number of PVC workers of Dow Chemical USA, conducted by Picciano *et al.* (*J. occup. Med.* 1977, **19**, 527), suggests that VC exposures below 15 ppm do not induce chromosomal aberrations. When lymphocyte cultures taken from a group of 209 workers who had worked in the plant for periods ranging from 1 to 332 months (average 48·3 months) were compared with those from a control group of 295 pre-employment examinees, no significant cytogenetic differences were observed. The workers had been exposed to maximum levels of 15·2 ppm VC before 1960, 11·4 ppm from 1960 to 1972 and 8·7 ppm between 1973 and 1974. The two groups were not comparable in age, the controls, averaging 25·1 yr, being some 14 yr younger than the VC workers, and apparently no attempt was made to standardize with respect to X-ray exposure. Again there was no indication of any VC-associated changes in the chromosomes when the data were analysed with respect to monomer exposure, the 209 workers being divided for this purpose into three sub-groups, those exposed on average to less than 1 ppm, 1–5 ppm and above 5 ppm VC, respectively.

Further support for VC as the real cause of the chromosomal damage comes from Hansteen *et al.* (*Mutation Res.* 1978, **51**, 271). In 1974, this group found that at 3·41%, the mean chromosome breakage frequency in lymphocyte cultures from a group of 39

workers from a PVC plant was significantly higher than that seen in the 16 controls (1·79%). However, when 35 of the original 39 workers were re-examined 2–2·5 yr later, a period during which they had only minimal VC exposure, the frequency of chromosome breakage was down to control levels, suggesting that visible chromosomal changes may reflect only recent industrial experience.

It has been shown that the frequency and type of somatic chromosomal aberration seen in any particular worker may vary markedly over a period of time during which there is continual exposure to VC (Kučerová *et al. loc. cit.*). A wide inter-subject variability has also been observed (Hansteen *et al. loc. cit.*). The biological implications of somatic mutations are as yet unknown—the time-scale of chromosomal change is an area particularly in need of further study—although such changes may eventually prove to be of value in the early identification of groups at high risk from cancer and they may have some potential for screening individuals (one subject with a high breakage frequency in this group's original study was subsequently found to have cancer). It is reassuring, however, that on the present evidence workers exposed to VC at levels up to the TLV of 5 ppm probably do not develop visible cytogenetic changes in the cells of the peripheral blood.

While somatic mutation is expressed in exposed individuals, alterations of the genetic material in the germ cells will not become apparent until the next generation and perhaps even later. One possible consequence of a chromosomal aberration in germ cells is the induction of dominant lethal mutations, which in many would be observed as early foetal loss. A study by Infante *et al.* (*Lancet* 1976, **I**, 734) of the pregnancy outcome among wives of workers exposed to VC suggests that the monomer may possess just such activity. A group of 95 polymerization workers (primary VC group) was compared with an age-matched control group made up of 158 rubber and PVC-fabrication workers (with very low or no VC exposure); these numbers reflected group participation rates of 62–77%. Age-adjusted foetal deaths per 100 pregnancies in the primary VC group were 6·9 before and 15·8 after VC exposure, compared with corresponding foetal losses of 6·9 and 8·8% in the controls. The statistically significant ($P < 0{\cdot}02$) difference between the primary VC group after VC exposure and the controls was found to reflect a greater foetal loss in the wives of men under 30. In this age group, foetal loss after VC exposure was 20%, compared with 5% in the controls, whereas the foetal loss of wives of men over 30 yr old was comparable in the exposed and control groups (13 and 12%, respectively). A VC-associated increase was still apparent when women with more than one abortion were excluded from the analysis. The lack of details of the method of data collection and the age-adjustment procedure used in this study were queried by Paddle (*ibid* 1976, **I**, 1079). Responding with details of their interview procedures and with a further explanation of the age-adjustment method, Infante *et al.* (*ibid* 1976, **I**, 1289) tentatively suggested that their findings for the under-30 age group might have reflected the placing of newly-hired personnel in categories involving the worst levels of VC exposure.

These findings in particular raise doubts over the possible genetic risks of VC. More studies of this type are badly needed. It is to be hoped that future studies will include direct information both on maternal age at conception and on occupational exposure levels.

[J. Hopkins—BIBRA]

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ABSTRACTS AND COMMENTS

FOOD CONTAMINANTS

Mercury in British fish too—but not too much

Haxton, J., Lindsay, D. G., Hislop, J. S., Salmon, L., Dixon, E. J., Evans, W. H., Reid, J. R., Hewitt, C. J. & Jeffries, D. F. (1979). Duplicate diet study on fishing communities in the United Kingdom: Mercury exposure in a 'critical group'. *Envir. Res.* **18**, 351.

Poisoning from methylmercury in fish is one area in which man has learned the hard way about the dangers. Previous official surveys in the UK, however, have indicated that average intakes here are well within recommended FAO/WHO limits (*Cited in F.C.T.* 1974, **12**, 139). The present survey involved a particularly susceptible group.

A study was carried out on fishermen and their families on the shores of the north-eastern Irish Sea, where mercury levels in fish and shellfish were above average and were associated with areas of known mercury discharges and where people generally consume greater than average amounts of fish. A similar group from the shores of the southern English Channel was used as a reference group. The authors aimed to determine the intakes of mercury and methylmercury among the two populations, to determine the effects of this on mercury concentrations in blood and hair and to measure selenium intakes.

The group from the Irish Sea shore comprised 150 individuals with a spread of fish consumption, estimated by interview at 20–400 g/day; the 75 reference-group members had similar intakes. When each individual supplied a duplicate of his diet for a week, true levels of fish consumption were all below 225 g/day and mainly between 20 and 125 g/day. Mercury concentrations in the fish were from 0·05 to 0·63 mg/kg with means of 0·27 and 0·22 mg/kg in the test and reference groups respectively. Total mercury intake ranged from 5 to 620 μg/wk and was significantly higher (relative to body weight) for the test community. The contribution to total mercury intake from the non-fish components of the diet is very low. Dietary methylmercury levels were determined for participants who consumed more than 150 μg mercury/wk; 97% of the mercury was in the methylated form. Overall, 13% of the participants exceeded the FAO/WHO tolerable weekly intake of 0·2 mg methylmercury/person (*ibid* 1973, **11**, 655).

Selenium levels in the diets averaged 0·10 and 0·09 mg/kg for the Irish Sea and English Channel groups respectively. There was no significant difference between the two groups. The average weekly intakes for individuals were 0·11–1·59 mg with a mean of 0·54 mg. There was no evidence of a correlation between the mercury and selenium levels.

Blood mercury levels are generally accepted as an index of short-term exposure. The concentrations of organic mercury in the blood varied from below 0·03 to 2·35 μg/100 ml with a mean of 0·45 μg/100 ml for the Irish Sea group compared with 0·03 to 1·09 μg/100 ml with a mean of 0·32 μg/100 ml for the reference group. The difference between the means was statistically significant but less significant than the difference for the mercury intakes. The mean concentrations of total mercury in the blood were 0·50 μg/100 ml for the Irish Sea group (range 0·04–2·58 μg/100 ml) and 0·35 μg/100 ml for the reference group (range 0·04–1·21 μg/100 ml). Total blood mercury concentrations were significantly related to mercury intakes but the data were too widely scattered to establish the form of the relationship.

The mean level of mercury in hair was 2 mg/kg in the Irish Sea group compared with 1·35 mg/kg in the reference group. A significant relationship was found between mercury in hair and mercury intake and between levels of mercury in blood and hair.

The highest intakes in the populations studied were of the order of 0·4–0·5 mg methylmercury/wk, more than twice the tolerable weekly intake recommended by FAO/WHO. However, such levels resulted in total blood-mercury concentrations of not more than 2·6 μg/100 ml, whereas blood levels associated with the earliest effects in the most sensitive adults are around 20–50 μg/100 ml. The authors conclude that fish consumers in the UK are unlikely to be adversely affected by current levels of methylmercury in fish in UK coastal waters.

Mouldy potatoes for pigs and rabbits

Sharma, R. P., Willhite, C. C., Wu, M. T. & Salunkhe, D. K. (1978). Teratogenic potential of blighted potato concentrate in rabbits, hamsters, and miniature swine. *Teratology* **18**, 55.

A few years ago, a theory was postulated that anencephaly and spina bifida were induced by the consumption of blighted potatoes, but subsequent epidemiological and experimental studies provided little support for the hypothesis (*Cited in F.C.T.* 1974, **12**, 772). Nevertheless the idea has continued to evoke some interest, and the above-cited study investigated the possible relationship between the ingestion of blighted potatoes and anencephaly in hamsters, miniature swine and rabbits.

The rabbits were fed throughout gestation and the swine during the first half of gestation on balanced diets containing 50% freeze-dried potatoes previously blighted by inoculation with *Phytophthora infestans* or *Alternaria solani*. These diets were also fed to pregnant hamsters for an undefined period. Control groups for each species were fed a balanced diet consisting of 50% unblighted potatoes and 50% commercial animal feed. Separate groups of hamsters were orally intubated on days 5–10 of gestation with mycelial extracts of these two fungi or with glycoalkaloids from potato sprouts, in each case in ethanolic solution. In all groups, the offspring were removed by caesarian section and the foetuses were examined for gross external and internal malformations. Examinations were performed under a dissecting microscope,

medial sectioning being performed to permit detailed examination of the brain and spinal cord and associated bone structures, but no tissues were examined histologically.

In the hamsters, no significant treatment-related differences were seen in food consumption, pregnancy rate or litter size, although there was some reduction in maternal and foetal weights following ingestion of the blighted-potato diets. There was no evidence that either the blighted-potato diets or the intubated solutions had any teratological or embryotoxic effects in this species. There was one case of anencephaly in the litters from the four miniature swine fed the fungal diets. The cerebral hemispheres and most of the midbrain were absent from this foetus, but its skeletal formation was normal. In five of the 60 foetuses taken from rabbits fed potato infested with one or other fungus, incomplete closure of the caudal vertebral column produced a condition similar to occult sacral spina bifida; a further seven of these foetuses were dead and abnormally small and the brain of one in the *A. solani* group was half the normal size, the cranial cavity being filled with fluid.

Unfortunately the small size of the control group (a total of nine foetuses from two does) precludes any judgement on the biological significance of these findings in the rabbit. Similarly little can be deduced from the single case of anencephaly among the four litters from sows fed blighted potato and the two control litters (giving totals of 34 and ten foetuses, respectively). Nevertheless, the authors claim that anencephaly is rare in swine, and they suggest that their studies provide sufficient indication of the susceptibility of the swine and rabbit to potato blight to make this possibility worthy of further study.

AGRICULTURAL CHEMICALS

ETU and the thyroid

Lu, M.-H. & Staples, R. E. (1978). Teratogenicity of ethylenethiourea and thyroid function in the rat. *Teratology* **17**, 171.

Newsome, W. H., Yagminas, A. P., Villeneuve, D. C. & Franklin, C. A. (1978). The effect of sodium bromide ingestion on the goitrogenic action of ethylenethiourea in the male rat. *Bull. envir. Contam. Toxicol.* **20**, 678.

Ethylene thiourea (ETU), a degradation product of ethylenebisdithiocarbamate fungicides, has been shown to be mutagenic in bacteria and teratogenic in rats (*Cited in F.C.T.* 1977, **15**, 361; *ibid* 1979, **17**, 177). Its transplacental passage into the foetus has been demonstrated (*ibid* 1977, **15**, 80), and it has been shown to be concentrated in the thyroid gland (*ibid* 1979, **17**, 416). The effects of ETU on thyroid function are described in the two papers cited above.

In the first cited study, female rats were given 40 mg ETU/kg/day by gavage on days 7–15 of gestation. The treated groups included normal euthyroid rats, rats rendered hypothyroid by operation, and others injected sc with 50 μg thyroxine/kg on days 7–15 of gestation. When the animals were killed on day 20, 84–100% of the foetuses in all groups receiving ETU showed malformations, regardless of the thyroid status of the dam, compared with 10% of foetuses of dams which had had thyroids and parathyroids removed at 75 days of age, but which had not been exposed to ETU. No malformations were observed among the foetuses of the other groups not treated with ETU. Thus, the teratogenicity of ETU did not involve alteration of the maternal thyroid status. However, ETU treatment did lower serum thyroxine concentrations, and hypothyroidism in itself increased the incidence of foetal malformations and slightly altered the pattern of specific malformations that occurred after ETU administration.

In the second study cited, groups of ten male rats were fed for 28 days on diets containing 200 or 2000 ppm sodium bromide (NaBr), with or without 25, 50 or 100 ppm ETU. After exposure, the thyroid glands were assayed for ETU, and serum thyroxine was determined by immunoradiometric assay. The concentration of ETU in the thyroid paralleled the dietary concentration at the 25 and 50 ppm ETU levels, but not at the 100 ppm ETU level, and was not significantly different in rats fed both NaBr and ETU. There was a biphasic response of serum thyroxine levels to the administration of 200 and 2000 ppm NaBr in combination with ETU. At lower levels of ETU the serum thyroxine level tended to increase while at higher ETU levels there was no increase in serum thyroxine, but the thyroid weight tended to increase. Thus, over the dose range used, the goitrogenic effect of ETU was only slightly augmented by the addition of NaBr, another known goitrogen, to the diet.

FEED ADDITIVES

DES metabolism in primates

Helton, E. D., Hill, D. E., Lipe, G. W., Sziszak, T. J. & King, J. W., Jr. (1978). The metabolism of diethylstilbestrol in the rhesus monkey and chimpanzee. *J. envir. Path. Toxicol.* **2**, 521.

The metabolism and excretion of diethylstilboestrol (DES) in livestock is prolonged, and since the safety of residues present in carcasses used as food has been questioned on carcinogenic and teratological grounds, a more accurate assessment of the metabolism of DES in various animal species is desirable.

Three female rhesus monkeys and one female chimpanzee were dosed orally with ^{3}H- or ^{14}C-labelled DES in aqueous ethanol, and one pregnant rhesus monkey was dosed iv. Irrespective of species and the route of administration, the excretion of radioactive metabolites was mainly in the urine. In the pregnant monkey dosed iv, 13·9 and 55·8% of the radioactivity had been excreted in the faeces and urine, respectively, after 72 hr. Faecal excretion was lower in the other animals. After 48 hr, the amounts of the dose excreted in the faeces and urine, respectively, were 3·0 and 25·4% for one animal and 0·5 and 61·1% for another. In a third monkey, after 144 hr, 23·7% of the dose had been excreted in the urine and 3·6% in the faeces. In the chimpanzee 36·5% of the dose was excreted in the urine and 18·4% in the faeces.

No non-polar labelled metabolites were found in the urine, suggesting that only conjugated DES was excreted by this route. Five fractions were separated by solvent extraction and chromatography; four were hydrolysable with β-glucuronidase, and the aglycones were characterized by gas chromatography and mass spectrometry as *cis*- and *trans*-DES and dienoestrol. In both species the faecal metabolites included both polar and non-polar compounds. Non-polar metabolites were numerous, and the primary non-polar faecal metabolite chromatographed as DES in both species. In one rhesus monkey the occurrence of DES and dienoestrol in the non-polar fraction was confirmed by mass spectrometry. The other non-polar metabolites were not identified. The polar metabolites resembled those detected in urine, but were less susceptible to enzymic hydrolysis.

[The results indicated much variation between animals, and may also have been affected by the differences between the doses given to each animal. There was a wide range in the total percentage of the dose recovered in the urine and faeces. In some respects these results differ from those of other workers. Metzler *et al.* (*J. Toxicol. envir. Hlth* 1977, **3**, 439) dosed monkeys and chimpanzees with 1 mg crystalline DES/kg contained in gelatin capsules. Although the principal route of excretion was the urine, the amounts of the ingested radioactivity excreted in the faeces were considerably greater than in the study described above, and the only faecal metabolite detected was DES. These discrepancies remain to be resolved.]

OCCUPATIONAL HEALTH

Chromium through the skin

Lidén, S. & Lundberg, E. (1979). Penetration of chromium in intact human skin in vivo. *J. invest. Derm.* **72**, 42.

Chromium (Cr), a common cause of skin allergy, can be absorbed through human skin in both trivalent and hexavalent forms (*Cited in F.C.T.* 1973, **11**, 507). Its absorption seems to be enhanced by surfactants and varies with the pH of the solution. That the formation of complexes and the nature of the vehicle may influence Cr penetration is evident from the present study.

Intact human skin from patients with suspected contact dermatitis was treated with an occluded filter-paper patch impregnated with potassium dichromate ($K_2Cr_2O_7$) in aqueous solution or in yellow petrolatum, or a Cr-glycine complex in aqueous solution. Test patches were left for 5, 24 or 48 hr and skin biopsy samples were taken at 5, 24 or 72 hr. Biopsy specimens were immediately frozen, paraffin-embedded, and sectioned parallel to the skin surface. Cr concentrations in the section were estimated by graphite-furnace atomic absorption spectroscopy. In addition to the high levels found in the stratum corneum, Cr was concentrated at two sites, at the dermal-epidermal junction and in the upper middermis, but the significance of this distribution was not clear to the authors. A steady state of distribution was evident by 5 hr, and penetration continued for up to 72 hr. Hexavalent Cr (Cr^{VI}) in petrolatum penetrated much more than Cr^{VI} in aqueous solution. The pronounced difference, roughly five-fold, between Cr^{VI} penetration in petrolatum and in water may have been due to the greater capacity of the lipophilic vehicle to penetrate through hair follicles and sebaceous glands. Relative penetration was greater with a 0·05% solution than with a 0·5% solution. The Cr applied as the Cr-glycine complex penetrated less than did that applied as the hexavalent salt. Cr-glycine elicited a weak positive percutaneous reaction in one of two Cr-sensitized patients.

Mutagenicity of impure DBCP

Biles, R. W., Connor, T. H., Trieff, N. M. & Legator, M. S. (1978). The influence of contaminants on the mutagenic activity of dibromochloropropane (DBCP). *J. envir. Path. Toxicol.* **2**, 301.

Dibromochloropropane (DBCP) has been implicated as a possible cause of sterility among industrially exposed workers, and studies in the rat have demonstrated that the testis is a target organ for DBCP (*Cited in F.C.T.* 1978, **16**, 498). Further studies of men with a history of direct exposure to DBCP have shown that spermatogenic activity may be reduced in proportion to the period of exposure to the compound (*ibid* 1979, **17**, 555). However the mechanism of DBCP's action on the testes is unknown. Animal tests have shown DBCP to be carcinogenic in rats and mice (*ibid* 1975, **13**, 144) and the mutagenicity of DBCP in *Salmonella typhimurium* strain TA1530 has also been demonstrated (*ibid* 1976, **14**, 505).

As in all problems concerning the effects of industrial chemicals on humans, the role of contaminants must be evaluated before a pure compound is incriminated. In the paper cited above, technical-grade DBCP containing 1% epichlorhydrin, 0·6% allyl chloride and 2·4% related halogenated hydrocarbons was compared with a 99%-pure sample of DBCP for

mutagenicity in the *Salmonella typhimurium* TA1535 method, with and without S-9 activation. The technical-grade sample produced a weak dose-related increase in the number of revertant cells at concentrations from 50 to 1600 μg/agar plate. Levels above 1600 μg/plate were toxic. The pure grade showed only a very slight sporadic increase in the numbers of revertants; it too was toxic at levels above 1600 μg/plate. Distillation of technical-grade DBCP yielded an initial fraction rich in epichlorhydrin, and even at levels as low as 100 μg/plate this fraction produced more than twice the number of revertants produced by the highest concentration of technical DBCP tested. The response of the technical-grade DBCP in the dose-response tests correlated with the response that would be expected from the equivalent amount of pure epichlorhydrin. Activation of both grades of DBCP with S-9 derived from Aroclor-pretreated rats, however, resulted in similar strong dose-related mutagenic responses in the range 20–200 μg/plate. It is clear that epichlorhydrin contributes very significantly to the direct mutagenic activity of commercial samples of DBCP, but that DBCP itself is also potently mutagenic after metabolic activation in the liver. The slight direct activity of the 'pure' DBCP may be attributable to the compound itself or to traces of impurities.

Sensitization to epoxy resin components

Thorgeirsson, A. (1978). Sensitization capacity of epoxy reactive diluents in the guinea pig. *Acta derm.-vener., Stockh.* **58**, 329.

Thorgeirsson, A. (1978). Sensitization capacity of epoxy resin hardeners in the guinea pig. *Acta derm.-vener., Stockh.* **58**, 332.

Epoxy resins and their hardeners are commonly responsible for contact allergy (*Cited in F.C.T.* 1974, **12**, 582). There is evidence that sensitizing capacity may reside only in compounds of lowest molecular weight (*ibid* 1978, **16**, 503) and diluents and hardeners may also play a part in sensitizing industrial handlers.

In the first study cited above, five reactive diluents containing epoxide groups and with molecular weights between 175 and 360, and a sixth, an aliphatic polyglycidyl ether of molecular weight 1700, were tested in a guinea-pig maximization test. 1,2-Epoxydodecane elicited reactions in 40% of the animals, the monoglycidyl ester of synthetic fatty acids in 87%, the monoglycidyl ether of isomeric alcohols in 80%, the diglycidyl ether of butanediol in 60%, and the diglycidyl ether of neopentylglycol in 87%. The high-molecular-weight polyglycidyl ether produced no reactions.

In the second paper cited, the results of a guinea-pig maximization test on a number of epoxy resin hardeners are described. The hardeners included seven aliphatic polyamines, three cycloaliphatic polyamines, two polyaminoamides, an aromatic amine and adducts of phenol-accelerated triethylenetetramine (TETA), of TETA and propylene oxide, of isophoronediamine and low-molecular-weight epoxy resin, and of diethylenetriamine and epoxy resin.

Reactivity to the aliphatic polyamines ranged from 55 to 93%, and diaminodiphenylmethane elicited reactions in 20%. There was no reaction to one of the cycloaliphatic polyamines, 3,3'-dimethyl-4,4'-diaminodicyclohexylmethane, but 100% of the tested animals reacted to the other two, isophoronediamine and *N*-aminoethylpiperazine. A polyaminoamide based on tetraethylenepentamine elicited reactions in 67% of the animals but that based on TETA in only 20%. The adduct of phenol-accelerated TETA produced reactions in 47%, but there was no reaction to the other triethylenetetramine adducts. The adduct of isophoronediamine and a low-molecular-weight epoxy resin gave reactions in 73% of the animals. The results indicated that amine-free polyaminoamides are probably not sensitizers and that certain adducts, also, are not sensitizers unless they contain some free amine.

In view of the sensitizing capacity of many of the diluents and hardeners tested in these studies, there is a need for further tests on these and other substances used in epoxy resins in order to identify, or if necessary develop, additional non-sensitizing compounds.

Combined solvents—effects in rats

Vainio, H., Savolainen, H. & Pfäffli, P. (1978). Biochemical and toxicological effects of combined exposure to 1,1,1-trichloroethane and trichloroethylene on rat liver and brain. *Xenobiotica* **8**, 191.

Trichloroethylene is a potentially toxic solvent, its harmful effects being attributed largely to its metabolite trichloroethanol (*Cited in F.C.T.* 1974, **12**, 163); among the toxic effects produced are liver lesions (*ibid* 1978, **16**, 491). Although 1,1,1-trichloroethane is relatively innocuous (*ibid* 1975, **13**, 402), it is apparently able to bind to cytochrome *P*-450 and so affect liver function (Pelkonen & Vainio, *FEBS Lett.* 1975, **51**, 11). Industrial exposure to such solvents rarely involves a single compound, and the results of an investigation into the combined toxicity of these two chlorinated hydrocarbons are therefore of interest.

Male rats were exposed to 500 ppm 1,1,1-trichloroethane and 200 ppm trichloroethylene together in the atmosphere of a 1 m^3 exposure chamber for 6 hr daily for 4 days. On day 5, two animals were killed immediately and two groups of four each were further exposed to the solvents for 2 hr or 6 hr before they were killed. Brain, liver, lung and perirenal fat samples were taken for estimation of the compounds.

After 4 days, marked accumulation of trichloroethane was found in perirenal fat, but no trichloroethylene appeared there until the exposure on day 5. Brain retention of the two compounds was in the ratio 8:1 (1,1,1-trichloroethane:trichloroethylene), and a similar relative distribution occurred in lung tissue. In liver, the trichloroethylene concentration decreased towards the end of the experiment on the fifth day.

The brain-protein content was not significantly altered by combined exposure, but in rats re-exposed on day 5 for 6 hr there was a reduction in brain RNA. Acid-proteinase activity was increased in rats after the 2-hr exposure on day 5 but was normal after the 6-hr exposure. Brain glutathione was not affected by the exposure. Microsomal drug-metabolizing activity in

the liver was enhanced by combined exposure, cytochrome *P*-450 increasing by about 40% and styrene mono-oxygenase some three-fold.

Microsomal UDP-glucuronosyl transferase activity in digitonin-activated microsomes was doubled. Following re-exposure on day 5, styrene mono-oxygenase activity declined, and it was apparent that a reversible decrease in the activity of this enzyme system may also have occurred during the first 4 days of exposure to the solvents.

ENVIRONMENTAL CONTAMINANTS

Ferritin in league with lead inclusions

Richter, G. W., Velasquez, M. J. & Shedd, R. (1979). Ferritin in rat kidneys with specific lesions due to a single dose of lead. *Am. J. Path.* **94**, 483.

A single parenteral injection of lead into the rat or mouse has been shown to result in a marked increase in protein synthesis in the kidneys and the rapid appearance of characteristic intranuclear inclusion bodies in the cells of the proximal tubules (*Cited in F.C.T.* 1976, **14**, 216). These inclusion bodies, which also occur in the cytoplasm but do not persist there as they do in the nucleus, were shown to be a lead–protein complex, apparently formed from protein synthesized as a direct result of the lead stimulation. Subsequently, Richter (*Am. J. Path.* 1976, **83**, 135) demonstrated that clusters of ferritin molecules were regularly found in close proximity to the cytoplasmic lead–protein bodies during the first 3 days after a lead injection, thus raising questions of the possible effects of lead on iron metabolism.

Further work has now indicated, however, that administration of lead affects the distribution of ferritin in the cytoplasm rather than its rate of synthesis. In rats of two strains given either distilled water or water enriched with 1% iron, it was shown by immunoradiometric assay that the kidneys of adult females contained significantly more ferritin than the males of comparable age. Injection of lead 48 hr before the rats were killed, followed 30 hr later by injection of ^{14}C-labelled amino acids, demonstrated that the synthesis of ferritin protein extractable from the kidneys was either unaffected or in some cases depressed by the administration of lead, whether or not supplemental iron was given. Moreover, in the absence of supplemental dietary iron, lead did not promote, and in fact tended to depress, the incorporation of the iron isotope ^{59}Fe into kidney ferritin. Nevertheless, electron microscopy confirmed the presence of ferritin clusters lying close to the dense lead–protein bodies in the cytoplasm of these rats.

This study did not establish whether the ferritin in these clusters was synthesized before or after the lead was injected, nor whether it was formed in the vicinity of the lead–protein microfibrils or only accumulated there. The findings do indicate, however, that this phenomenon reflects a selective effect of lead on ferritin distribution in the cytoplasm rather than the previously postulated formation of ferritin from iron diverted by lead from its normal utilization in metabolic pathways.

COSMETICS, PHARMACEUTICALS AND HOUSEHOLD PRODUCTS

Flame retardants—another washout?

Ahrens, V. D., Maylin, G. A., Henion, J. D., St. John, L. E., Jr. & Lisk, D. J. (1979). Fabric release, fish toxicity and water stability of the flame retardant, Fyrol FR-2. *Bull. envir. Contam. Toxicol.* **21**, 409.

The leaching of flame retardants from fabrics on laundering and their toxicity to aquatic species are among the problems of selecting substitutes for the now widely disclaimed tris-(2,3-dibromopropyl) phosphate (TRIS). We have previously reported that tris-(2,3-dichloropropyl) phosphate (Fyrol FR-2; *Cited in F.C.T.* 1978, **16**, 504) is toxic to goldfish and comparable with TRIS in this respect. Further studies including the effects of laundering have now been carried out on Fyrol FR-2.

Pairs of children's pyjamas, treated with Fyrol FR-2 or TRIS or untreated, were laundered by a standard test method using soft water and 60 g phosphate detergent. After specific numbers of successive launderings, circular discs of the fabrics were removed and analysed for chloride or bromide to determine the amount of flame retardant remaining. The rates of release of Fyrol FR-2 and TRIS were similar over 20 launderings; the total quantities released were respectively 37 and 46% (of the original amount on the garments). The possibility of absorption of released TRIS by unfinished polyester fabric laundered simultaneously was also investigated. No such absorption was found.

When a piece of unlaundered polyester nightwear, measuring 38 × 64 cm and pretreated with Fyrol FR-2, was immersed in 20 litres of well-water containing six goldfish, the goldfish became progressively more sluggish and all died within 3 hr; analysis of this water revealed 30 ppm Fyrol FR-2. After a single laundering of the piece of fabric, there were no signs of toxicity among fish similarly exposed for 96 hr.

Fyrol FR-2 appeared to be stable after 24 hr in water. By contrast TRIS has previously been reported to undergo dehydrobromination in water (*ibid* 1978, **16**, 504).

TEST PROCEDURES

Feeding experimental error?

Edwards, G. S., Fox, J. G., Policastro, P., Goff, U., Wolf, M. H. & Fine, D. H. (1979). Volatile nitrosamine contamination of laboratory animal diets. *Cancer Res.* **39**, 1857.

The carcinogenicity of *N*-nitrosamines in laboratory animals is well established (*Cited in F.C.T.* 1979, **17**, 167). These compounds have been detected in a wide range of environments, but it is also known that they can be formed inadvertently during analysis (*ibid* 1979, **17**, 535). Results from the study cited above draw attention to another possible source of experimental error, the presence of low levels of volatile *N*-nitrosamines in laboratory-animal feeds.

Samples of nine commercially available pelleted laboratory-rodent feeds and one canine feed were analysed for nitrosamines using a gas chromatograph interfaced to a thermal energy analyser, and various procedures were used to avoid the formation of artefacts. All of these feeds were labelled as containing unspecified amounts of fish meal, and *N*-nitrosodimethylamine (NDMA) was detected in all of them at levels between 0·9 and 3·7 ppb ($b = 10^9$). In addition, one sample of rabbit feed contained 1·3 ppb NDMA and 3·2 ppb *N*-nitrosopyrrolidine (NPYR) but another sample contained no detectable volatile nitrosamines (<0·1 ppb). Fish meal was not listed as an ingredient in either of the samples of rabbit feed.

NDMA was also detected in all seven samples of NIH (National Institutes of Health) open formula rat and mouse ration that were tested. One sample contained 52 ppb NDMA, while in the other six samples the levels ranged from 1 to 8 ppb. NPYR was detected in five of the samples of the NIH ration. Again fish meal was implicated as the major source of the nitrosamine. Analysis of the corn- and fish-meal components of the NIH rat and mouse ration showed that NDMA was present in sufficient amounts in the fish meal to account for the nitrosamine contamination of the feed.

The authors consider it improbable that even the highest level of nitroamines detected in these feed samples would cause a significant increase in the incidence of cancer in animals used in a typical study (one involving a few hundred animals). However they cite a recent study that indicates that such low nitrosamine levels may have an effect (Anderson *et al. J. natn. Cancer Inst.* 1979, **62**, 1553). These workers found that the lung-tumour incidence was significantly increased in the progeny of strain A female mice that had been given 10 ppb NDMA in their drinking-water from 4 wk before mating until parturition. The offspring themselves were dosed at this level until they were 22 wk old. It is also possible that dietary contaminants could act synergistically with test carcinogens or co-carcinogens to increase tumour incidence or alter the target organ. Such synergistic effects have been demonstrated with higher levels of NDMA (Hoch-Ligeti *et al. ibid* 1968, **40**, 535). It is concluded that these results indicate the need to minimize the nitrosamine content of animal diets as far as possible, so as to avoid a possible source of experimental error in carcinogenicity testing.

CARCINOGENICITY AND MUTAGENICITY

Co-carcinogenic role of cholesterol in colon cancer

Cruse, P., Lewin, M. & Clark, C. G. (1979). Dietary cholesterol is co-carcinogenic for human colon cancer. *Lancet* **I**, 752.

Epidemiological studies of colon cancer provide strong indications of the involvement of some environmental factor or factors. The disease is most common in north-west Europe and the USA and is relatively rare in Africa, Asia and Japan (*Cited in F.C.T.* 1976, **14**, 209). Several theories have been put forward postulating the roles of dietary components. Colon cancer has been linked to a low intake of fibre in the diet and to the consumption of large amounts of animal protein. The theory of a relationship between the consumption of large amounts of fat, particularly animal fats, and colon cancer has also received much support, and there have been investigations into the effects of the acid and neutral steroids and their dietary metabolites and of cholesterol (*ibid* 1976, **14**, 211). Cruse *et al.* (cited above) have put forward a hypothesis, outlined below, of the role of cholesterol as a co-carcinogen for human colon cancer. They base their view on evidence from a large number of epidemiological and human studies, on the results of animal studies and on the knowledge of the biochemical effects of cholesterol.

Epidemiological studies have implicated high intakes of meats and animal fats in the pathogenesis of colon cancer, and there is a significant correlation between a high consumption of cholesterol-containing foods and the occurrence of the disease. Additionally, an overlap is observed between populations with a high incidence of colon cancer and those with a high incidence of arteriosclerotic heart disease. This overlap suggests shared aetiological factors for the two diseases, and cholesterol is known to be involved in the latter. Faecal levels of cholesterol and cholesterol metabolites are higher in patients with colon cancer or with diseases that are known to predispose to colon cancer than they are in healthy people from the same population group. Furthermore population groups with a high incidence of colon cancer excrete higher levels of cholesterol and cholesterol metabolites than those with a low incidence of the disease. Studies in animals have also shown a close relationship between cholesterol intake and the incidence of colon tumours.

Cruse *et al.* suggest that cholesterol acts as a co-carcinogen, promoting the development of an already

initiated incomplete carcinogenic process. Possible mutagenic initiating agents are intrinsic genetic factors, and chemical or other agents present in industrialized environments. There are several mechanisms by which cholesterol might promote carcinogenesis. It is essential in membrane structure and function and is involved in membrane-bound enzyme activity and steroid-hormone synthesis. It could therefore determine the rate of development or spread of a carcinoma or precancerous lesion. Cholesterol or closely related compounds have also been shown to affect protein synthesis and the stabilization of the DNA helix.

The cholesterol hypothesis accounts for the observed geographical distribution of the incidence of colon cancer. The disease is rare among rural Africans who are exposed to few mutagenic initiators and consume relatively little cholesterol. It is also uncommon among the Japanese, who are industrialized and therefore exposed to initiators, but who eat little cholesterol. On the other hand, Eskimos, another group with a low incidence of colon cancer, eat much cholesterol but are not industrialized and so are not exposed to major initiators.

This hypothesis is claimed to differ in several ways from earlier theories linking dietary fat and human colon cancer. It makes cholesterol specifically responsible for fat-linked epidemiological associations, it emphasizes the importance of ingested cholesterol and it suggests a direct mechanism of action—that cholesterol is a co-carcinogen. If the hypothesis is correct, a low-cholesterol or cholesterol-free diet might decrease the risk of colon-cancer development. In patients who have already had a colon carcinoma resected such a diet might also inhibit the growth of residual tumour cells or retard metastases.

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MEETING ANNOUNCEMENTS

FOODBORNE INFECTIONS AND INTOXICATIONS

Following an earlier brief announcement (*Fd Cosmet. Toxicol.* 1980, **18,** 111), further details have been made available about the World Congress on Foodborne Infections and Intoxications to be held in the International Congress Centre, Berlin (West), from 29 June to 3 July 1980. The programme will include papers on the toxic and carcinogenic potential of natural toxins and other contaminants in food, as well as on the transmission of infections by foodstuffs. The influence of social and economic factors, of dietary habits and of developments in technology will also be considered in this context.

Further information may be obtained from Generalsekretariat Weltkongress, c/o Institut für Veterinärmedizin, Thielallee 88–92, Postfach 33 00 13, D–1000 Berlin 33 (telephone no. (030) 83 08–763; Telex 01 84 016).

DRUGS AND THE LIVER

The Liver Unit, King's College Hospital and Medical School, London, is organizing an international symposium on "Drug Reactions and the Liver: Mechanisms and Measures for Control". The meeting will be held at The Royal Society, London, on 3 and 4 July 1980. Current knowledge of the epidemiology, pathogenesis, management and prevention of hepatic drug reactions will be presented by clinicians, research scientists and members of the drug industry. Sessions will generally involve short presentations followed by panel discussions with free audience participation, and the numbers attending will be limited to facilitate discussion.

Correspondence relating to the symposium should be addressed to: Advisory Services Medical Symposia Ltd., 79 Wimpole Street, London W1M 7DD (telephone no. 01–935 9962).

NUTRITION, CANCER AND TOXICOLOGY

The Nutrition Society is to hold two related symposia in Cambridge (in the Physiology Lecture Theatre) on 22 and 23 July 1980. On the first day, a symposium on "Nutrition and Cancer" is scheduled to include papers by Prof. J. V. B. Joosens, Dr. M. J. Hill, Dr. J. H. Cummings, Prof. M. R. Alderson and Prof. J. W. T. Dickerson, who will consider diet, and specifically dietary fats and dietary fibre, in relation to cancer, epidemiological studies and the nutrition of the cancer patient. The final paper, on the toxicity and carcinogenic potential of food additives, will provide a link with the symposium on "Nutrition and Toxicology" to be held on the following day. This paper will be given by Dr. F. A. Fairweather (DHSS), who will also chair the second symposium. The programme for the latter includes papers on the safety evaluation of foods (Mr. J. McL. Philip, Unilever Ltd.), the nutritionists' attitude to tumour incidence in laboratory animals (Dr. F. J. C. Roe), the influence of gut microflora on food toxicity (Dr. I. R. Rowland, BIBRA), *in vitro* tests for chemical carcinogens (Dr. R. C. Garner, University of York) and new approaches to safety screening (Dr. D. M. Conning, BIBRA).

Persons who are not members of the Nutrition Society may attend the meetings if introduced by a member. The Society's Hon. Programmes Secretary is Mr. M. I. Gurr, National Institute for Research in Dairying, Shinfield, Reading RG2 9AT (telephone no. 0734–883103).

CO-CARCINOGENS AND TUMOUR PROMOTERS

The German Cancer Research Centre and the International Agency for Research on Cancer are arranging a symposium on Co-carcinogenesis and Biological Effects of Tumour Promoters to be held in the Castle of Elmau (in the Bavarian Alps) from 13 to 16 October 1980. It is expected that attendance will be limited. The programme will consist of invited lectures and selected papers; the language of the meeting will be English. Further information may be obtained from the Secretariat: Cocarcinogenesis and Biological Effects of Tumor Promotors, German Cancer Research Center, DKFZ, Im Neuenheimher Feld 280, D–6900 Heidelberg 1, Federal Republic of Germany.

SOIL POLLUTION CONGRESS

The Faculty of Agricultural Sciences of El-Zagazig University, Egypt, is arranging the 1st International Congress for Soil Pollution and Methods of Protection from Pesticide Residues. The suggested date for the Congress, which will be held at El-Zagazig, is 1–7 September 1981.

Topics to be considered will include pesticide residues in soil, plants, air, water and foods, their persistence and degradation, their effects on soil fertility and on useful plants and insects, and the problem of insect resistance. Also to be considered are new approaches to pest control, aimed at reducing the pollution of soils by pesticide residues. Papers relating to these subjects are being invited from interested organizations and manufacturing companies, and provision is being made for about 1000 scientists to attend.

Further enquiries, and suggestions regarding participation, should be addressed to: Prof. Dr. Ahmed A. Abdel-Gawaad, El-Zagazig University, President's Office, El-Zagazig, Egypt.

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FORTHCOMING PAPERS

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

Screening of food dyes for genotoxic activity. By R. B. Haveland-Smith and R. D. Combes.

Genotoxicity of the food colourings Red 2G and Brown FK in bacterial systems. Use of structurally related dyes and azo-reduction. By R. B. Haveland-Smith and R. D. Combes.

Species differences in the haemorrhagic response to butylated hydroxytoluene. By O. Takahashi, S. Hayashida and K. Hiraga.

Evaluation of the mutagenicity of sorbic acid–sodium nitrite reaction products produced in bacon-curing brines. By M. C. Robach, V. G. DiFate, K. Adam and L. D. Kier.

Mutagenicity of Chinese wine treated with nitrite. By J.-Y. Lin and M.-W. Tai.

Studies of the effect of 2-(2'-hydroxy-5'-methylphenyl)benzotriazole on rat liver. By K. Schmid, W. Schweizer, W. Stäubli and F. Waechter.

Metabolism and tissue distribution of [^{14}C]aflatoxin B_1 in pigs. By J. Lüthy, U. Zweifel and Ch. Schlatter.

In vivo covalent binding of aflatoxin metabolites isolated from animal tissue to rat-liver DNA. By W. Jaggi, W. K. Lutz, J. Lüthy, U. Zweifel and Ch. Schlatter.

Effects of mycotoxins on mixed function oxidase and adenosine triphosphatase systems in neonatal rats. I. Aflatoxin B_1/rubratoxin B. By T. D. Phillips, M. Y. Siraj and A. W. Hayes.

Effects of feeding T-2 toxin to rats and monkeys. By C. Rukmini, J. Siva Prasad and K. Rao.

Investigation on the toxicology and safety of algal diets in albino rats. By L. V. Venkataraman, W. E. Becker, T. Rajasekaran and K. R. Mathew.

Effect of betel-quid chewing on nitrite levels in saliva. By N. M. Shivapurkar, A. V. D'Souza and S. V. Bhide.

Chemistry of nitroso-compounds. Part 16. Formation of *N*-nitrosamines from dissolved NOCl in the presence of alkanolamines and related compounds. By B. C. Challis and D. E. G. Shuker.

Studies of *in vitro* cell transformation and mutagenicity by surfactants and other compounds. By K. Inoue, T. Sunakawa and S. Takayama.

Volatile nitrosamines in human blood before and after ingestion of a meal containing high concentrations of nitrate and secondary amines. By M. Yamamoto, T. Yamada and A. Tanimura. (Short paper)

CIIT dedication: Address of welcome and introduction of the main speaker. By L. Golberg. (Review paper)

New directions in toxicology. By P. H. Abelson. (Review paper)

ADDITIVE MIGRATION FROM PLASTICS INTO FOOD

T. R. Crompton

This book deals with the problems that may arise where direct contact occurs between the packed commodity and the plastic wrapping. The various types of plastics used in packaging are discussed as well as the types of substances present in the plastic which may migrate to food. Much of the book analyzes, for the first time, the world literature on the subject, covering extraction testing and the analysis of extractants. Much of interest is included for legislators who are involved in formulating regulations concerning packaging in plastics and for medical and public health authorities, too.

Contents

Types of polymers used in food packaging — Non-plastics components of plastics — Principles of extractability testing of plastics — Determination of additives in differing extractants: aqueous, alcoholic, edible oils and fatty foodstuffs — Determination of additives and their degradation products — Determination of antioxidants in foods — Legislative aspects of the use of additives in foodgrade plastics.

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

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