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Research Section

N-NITROSODIMETHYLAMINE IN DRIED DAIRY PRODUCTS

L. M. LIBBEY, R. A. SCANLAN and J. F. BARBOUR
Department of Food Science and Technology, Oregon State University, Corvallis, OR 97331, USA

(*Received 26 December 1979*)

Abstract—Commercially available non-fat dried milk and dried buttermilk were analysed for volatile nitrosamines using a chemiluminescence detector. *N*-Nitrosodimethylamine, the only volatile nitrosamine that could be confirmed to be present by mass spectrometry, was detected in eight out of nine samples analysed. The range of levels was 0 to 4·5 ppb (μg/kg) while the mean was 1·9 ppb. The identity of *N*-nitrosodimethylamine was confirmed by mass spectrometry in five of the samples.

INTRODUCTION

Analysis of foods for trace levels of the carcinogenic volatile *N*-nitrosamines has been a subject of intense study for over 10 yr. The recent advent of a very sensitive and quite specific chemiluminescence detector (Thermal Energy Analyzer; TEA) for *N*-nitroso compounds (Fine, Rufeh, Lieb & Rounbehler, 1975) represents a breakthrough in the analysis of these compounds. Until quite recently it was thought that only foods containing nitrite and nitrate (either naturally or as additives) would contain measurable amounts of *N*-nitrosamines, since it was assumed that the only significant route for nitrosamine formation was nitrosation by nitrosating agents derived from nitrite. On the basis of the low levels of nitrite and nitrate present, a non-fat dried milk would seem an unlikely source of *N*-nitroso compounds, yet it was analysed for nitrosamines by Reineccius & Coulter (1972). The sensitivity of their method was 10 ppb (μg/kg) at best, and they failed to find volatile *N*-nitrosamines in dried milk prepared by either direct gas firing or by indirect steam heat drying. More recently Terplan (1978) examined milk and milk products for *N*-nitrosamines, but dried milk was not examined, and the methods used precluded definitive results on the presence of *N*-nitrosodimethylamine (NDMA) in the samples examined. Although Sen, Seaman & McPherson (1979) list non-fat dried milk as a food presumably containing NDMA, their TEA finding was not confirmed by mass spectrometry. At the recent Budapest conference on *N*-nitrosamines very brief mention (Hotchkiss, Libbey, Barbour & Scanlan, 1979) was made of our confirmed finding of NDMA in several samples of dried dairy products; the purpose of this report is to document and amplify these findings.

EXPERIMENTAL

Seven non-fat dried milks and two dried buttermilks were purchased from local retail outlets. These products were sampled and examined for their content of volatile *N*-nitrosamines as described by Hotchkiss, Scanlan & Libbey (1980) except that 50 g of sample and 70 ml of mineral oil were used in the vacuum distillation procedure. The distillate extracts were quantitatively analysed on a gas chromatograph (GC) interfaced to a TEA (Thermo Electron Corp., Waltham, MA). It was necessary to scale-up the distillation and extraction procedure three-fold in order to obtain sufficient amounts of nitrosamine for mass spectral confirmation. The trapping of the *N*-nitrosamines from the GC–TEA for GC-mass spectrometric (GC–MS) confirmation was also carried out by the method described previously (Hotchkiss *et al.* 1980), except that instead of the specified Carbowax 20 M packed column, a stainless-steel column (3·05 m × 3·18 mm OD) packed with 5% OV-275 on Chromosorb W-AW, 80/100 mesh was used. The column was operated at 100°C and the injector temperature was 150°C. In essence, a minivolume valve (Carle Instrument Co., Fullerton, CA) installed between the end of the packed column and the TEA allowed the diversion of the NDMA for collection in a dry-ice-chilled coil of 25·4 cm × 1·59 mm OD nickel tubing: several injections were required to collect enough nitrosamine (50–100 ng) for full-scan mass spectra.

The trapped NDMA was analysed using a GC–MS–data system. The GC inlet was fitted with a valving system that allowed the trapped nitrosamines to be transferred directly onto the GC column. The GC conditions: column, 152 m long and 0·75 mm ID Carbowax 20 M wall-coated open tubular stainless-steel column; column temperature, 120°C; carrier gas, helium, 15 ml/min. The GC was a Varian model 1400 (Varian Instruments, Palo Alto, CA) interfaced to a Finnigan model 1015C MS (Finnigan Corp., Sunnyvale, CA). Conditions for the MS were: filament current 450 μA; electron voltage, 70 eV; analyser pressure, 10^{-6} Torr; mass scans covered *m/z* 10–100. The MS was interfaced to a Riber 400 data system, (Riber Data Systems, Inc., Palo Alto, CA) and data were acquired by the 'integration as a function of signal strength' (IFSS) program. The data output used programs for limited mass searches and usually *m/z* 74 was used to locate NDMA in the chromatogram. Clean mass spectra were obtained by another program called MSSOUT which allowed averaging of both nitrosamine and background spectra.

RESULTS

The results from the analysis of the nine samples of dried dairy products by GC–TEA are summarized in Table 1. All but one sample contained detectable levels of NDMA, the only volatile nitrosamine that was confirmed to be present. Small GC–TEA peaks with retention times corresponding to those of *N*-nitrosopyrrolidine and *N*-nitrosopiperidine were sometimes observed, but the presence of these two *N*-nitrosamines was not confirmed by MS. The mean level of NDMA for all samples was 1·9 ppb, and the range was 0 to 4·5 ppb. Values in Table 1 are uncorrected for recovery which was approximately 75% for NDMA. The chemiluminescence (TEA) detection limit for NDMA in these products was approximately 0·1 ppb.

A low-resolution mass spectrum confirming the presence of NDMA (4·5 ppb) in a sample of non-fat dried milk is shown in Fig. 1a, while Fig. 1b shows the spectrum of authentic NDMA obtained on the same instrument. The mass-spectral data unequivocally establish the identity of NDMA in the non-fat dried milk. Mass-spectral confirmation was obtained on five of the dried dairy product samples.

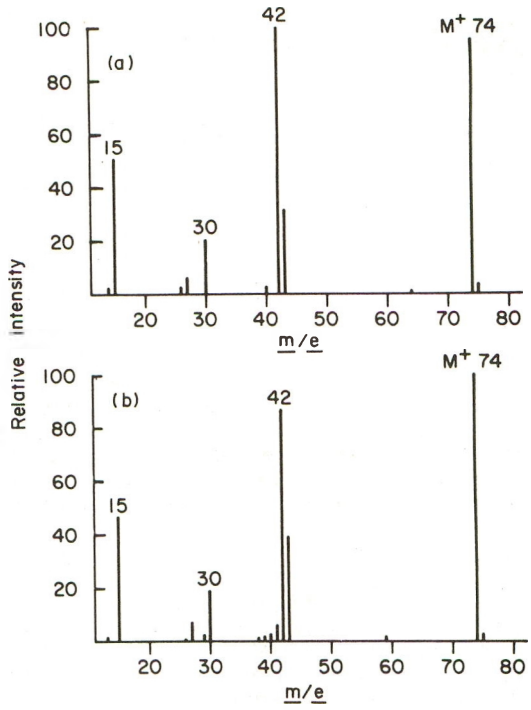

Fig. 1. Mass spectra of *N*-nitrosodimethylamine (NDMA) from (a) non-fat dried milk (determined by gas chromatography-thermal energy analysis to contain 4·5 ppb NDMA) and (b) authentic compound.

DISCUSSION

The results of our limited study indicate that the presence of low levels of NDMA in dried dairy products may be general. It should be mentioned that the low levels of NDMA reported here would be made even lower when the dried dairy product was rehydrated with about 10 volumes of water, as is usual before use. Although NDMA has been found to be a potent carcinogen in animal feeding studies, the hazard posed to humans by the ingestion of foods containing trace (ppb) levels of NDMA is unknown. However, until more is known about the effects of exposure to very low doses, it seems prudent to minimize human exposure to *N*-nitroso compounds. Investigations of nitrosamine formation and occurrence in foods initially focused on cured meats (Scanlan, 1975). Nitrosamine formation in these products was suspected because sodium nitrite is added during the manufacture of cured meats. More recent work indicates that *N*-nitrosamine formation is possible in foods that are dried in a direct-fired dryer. In such a dryer the products of combustion come into direct contact with the food being dried. Nitrosamine formation is probably due to the reaction between secondary and/or tertiary amines in the food and the oxides of nitrogen that are produced during fuel combustion. Fish meal is often prepared by direct-fired drying. Sen, Schwinghamer, Donaldson & Miles (1972) reported the presence of NDMA in fish meal, presumably prepared without nitrite, and suggested the involvement of oxides of nitrogen from the fire gases in the formation of the nitrosamine.

More recently Spiegelhalder, Eisenbrand & Preussmann (1979a) and Scanlan, Barbour, Hotchkiss & Libbey (1980) reported NDMA levels of a few ppb in European and U.S. beers respectively. It has been verified (Kann, Tauts, Kalve & Bogovski, 1979; R. A. Scanlan and J. F. Barbour, unpublished data, 1979; Spiegelhalder, Eisenbrand & Preussmann, 1979b) that malt produced by direct-fired drying is the major if not the sole source of NDMA in beer. Although the mode of formation of NDMA in the dried dairy products analysed in this study has yet to be established, direct-fired drying seems a likely possibility. At this point one might ask whether nitrosamine formation is common in direct-fired dried foods. Future research in our laboratory will be directed to answer that question and to investigate means by which NDMA formation can be prevented.

Table 1. N-*Nitrosodimethylamine content of dried dairy products*

Type of product	Sample no.	NDMA content (ppb)
Non-fat dried milk	1	4·5*
	2	ND
	3	2·2*
	4	3·6*
	5	1·6
	6	2·2*
	7	0·4
Dried buttermilk	1	0·9
	2	1·8*

NDMA = *N*-Nitrosodimethylamine

The values are uncorrected for the level of recovery of NDMA, which was about 75%. An asterisk indicates that the presence of NDMA was confirmed by mass spectrometry.

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SYNERGISTIC EFFECT OF CHLOROGENIC ACID AND THIOCYANATE ON *IN VITRO* FORMATION OF *N*-METHYL-*N*-NITROSOANILINE UNDER PHYSIOLOGICAL CONDITIONS

D. LATHIA and U. FRENTZEN

Fachhochschule Niederrhein, Fachbereich Ernährung/Hauswirtschaft, Richard-Wagner-Str. 101, 4050 Mönchengladbach, Bundesrepublik Deutschland

(*Received 11 January 1980*)

Abstract—The rate of formation of *N*-methyl-*N*-nitrosoaniline (NMNA) from nitrite and *N*-methylaniline in the presence of chlorogenic acid and/or thiocyanate at levels likely to be encountered *in vivo* was studied in a series of *in vitro* reactions. Chlorogenic acid, a phenolic component of coffee, had a marked catalytic effect, which was directly related to its concentration. Thiocyanate also catalysed NMNA formation, and together the two compounds had a cumulative effect on the reaction although in the presence of a constant level of thiocyanate, the increase in NMNA yield was indirectly proportional to the chlorogenic acid concentration.

INTRODUCTION

N-Nitrosamines are known for their powerful carcinogenicity (Magee & Barnes, 1956 & 1967). There is good evidence that they may be formed under acidic conditions in the gastro-intestinal tract by the interaction of nitrite and secondary amines, which are common constituents of human food (Wogan & Tannenbaum, 1975). Such nitrosamine formation can be catalysed by a number of other food constituents both *in vitro* and *in vivo*, including halides like iodide and bromide and the pseudohalide thiocyanate (Boyland, Nice & Williams, 1971; Lathia & Rütten, 1979). It is also known that smokers' saliva contains some three or four times more thiocyanate than that of non-smokers (Boyland & Walker, 1974; Lathia & Brendebach, 1978). The results of recent studies of *in vitro* nitrosamine formation have also shown the catalytic effect of some naturally occurring polyphenols like chlorogenic acid and gallic acid, which are essential constituents of coffee (Challis & Bartlett, 1975) and gallotannins (Walker, Pignatelli & Castegnaro, 1975), respectively.

So far, however, studies of the catalysis of nitrosamine formation *in vitro* have been concerned only with single catalysts. The likelihood that more than one catalyst may be present simultaneously in human food and body fluids, with the consequent possibility of a cumulative catalytic influence on *in vivo* formation of nitrosamines, requires careful attention.

In our early experiments (Lathia & Rütten, 1979; Lathia, Rütten & Jäger, 1979) we demonstrated the cumulative catalytic effect of thiocyanate and iodide on the rate of formation of *N*-methyl-*N*-nitrosoaniline (NMNA) *in vitro*. This paper reports the catalytic effect of chlorogenic acid in physiologically relevant concentrations on the rate of NMNA formation *in vitro*. It also demonstrates the strength of the combined catalytic effect of chlorogenic acid and thiocyanate on this reaction.

A preliminary report of this work was given at the XVI Nutrition Science Congress held by the West German Nutrition Society in Giessen in 1979 (Lathia & Frentzen, 1979).

EXPERIMENTAL

Materials. The following stock solutions were prepared in double-distilled water: 0·02 M-KNO_2, 0·02 M-*N*-methylaniline (NMA), 0·0103 M-KSCN, 20% (w/v) perchloric acid and 5·65 mM-chlorogenic acid. All chemicals were of analytical grade and stock solutions were diluted as necessary before use with double-distilled water.

Reaction mixtures. The experimental conditions have been described previously (Boyland *et al.*, 1971; Lathia & Rütten, 1979). The reactants and reaction mixtures were all maintained at 37°C, and immediately before each reaction the pH of all diluted solutions was adjusted to pH 2 with 20% (w/v) perchloric acid. The nitrosation reactions were carried out with final concentrations of 0·1 mM-KNO_2 and 0·1 mM-NMA.

Procedure. To study the rate of NMNA formation, 1·5 ml 0·2 mM-NMA was added to 1·5 ml 0·2 mM-KNO_2 solution. The reaction mixture was kept in a well-stoppered vessel at 37°C for 1, 5 or 10 min. At the end of the incubation the reaction was stopped by the addition of 2 ml 0·1 N-sodium hydroxide. In accordance with the extraction method described by Mirvish, Wallcave, Eagen & Shubik (1972) and by Tanaka, Chung, Hayatsu & Kada (1978), the alkaline reaction mixture was extracted, after addition of 1 g NaCl, with dichloromethane (5 ml × 2) and the organic fraction was dried with sodium sulphate. The absorbance was then measured in a Hitachi Double Beam 100-60 spectrometer at 269 nm and the NMNA yield (μM) was calculated with the aid of a calibration curve. A blank reading was taken in the absence of NMA by adding 1·5 ml dilute perchloric acid at pH 2. All determinations were performed five times.

Catalysis of in vitro *NMNA formation.* To study the influence of chlorogenic acid alone on the rate of *in vitro* formation of NMNA, the same procedure was used and chlorogenic acid was added to the reaction mixture to provide final concentrations of 0 (control), 0·0565, 0·141 or 0·282 mM. Similar incubations, containing in addition 0·025 mM-KSCN, were set up to study the cumulative catalytic effect of chlorogenic acid and thiocyanate on NMNA formation.

RESULTS

The NMNA yield increased rapidly with increasing chlorogenic acid concentration (Table 1). The NMNA concentration after a 10-min reaction time was 0·10 μM higher in the presence of 0·282 mM-chlorogenic acid than in the control reaction. After reaction for 1 and 5 min, the corresponding differences were nearly 0·02 and 0·07 μM respectively. Thus after 10 min, NMNA formation in the presence of 0·282 mM-chlorogenic acid showed an increase of 67% over the chlorogenic acid-free control reaction. Even at the lowest chlorogenic acid concentration (0·0565 mM) the catalytic effect on NMNA formation was considerable (Table 1).

Table 1 also shows the cumulative effect of both chlorogenic acid and thiocyanate on *in vitro* NMNA formation. Thiocyanate alone was a more active catalyst than chlorogenic acid alone, and together the two compounds led to remarkably high yields of NMNA. For example, with 0·025 mM-KSCN and 0·0565 mM-chlorogenic acid, the yield of NMNA was 24% higher than that obtained with thiocyanate alone, and 206% higher than that of the control reaction. Higher concentrations of chlorogenic acid with this level of thiocyanate resulted in slightly lower yields of NMNA, but in each case the percentage yield was markedly higher in the presence of the two compounds than with chlorogenic acid alone or in the control reaction.

DISCUSSION

Polyhydric phenols with hydroxyl groups in the *o-* or *p-* positions are normally easily oxidized to the corresponding quinones. This oxidizing property leads, for example, to the formation of nitric oxide when dilute HNO_2 is reacted with 4-methylcatechol in aqueous medium (Challis & Bartlett, 1975). This nitric oxide then reacts with excess HNO_2 to form N_2O_3, which may subsequently promote the formation of nitrosamines from secondary amines. Challis & Bartlett (1975) achieved, in model experiments, 56 and 49% increases in *N*-nitrosopiperidine yield when piperidine was reacted with 100 mM-nitrite at pH 4 in the presence of 0·01 M-chlorogenic acid or of 0·01 M-4-methylcatechol, respectively.

Our study confirms, in general, this catalytic effect of chlorogenic acid on the *in vitro* formation of nitrosamines. However, the concentrations of chlorogenic acid used in these experiments were significantly lower than that used by Challis & Bartlett (1975) and were very similar to the levels that may actually be present in the human stomach following the ingestion of one or two cups of coffee, assuming 250 mg chlorogenic acid per cup of coffee (Eichler, 1976) and also taking into account a dilution of some five- to tenfold in the stomach contents. The results show clearly a significant increase in the rate of NMNA formation as the chlorogenic acid concentration was increased.

The experimental data also demonstrate the synergistic effect of both catalysts on the *in vitro* formation of NMNA, but it is also of interest to note the gradual decrease in NMNA yield with increasing chlorogenic acid in the presence of 0·025 mM-KSCN, in contrast to the experiments without thiocyanate. This slight decrease in NMNA yield may have been due to competitition for the available nitrosating agent between chlorogenic acid molecules at the higher concentration and NMA. A similar decrease in nitrosamine formation was also found by Walker *et al.* (1975) with increasing concentrations of gallic acid.

Although the combined effect of the two compounds on the NMNA yield became less marked as the chlorogenic acid concentration increased, the experimental data as a whole indicate higher yields in the presence of both catalysts than with a single catalyst at any given concentration.

Although these results are in no way conclusive, they do indicate that chlorogenic acid and thiocyanate may have some synergistic effect on *in vivo*

Table 1. *Effects of chlorogenic acid and/or thiocyanate on yields of* N-*methyl*-N-*nitrosoaniline* (*NMNA*) *from nitrite and* N-*methylaniline* (*NMA*) in vitro

Concn* (mM) of		Formation of NMNA after 10 min		
			Increase (%) over	
Chlorogenic acid	KSCN	Net yield† (μM)	Control	KSCN alone
0 (control)	0	0·15	100	—
0·0565	0	0·18	120	—
0·141	0	0·21	140	—
0·282	0	0·25	167	—
0	0·025	0·37	246	100
0·0565	0·025	0·46	306	124
0·141	0·025	0·44	293	119
0·282	0·025	0·40	266	108

*Final concentration in reaction mixture, which contained also 0·1 mM-KNO_2 and 0·1 mM-NMA.
†Figures are means of five separate reactions.

nitrosamine formation if ingested simultaneously, and may thus significantly increase human exposure to carcinogenic nitrosamines.

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MUTAGENICITY OF PEANUT OILS AND EFFECT OF REPEATED COOKING

L. Y. Y. FONG, C. C. T. TON and P. KOONANUWATCHAIDET*
Department of Biochemistry, University of Hong Kong

and

D. P. HUANG
Institute of Oncology and Radiology, Queen Elizabeth Hospital, Hong Kong

(*Received 7 January 1980*)

Abstract—Four samples of peanut oils obtained from local markets in Hong Kong were tested for mutagenicity using the Salmonella/microsomal test system. In the presence of microsomes, dimethylsulphoxide (DMSO) extracts of all of the samples were mutagenic to *Salmonella typhimurium* strains TA98 and TA100, but the mutagenic activity of the oils decreased after they had been repeatedly cooked. However, when the extracts were pre-incubated with the bacteria (TA100) and microsomes the mutagenic activity of the cooked oils was greater than that of the uncooked oils, suggesting the possible contamination of the cooked oils by *N*-nitrosamines. Mutagenic activity was also detected in random samples of seven other brands of uncooked oils obtained from local markets. By absorption spectrophotometry aflatoxin B_1 levels in three of these oils were found to range from 98 to 150 μg/kg. DMSO-extracts of ten samples of poor-grade market peanuts were also mutagenic and the peanuts contained 95–1055 μg aflatoxin B_1/kg. The contamination of local peanut oils by mutagens before and after repeated cooking is concerning, since in more than 90% of Chinese households in Hong Kong peanut oils are used daily for cooking and are habitually saved and re-used repeatedly.

INTRODUCTION

There have been a number of studies on the relationship between the ingestion of aflatoxins and primary liver cancer in man in regions of Africa and Asia (Peers & Linsell, 1973; Shank, Bhamarapravati, Gordon & Wogan, 1972). However, although the association is apparently strong, it remains inconclusive.

In Hong Kong the incidence of primary liver cancer is high, the age standardized rate for males being 35·6/100,000/yr (*Cancer Registry*, 1976). A study by Shank, Wogan, Gibson & Nondasuta (1972) indicated that aflatoxin contamination of Hong Kong foods and foodstuffs is low; of 878 samples analysed, only 22 contained these mycotoxins. Peanut oil was not tested. Since peanut oil is used as a cooking oil by over 90% of the Chinese households in Hong Kong, we feel that it is the most likely source of aflatoxin ingestion for the local people. In this study, we assayed 11 randomly-selected samples of peanut oil for aflatoxins by a chemical method (*Association of Official Analytical Chemists*, 1975) and for mutagenicity by the Ames test (Ames, McCann & Yamasaki, 1975) which has been used for a wide range of compounds and shows a high level of accuracy in the prediction of carcinogenicity (Purchase, Longstaff, Ashby, Styles, Anderson, Lefevre & Westwood, 1978). Since many people re-use their frying oils the Ames test was carried out on both uncooked and repeatedly cooked oils. For comparison, samples of peanuts were also analysed by the two methods.

* Present address: Department of Biochemistry, Khon-Kaen University, Thailand.

EXPERIMENTAL

Chemicals. Aroclor 1254 was a gift from Monsanto Chemical Co., St. Louis, MO, USA. Spectrophotometric-grade dimethylsulphoxide (DMSO) was purchased from Riedel de Haen, Seelze-Hanover, Federal Republic of Germany, aflatoxins B_1, B_2 and G_1 were obtained from Calbiochem, San Diego, CA, USA and nicotinamide adenine dinucleotide phosphate (NADP) and glucose-6-phosphate (G-6-P) were obtained from Sigma Chemical Co., St. Louis, MO, USA.

Samples of peanut oils and peanuts. Fifteen peanut-oil samples were studied. Seven were samples of different brands (1–7) obtained from the local market. Four pairs (A–D) of samples of peanut oil were obtained from different families, each pair consisting of an uncooked (market) sample and a sample of the same oil after repeated cooking. Cooking generally involved frying traditional Chinese foodstuffs. Two samples of corn oil were also studied. Ten samples of commonly consumed peanuts, deliberately selected for their poor quality, were obtained from local markets.

Chemical assay for aflatoxin B_1. Aflatoxins were extracted from peanut oil and peanuts, by the official method of the Association of Analytical Chemists (1975). Samples of oil (500 ml) and peanuts (100 g) were extracted with chloroform and water, cleaned-up on a silica-gel column and estimated semi-quantitatively by determining fluorescence on thin-layer chromatograms. Although traces of aflatoxins B_2 and G_2 were detected along with aflatoxin B_1, their levels were not estimated because of their low concen-

trations and reportedly weak carcinogenicity (McCann, Choi, Yamasaki & Ames, 1975). Quantitative estimation was carried out by scraping the bands of aflatoxin B_1 off the chromatographic plate and eluting the silica-gel coating with methanol. Each eluant was made up to a standard volume and its optical density was measured at 363 nm (Nabney & Nesbitt, 1965).

Preparation of samples for mutagenicity assay. Peanuts were minced finely and suspended in DMSO (2 ml DMSO/g sample), and peanut oil–DMSO (10:1, v/v) mixtures were prepared. The suspension or mixture was shaken vigorously for 3 hr and then centrifuged at 2000 **g** for 10 min at room temperature. The supernatant was used in the mutagenicity test.

Bacterial strains. Tester strains of *Salmonella typhimurium*, TA98 for the detection of frameshift mutations and TA100 for base-pair substitution mutations, were generously supplied by Prof. Bruce N. Ames, University of California, Berkeley, CA, USA.

Preparation of the S-9 mix. A 9000-**g** supernatant (S-9) of liver homogenate was prepared by the method of Ames *et al.* (1975) from a male Sprague–Dawley rat (body weight 200 g) that had been induced with a single ip injection of a polychlorinated biphenyl mixture (500 mg Aroclor 1254/kg) and had been killed 5 days later. The S-9 mix contained (per ml): S-9 (0·15 ml), $MgCl_2$ (8 μmol), KCl (33 μmol), G-6-P (5 μmol), NADP (4 μmol) and phosphate buffer, pH 7·4 (100 μmol).

Mutagenesis assay. The assays were carried out as described by Ames *et al.* (1975), 0·1 ml of the bacterial tester strain, the sample to be tested and, where appropriate, 0·5 ml S-9 mix being added to 2 ml molten top agar. The contents were mixed and poured onto minimal-glucose agar plates containing a limited amount of L-histidine. After incubation for 2 days at 37°C, the colonies that had reverted to histidine prototrophy were counted. For increased sensitivity in the detection of the mutagenicity of *N*-nitrosamines, the 'S-9 + P' procedure described by Yahagi, Nagao, Seino, Matsushima, Sugimura & Okada (1977) was used: the test substance S-9 mix and bacteria (TA100 strain only) were preincubated at 25°C for 20 min and then mixed with 2 ml molten top agar and finally poured onto a minimal-glucose agar plate.

RESULTS AND DISCUSSION

With peanut oils, mutagenic activities were found only when microsomal enzymes (S-9) were incorporated in the test system. When these activities were expressed as aflatoxin B_1 equivalents (by comparison with an authentic standard) they corresponded well with the results of spectrophotometric measurements on the uncooked market samples 1, 2 and 3 (Table 1). This correlation would suggest that aflatoxin B_1 is the predominant mutagen in these oils. This conclusion is supported by the fact that the mutagenic activities of aflatoxins B_2, M_1, G_1 and G_2 have been reported to be very weak compared with the activity of aflatoxin B_1 (McCann *et al.* 1975). Thus it appears that the Ames Salmonella test, besides serving as a mutagenicity screening method, can also be used as an assay for aflatoxin B_1 in peanut oil. Mutagenic activity was not detected in either of the two samples of corn oil examined.

The effects of cooking on the mutagenicity of peanut oils are exemplified by the results for sample A (Fig. 1). Repeated cooking decreased the mutagenic activities in assays that did not use the modified 'S-9 + P' procedure of Yahagi *et al.* (1977). By comparison with the activities of authentic aflatoxin B_1 standards it was determined that samples A and B contained 68 and 45 μg/kg aflatoxin B_1 equivalents, respectively, before use and 17 and 11 μg/kg, respectively, after repeated cooking. Similarly, samples C and D contained 10–20 μg/kg aflatoxin B_1 equivalents before use, but less than 2 μg/kg after repeated cooking. Aflatoxins have been found to be stable up to their melting points of about 250°C (Feuell, 1966). However, Mann, Codifer & Dollear (1967) found that about 80% of aflatoxin B_1 in contaminated oilseed meals was lost by heating for 2 hr at 100°C at a meal-moisture level of 20%. During cooking, some moisture must be introduced into the oil and this might account for the decreased levels of aflatoxin in oils that have been repeatedly cooked.

Table 1. *Aflatoxin B_1 levels in peanut oils as determined by absorption spectrophotometry or estimated by the Ames Salmonella mutagenicity test*

Sample no.	Aflatoxin B_1 determined by absorption spectrophotometry*, μg/kg	Aflatoxin B_1 equivalents†, μg/kg, estimated by the mutagenicity test using *Salmonella typhimurium* strain	
		TA98	TA100
1	98	106	112
2	114	121	127
3	150	146	158
4	ND	48	41
5	ND	58	67
6	ND	131	120
7	ND	20	15

ND = Not determined

*Optical density at 363 nm was determined. For method, see text.

†Aflatoxin B_1 equivalents were calculated by comparison with the mutagenic activities of authentic standards under identical experimental conditions.

Values are means of two observations.

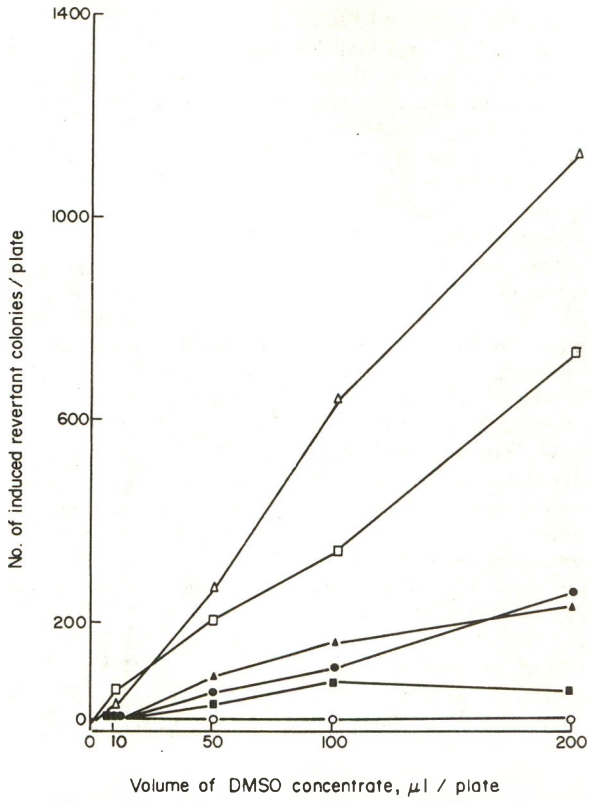

Fig. 1. Mutagenicity dose-response curves of a dimethylsulphoxide (DMSO) concentrate of a sample (sample A) of uncooked (open symbols) and cooked (solid symbols) peanut oil tested by the Ames Salmonella test using *S. typhimurium* strain TA98 with S-9 mix (□, ■), TA100 with S-9 mix (△, ▲), and strain TA100 with 'S-9 + P', the modified procedure of Yahagi *et al.* 1977 (○, ●).

Table 2. *Mutagenicity* in vitro *and aflatoxin* B_1 *levels of samples of peanuts obtained from local markets in Hong Kong*

Sample no.	Aflatoxin B_1, μg/kg*	Induced revertants/200 μl DMSO concentrate/plate† using *Salmonella typhimurium* strain			
		TA98		TA100	
		−S-9	+S-9	−S-9	+S-9
1	290	NS	955	NS	1643 (100)
2	179	NS	427	NS	547 (100)
3	463	NS	579	NS	1144
4	450	NS	682	NS	995
5	95	142	585	NS	908
6	345	127	535	NS	759
7	622	NS	690	NS	936
8	983	101	1088	NS	2186
9	1047	278	1591	348	2064
10	1055	384	1935	424	2534

DMSO = Dimethylsulphoxide NS = not significant.

*Determined by absorption spectrophotometry, with optical density measured at 363 nm. For method, see text.

†Spontaneous revertant colonies on control plates without mutagen and S-9 mix were subtracted (these were about 40 for TA98 and 180 for TA100; slightly larger numbers arose on plates containing S-9 mix). Values greater than twice the number of spontaneous revertant colonies were interpreted as significant and recorded. Numbers in brackets indicate volume (μl) of DMSO concentrate that gave the maximum number of induced revertants. Where no such number is given, the volume inducing the greatest response was generally 200 μl.

Values are means for two plates.

It was interesting to note that in the TA100 'S-9 + P' system, the mutagenic activity of repeatedly-cooked oils A (Fig. 1) and B was two to three times greater than that of the corresponding uncooked oils when tested at a level of 200 μl DMSO extract/plate. It is possible that during repeated frying, these oils may have extracted some *N*-nitrosamines from traditional Chinese foodstuffs such as dried shrimps, salted fish and Chinese sausages. These foods have been found to contain *N*-nitrosopyrrolidine and *N*-nitrosodimethylamine (Fong & Chan, 1973 & 1977).

Aflatoxin B_1 was detected by chemical analysis in each of the ten samples of peanuts at concentrations ranging from 95 to 1055 μg/kg (Table 2). All ten samples had mutagenic activities with both tester strains in the presence of S-9 mix. In some cases, mutagenic activity was observed in the absence of S-9 mix. This indicated that mutagens other than aflatoxins were present in peanuts.

In Western countries peanut oils are relatively free from aflatoxins regardless of the quality of the nuts from which they are expressed (Barnes, 1970) since the hot alkali that is used in the clarification step removes all residual aflatoxins (Parker & Melnick, 1966). On the other hand our data indicate that aflatoxin-contaminated peanut oils are common in local markets in Hong Kong. Although peanut oils may be a major source of aflatoxins in the local diet, the effects of daily ingestion of oils containing small amounts of these toxins are not known. Long-term experiments on the effects on rats of feeding synthetic diets containing aflatoxin-contaminated peanut oils obtained from local markets are in progress in our laboratory. In order to shed more light on the relationship between liver cancer and the ingestion of such oils, extensive epidemiological studies of local populations should be conducted.

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AN INVESTIGATION OF THE GENETIC TOXICOLOGY OF IRRADIATED FOODSTUFFS USING SHORT-TERM TEST SYSTEMS—II. SISTER CHROMATID EXCHANGE AND MUTATION ASSAYS IN CULTURED CHINESE HAMSTER OVARY CELLS

B. J. PHILLIPS*, E. KRANZ and P. S. ELIAS

International Food Irradiation Project, Federal Research Centre for Nutrition, Postfach 3640, D-7500 Karlsruhe, Bundesrepublik Deutschland

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Abstract—As part of a programme of short-term tests used to detect possible genetic toxicity in irradiated foodstuffs, cultured Chinese hamster ovary cells were exposed to extracts and digests of irradiated and unirradiated dates, fish and chicken and subjected to tests for cytotoxicity, sister chromatid exchange induction and mutation to thioguanine resistance. The results showed no evidence of genetic toxicity induced in food by irradiation. The general applicability of cell culture tests to the detection of mutagens in food is discussed.

INTRODUCTION

The first paper in this series (Phillips, Kranz, Elias & Münzner, 1980) outlined a programme for testing irradiated foodstuffs with a battery of short-term tests for genetic toxicity. This paper presents the results of two assays, the sister chromatid exchange (SCE) test and the hypoxanthine–guanine phosphoribosyl transferase (HGPRT) mutation assay in Chinese hamster ovary cells (CHO cells) in culture. As far as we are aware, these tests have not previously been used in the study of irradiated food or indeed of any other processed foods.

The *in vitro* SCE test has proved to be a highly sensitive indicator of chemical damage to chromosomes, detecting many known mutagens at concentrations well below those needed to cause other types of chromosomal aberration (Perry & Evans, 1975; Takehisa & Wolff, 1977). Although the exact mechanism of SCE formation is not understood, the test is becoming very widely used for mutagen detection.

Several mutation assays using mammalian cells in culture are now available. One of the most widely used involves mutation at the HGPRT locus, leading to loss of the enzyme and consequent resistance to toxic guanine analogues. We have used a modification of the technique described by O'Neill, Brimer, Machanoff, Hirsch & Hsie (1977), which has been shown to give a sensitive and quantitative response to many chemical mutagens (Hsie, O'Neill, Couch, San Sebastian, Brimer, Machanoff, Fuscoe, Riddle, Li, Forbes & Hsie, 1978). This test utilizes CHO cells, which are also ideal for use in the SCE test. The advantages of using the same mammalian cell line in tests for detecting forward mutation on the one hand and chromosomal damage on the other are obvious.

The problems associated with the use of *in vitro* methods for testing foodstuffs were discussed in the previous paper (Phillips *et al.* 1980). The products we have examined (dates, fish and chicken) cannot simply be added to a cell culture medium. The use of simple extracts of food is not an entirely satisfactory solution to this problem, and to provide samples for the cell culture tests we therefore used the *in vitro* method of enzymatic digestion that we used for bacterial mutagenicity testing and described earlier (Phillips *et al.* 1980).

The results presented here show that no genetic toxicity can be detected by cell culture methods in three foodstuffs irradiated under practical conditions. However, the use of mammalian cell systems to test for unknown mutagens in food presents certain problems, particularly with regard to sample preparation.

EXPERIMENTAL

Cells and cell culture. A sample of CHO-K_1-BH_4 cells was kindly supplied by Dr. J. C. Asquith of the Environmental and Medical Sciences Division, AERE, Harwell, UK. This line was derived by selection in medium containing aminopterin (O'Neill *et al.* 1977), from CHO cells originally isolated by Kao & Puck (1967). Very few pre-existing thioguanine resistant cells are present in this line. Cell stocks were kept frozen in liquid nitrogen and were thawed about 1 wk before use in the tests. The cells were grown in McCoy's 5 A medium supplemented with foetal calf serum (10%), penicillin (50 units/ml) and streptomycin (50 μg/ml), in an atmosphere of 5% CO_2 in air at 100% humidity.

Test chemicals and food samples. Cyclophosphamide (CPA) and ethyl methanesulphonate (EMS) were purchased from ICN Pharmaceuticals Inc., Cleveland, OH, USA, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) from Serva, Heidelberg, and

*Present address: British Industrial Biological Research Association, Woodmansterne Road, Carshalton, Surrey SM5 4DS, England.

benzo[*a*]pyrene (BP) from Carl Roth KG, Karlsruhe. Chlorambucil (CHL) was synthesized at the Chester Beatty Research Institute, London, and was kindly supplied by Dr. T. A. Connors. These chemicals were dissolved in dimethylsulphoxide (DMSO) and added to the medium to give a final concentration of 1% DMSO.

Food samples were prepared as described by Phillips *et al.* (1980). Unirradiated and irradiated dates, fish and chicken were either extracted with distilled water or digested with pepsin and pancreatin. Extracts and digests were ultrafiltered and the filtrates were made isotonic by the addition of either NaCl or distilled water. Sodium bicarbonate was added to give a final concentration of 2·2 mg/ml. The samples were sterilized by filtration and without further dilution were added to the cell cultures.

Treatment of cultures. Each 9-cm plastics Petri dish was seeded with 5×10^5 cells, 24 hr before the beginning of treatment. During treatment the cell number was approximately $1{\cdot}5 \times 10^6$/dish. Medium was poured off and replaced by the test solution (extract, digest or full medium containing a known mutagen), and the plates were incubated at 37°C for 2 hr. After this time, the plates were drained and washed with fresh medium.

In order to detect mutagens requiring metabolic activation, S-9 mix was added to the cultures during treatment at a concentration of 10%. The S-9 mix was prepared as described by Ames, McCann & Yamasaki (1975), and contained 10% S-9 fraction derived from Aroclor-treated male Sprague–Dawley rats.

Sister chromatid exchange (SCE). 5-Bromodeoxyuridine (BUdR) was dissolved in water at 1 mg/ml and added to SCE plates at the beginning of treatment to give a final concentration of 10 μg/ml. After treatment, the cells were incubated in the dark for 24 hr in the presence of BUdR. Mitotic cells were accumulated by treatment with 2·5 μg colchicine/ml for 2 hr. After hypotonic treatment, fixation and spreading, chromosome preparations were stained by the fluorescence-plus-Giemsa technique (Perry & Wolff, 1974). Hoechst-stained slides were submerged to a depth of 1 cm in phosphate-buffered saline and exposed to a 15-W ultraviolet (2537 Å) lamp at a distance of 10 cm for 30 min before incubation in 2 × SSC. At least 25 well-spread diploid metaphases were evaluated for each treatment.

Cell survival and mutation. Immediately after treatment, the plates were trypsinized and the cells were suspended in medium. After the cells had been counted, a small sample was diluted to give approximately 20–100 cells/ml, and 5-ml aliquots were dispensed into 5-cm dishes. These cultures were incubated for 7 days and colonies were stained and counted. Meanwhile 5×10^5 treated and suspended cells were seeded on a fresh 9-cm plate and grown in full medium. These cells were subcultured every 2 days, to maintain them in log phase during the 8-day expression time, as recommended by O'Neill *et al.* (1977). After 8 days, the cells were suspended and counted. Plating efficiency was determined by plating 100 cells in each of five 5-cm dishes and counting colonies after 7 days. The remaining cells were seeded at a density of 2×10^5 cells/dish in three 9-cm dishes in culture medium containing dialysed foetal calf serum and 2 μg thioguanine/ml. After 10 days, colonies of growing cells were counted. The frequency of thioguanine-resistant cells in the population surviving treatment was calculated, correction being made for differences in plating efficiency after expression.

RESULTS

Treatment of cell cultures with food samples

Preliminary experiments were carried out to determine how maximal exposure of cells to the food extracts and digests could be achieved. It was found that if the osmotic pressure of the sample was adjusted and sodium bicarbonate was added to give a neutral pH in equilibrium with 5% CO_2, then little or no non-specific toxicity was observed when cells were incubated for up to 4 hr in any sample. Longer exposures were also attempted, but as it was then necessary to add serum, salts and nutrients to the solution to maintain cell viability and growth, it was decided to use a 2–4 hr exposure. The possible advantage gained in test sensitivity with longer treatment times would probably have been outweighed by the necessary dilution and addition of substances likely to react with mutagens.

Table 1 shows the final concentrations of dissolved, food-derived material to which the cells were exposed. These figures were obtained from dry-weight determinations of the extract or digest. A correction was made for material not derived from the foodstuff but

Table 1. *Concentration of food-derived solids to which the cells were exposed*

Sample		Food-derived dry weight* (mg/ml of test soln)	Wet weight† of source material (mg)
Date:	extract	40	60
	digest	35	50
Fish:	extract	10	360
	digest	22	150
Chicken:	extract	9	330
	digest	21	133

*The results of the dry-weight determinations have been corrected to take account of material added during digestion or sample preparation.

†The weight of original food sample that gave rise to the food-derived solids present in 1 ml of test-solution.

added during digestion or sample preparation. When S-9 mix was added during treatment, the concentrations of food-derived material were reduced by 10%.

Effect of treatment

Table 2 shows the effects of treating cells with samples of extracts or digests of irradiated or non-irradiated dates, fish or chicken. No effect on cell morphology or survival was observed with date or chicken samples. Fish, however, had an immediate effect on cell shape, which was apparently associated with a loss of cell adhesion to plastic. In some cases, cell detachment was observed. This effect was reversible, however, and no effect on cell viability was detected.

In no case was any significant increase in SCE frequency observed. Each sample was tested only once for mutation induction but no treatment resulted in a yield of mutants outside the range of mutation frequencies observed in controls. Ten independent control experiments gave mutant yields in the range of 0–18/million with a mean of 5·0.

Positive controls

Five known mutagens were tested, normally concurrently with food tests, in order to demonstrate the sensitivity of the test methods. The results of treatment of cells with these compounds for 2 hr in full medium are shown in Table 3. Three of the compounds tested (EMS, CHL and MNNG) are direct-acting mutagens, and required no S-9 mix. All induced cell death, SCEs and mutation, although over different concentration ranges. CPA and BP were effective only in the presence of S-9 mix. A dose-response relationship was not obtained with BP, possibly because of its limited solubility or inadequacies in the activation system. Nevertheless, a response to BP was detectable at a low concentration.

Experiments were also carried out with CPA, using the SCE test, in order to investigate the effects on sensitivity of the presence of food extracts and digests during treatment. The effects of the extraction and digestion procedures on the detectability of this mutagen in fish were studied in a similar way. CPA was dissolved in water and various concentrations were added either to the fish homogenate before processing

Table 2. *Effects of treatment with date, fish or chicken samples on survival, sister chromatid exchange frequency and mutation in Chinese hamster ovary cells*

Sample		S-9 mix	Survival (%)	SCE/cell*	Mutants/10^6 survivors†
Date study					
None (control)		–	100	10·6 ± 3·0	3·7
		+	100	10·7 ± 3·2	4·2
Unirradiated:	extract	–	96	10·5 ± 2·4	3·9
		+	79	10·7 ± 3·0	3·0
	digest	–	92	10·5 ± 3·5	4·6
		+	105	10·2 ± 2·8	4·2
Irradiated:	extract	–	93	10·7 ± 2·1	3·0
		+	88	10·7 ± 2·0	5·0
	digest	–	96	10·1 ± 2·2	3·7
		+	113	10·9 ± 2·4	5·0
Fish study					
None (control)		–	100	11·6 ± 3·0	6·7
		+	97	11·7 ± 2·8	13·0
Unirradiated:	extract	–	102	11·6 ± 2·3	16·7
		+	89	11·4 ± 3·0	9·5
	digest	–	103	11·8 ± 3·5	1·7
		+	80	12·0 ± 3·0	2·1
Irradiated:	extract	–	88	12·5 ± 2·6	6·7
		+	94	12·2 ± 2·9	5·6
	digest	–	82	11·1 ± 2·5	10·0
		+	89	11·5 ± 2·7	5·2
Chicken study					
None (control)		–	100	11·7 ± 3·1	10·5
		+	98	11·5 ± 3·0	7·8
Unirradiated:	extract	–	81	11·8 ± 3·0	12·5
		+	101	11·4 ± 2·9	7·5
	digest	–	83	10·9 ± 2·8	6·6
		+	94	11·5 ± 3·2	3·0
Irradiated:	extract	–	79	12·2 ± 3·4	18·0
		+	109	11·2 ± 2·4	4·5
	digest	–	101	11·4 ± 3·0	2·0
		+	86	11·8 ± 3·3	5·2

*Values are means ±1 SD of at least 25 metaphases.
†Results of single experiments.

Table 3. *Effects of five mutagens on survival, sister chromatid exchange frequency and mutation in Chinese hamster ovary cells*

Compound	Dose (μg/ml)	Survival (%)	SCE/cell*	Mutants/10^6 survivors†
None	–	100	11·6 ± 3·0	5(0–18)
MNNG	0·025	60	23·3 ± 6·4	50
	0·05	40	33·4 ± 8·3	76
	0·1	20	39·4 ± 6·7	114
CHL	0·4	100	13·6 ± 3·0	10
	2·0	100	24·3 ± 4·4	48
	10	50	43·2 ± 3·8	130
EMS	14	100	12·2 ± 3·6	5
	80	99	16·3 ± 3·7	25
	500	82	30·4 ± 4·4	128
	2900	7	–	670
CPA	1·0	100	18·5 ± 2·8	50
	3·0	85	33·6 ± 4·4	120
	10	70	52·2 ± 8·2	260
	30	16	–	550
BP	3·0	63	19·8 ± 2·2	103
	10	61	19·4 ± 4·0	57
	30	55	19·6 ± 3·7	48

MNNG = *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine
CHL = Chlorambucil EMS = Ethyl methanesulphonate
CPA = Cyclophosphamide BP = Benzo[*a*]pyrene

*Values are means ±1 SD of at least 25 metaphases. Controls were pooled from ten separate experiments.

†The control value is the mean of ten separate experiments, with the observed range in parenthesis. Values for the test compounds are each the result of one experiment.

or to the finished samples before testing. The concentrations of CPA required to give a threefold increase in SCE frequency were 9·5 and 13·5 μg/ml in fish extract and digest, respectively, compared with 3·0 μg/ml in the culture medium. The doses that had to be added to fish homogenate to induce a similar response in the extract and digest were 20·0 and 105 μg/ml, respectively.

Thus the activity of CPA, as measured in culture medium, was reduced by a factor of 3·2 or 4·5 when the compound was tested in the presence of fish extract or digest, respectively. Activity was reduced by a factor of 6·7 when CPA was added to fish before extraction and by 35 when the treated fish was digested. When the effect of the presence of fish components during cell treatment is taken into account, it can be calculated that about 50% of the added CPA was lost during extraction and about 90% during digestion.

DISCUSSION

The results presented here are in agreement with those reported in the previous paper (Phillips *et al.* 1980) showing no evidence of genetic toxicity in the three irradiated foodstuffs tested.

The main problem encountered in carrying out these experiments was the choice of a method for reducing solid foodstuffs to a form suitable for addition to cell cultures. The rationale for the choice of digestion *in vitro* has been discussed (Phillips *et al.* 1980). In brief, it was accepted that no assumptions could be made about the nature or properties of any mutagens produced as a result of irradiation and that the whole food should be made available to the cells by means of procedures that mimicked, as far as possible, the ways in which food is processed *in vivo*. It was hoped that this procedure would permit, to some extent at least, any significant interactions that might occur between radiolysis products of food and components of the digestive system. It was considered possible, for example, that mutagenic substances, if produced, might either be bound to, or form integral subunits of, macromolecules and might only be released when the carrier molecules were broken down. Similarly, the digestion procedure might allow the activation of potential mutagens by other mechanisms such as nitrosation. On the other hand, the possibility was also admitted that mutagens might be destroyed by the preparative procedure or eliminated by direct reaction with food components. It can be argued, however, that since the *in vitro* digestion method is similar (in its main features) to normal *in vivo* mammalian digestion, these effects would also influence the activity of mutagens ingested by animals and that the results of the tests would therefore be more, rather than less, meaningful.

Before the results presented here can be accepted as valid, it is important to establish whether any mutagens present in the food would in fact have been detected and at what level. It has been shown by many workers, and to a certain extent here, that the SCE and mutation tests are sensitive to a wide range of mutagens in culture medium. Whether mutagens can be detected in food, however, depends on a number of factors, including the method of sample preparation and the nature of the food constituents present during testing.

It is extremely difficult to devise meaningful tests for the sensitivity of this system because the influence of the factors mentioned, and the overall sensitivity of the tests, depends very strongly on the properties of both the mutagen and the foodstuff in which it is found. For example, a twofold increase in SCE frequency is observed when 200 μg EMS/ml is dissolved in culture medium, while only 0·025 μg MNNG/ml is required for the same effect—an 8000-fold difference. Similarly, the stability of mutagens at pH 2 differs widely. Removal of mutagens from the sample by reaction with food constituents would also be expected to be influenced by the relative protein, fat and sugar content of different foods.

The experiments reported here on CPA in fish serve only to demonstrate some of the factors affecting the sensitivity of the system, It was shown, for example, that CPA dissolved in fish extract or digest had much less effect than when it was tested in culture medium. This may have been due to influences of components of the fish extract or digest on microsomal enzymes or to reaction between the active mutagen and peptides or amino acids in the sample.

In addition, it was found that extraction of fish containing CPA with water gave only about 50% of the expected activity, while digestion apparently removed 90% of the mutagenic activity.

Taking all the effects into account, it can be calculated that if CPA were a contaminant of fish, it would be detected by our methods at levels of 50 ppm by extraction and 200 ppm by digestion. Sensitivity to other compounds may of course be totally different.

In fairness, however, it should be reiterated that the factors that decrease the activity of mutagens in food tested by our methods may also operate *in vivo*. Interactions occurring during digestion or in blood may significantly reduce the adverse activities of mutagens in food. In this sense, the results of tests on digests of contaminated food may be a more realistic reflection of eventual human hazard than tests carried out under standard, simplified conditions. Obviously, this point requires much more extensive investigation.

Our attempts to use mammalian cells to examine the genetic toxicology of irradiated foods have served to highlight a number of difficult problems associated with demonstrating the safety of foodstuffs suspected of containing unknown mutagens either naturally or as a result of such processing as preservation by irradiation. We believe, however, that in relation to assessing the hazards to human health this work represents the best that can be achieved at present with these test systems in the field of food irradiation. In this context, mutagenesis has been used as an indicator of both mutagenic potential and carcinogenic potential, on the basis of the established close correlation between mutagenic activity as detected by the short-term tests used in this investigation and genotoxic carcinogenic activity observed in lifespan animal bioassays.

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INFLUENCE OF SOLVENTS AND ADSORBENTS ON DERMAL AND INTESTINAL ABSORPTION OF TCDD

H. POIGER and CH. SCHLATTER
Institute of Toxicology, Federal Institute of Technology and University of Zurich, Zurich, Switzerland

(*Received 21 November 1979*)

Abstract—The liver concentration of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was measured as a means of following the uptake of this chemical by the intestinal and dermal routes. Accumulation of the (radiolabelled) compound in the liver was found to provide a good and reproducible method of comparing TCDD uptake from different formulations. After oral administration of 14·7 ng TCDD using 50% ethanol as vehicle, 36·7% of the total dose was found in the liver after 24 hr. When the compound was administered in a mixture with soil particles, adsorption occurred and only about half of this amount was found in the liver. The liver level also decreased with increasing duration of contact between the soil and the dioxin. Adsorption onto activated carbon almost completely prevented uptake of the compound. Similar effects were observed after dermal application of TCDD in the various formulations. The highest liver content, 14·8% of the dose, was found after contact of the pure compound with the skin surface. The inhibiting effects of soil and activated carbon were even more pronounced. After incorporation of the dioxin into vaseline (a lipophilic ointment), 1·4% of the dose was found in the liver, whereas, after incorporation into polyethylene glycol 1500 (a hydrophilic ointment) containing 15% water, 14·1% was found in the liver. The potency of TCDD (applied in similar formations) to produce chloracne in the rabbit ear was tested. Threshold levels for the induction of lesions were between 1 μg for the pure compound and 160 μg when the compound was adsorbed onto charcoal.

INTRODUCTION

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the most toxic of the known chlorodioxins and is formed as an unwanted trace contaminant in the manufacture of 2,4,5-trichlorophenol. It has also been produced accidentally in relatively high amounts, e.g. 1953 in Germany (Goldmann, 1973) and 1976 in Seveso, Italy (Reggiani, 1978) causing toxic effects, particularly skin diseases in man. Chloracne-like lesions were the first apparent symptoms (Bauer, Schulz & Spiegelberg, 1961; Goldman, 1973; May, 1973) in humans after contact with the compound. Chloracne has also been demonstrated in the rabbit-ear test (Jones & Krizek, 1962; Kimmig & Schulz, 1957; Schwetz, Norris, Sparschu, Rowe, Gehring, Emerson & Gerbig, 1973). Previous investigations have been mainly concerned with the toxicity of this dioxin rather than its absorption. In the present work percutaneous and intestinal uptake of the compound was studied in the rat. Contact with TCDD in the environment would most often involve a mixture containing TCDD rather than the pure chemical. The effect of using different formulations of TCDD on its absorption was studied. The liver appears to be the main target organ for TCDD in the rat (Piper, Rose & Gehring, 1973; Rose, Ramsey, Wentzler, Hummel & Gehring, 1976; van Miller, Marlar & Allen, 1976) presumably because of accumulation of the dioxin. We therefore used the liver concentration of TCDD as a measure of the uptake of the compound. The influence of different formulations on the acnegenic potency of the compound was also studied.

EXPERIMENTAL

The tritium-labelled TCDD (specific activity 46·4 Ci/mmol) was obtained from A. Kende, Rochester, NY, USA; the TCDD originated from the Dow Chemical Company. Soil samples free from TCDD contamination were taken from the Seveso region of Italy. An aqueous slurry of the soil was passed through a metallic sieve with a mesh width of 160 μm and then dried at 60°C and ground in a mortar to give a powder of homogeneous particle size. Activated carbon (Norit SX I) was supplied by the Norit N.V. Amersfoort, Netherlands. All the other chemicals used were of analytical grade. To obtain a uniform distribution of the dioxin in the soil or activated carbon, the dry powders were suspended in methanolic solutions of TCDD. The solvent was immediately evaporated in a Rotavapor (Büchi Laboratoriums-Technik AG, Flawil).

The stability (over several days) of the tritium label was tested in aqueous solution (phosphate-buffer pH 7·4, 1 M-HCl and 1 M-NaOH) and in aqueous suspensions of soil and activated carbon at a temperature of around 30°C. The batches were extracted with dichloromethane and the radioactivity was measured in both the aqueous and the organic phases. After storage in both alkaline and acid conditions less than 5% of the radioactivity remained in the aqueous phase and could not therefore be attributed to TCDD.

To examine the adsorption of dioxin onto the activated carbon, a glass column (8 × 5 mm) was filled with adsorbent and then loaded with about 11 ng of

labelled TCDD. Only traces of radioactivity were found in the washings after attempting elution with 2 ml portions of acetone, hexane–acetone (4:1, v/v) ethyl acetate, dichloromethane, methanol, petrol ether, water or 1 M-HCl. Some of the radioactivity could be eluted using methyl isobutyl ketone (1%), a 1% aqueous solution of Triton X-100, or toluene (5%). A similar method was used to examine adsorption to each batch of soil. From an aqueous suspension (37%, w/w) about 66–70% of the added radioactivity could be recovered with hexane–acetone (4:1, v/v). Considerably lower values were obtained, using dichloromethane, ethyl acetate or methanol as extractants. Storage of the spiked slurry for 14 days at a temperature of 30–40°C reduced dioxin recovery using the hexane–acetone mixture to about 58% of the amount added.

TCDD uptake after oral administration. Female rats of the Sprague–Dawley derived ZUR: SIV-Z strain (supplied by Tierspital, Zurich), weighing 180 to 220 g were fasted overnight. The animals were kept in Makrolon cages on wire gratings during the experiments. Feed was provided not earlier than 6 hr after dosing. As an initial check on the method, animals were dosed orally with 14·7 ng TCDD (in ethanol)/rat, killed after 8, 24, 48, 72, 96 or 102 hr and their livers were analysed for TCDD content.

The tritiated dioxin was administered by stomach tube in 0·5 ml of one of the formulations listed below/rat.

1. Dissolved in 50% ethanol.
2. In an aqueous suspension of soil (37% w/w). The soil was in contact with the dioxin (in the dry state) for 10–15 hr at room temperature or over a period of 8 days at a temperature of about 40°C. During the storage period the sample was dried and moistened several times in an attempt to simulate natural conditions.
3. In an aqueous suspension of activated carbon (25% w/w). The carbon had been stored with the methanolic dioxin solution for 15–20 hr at room temperature.

TCDD uptake after dermal administration. Hairless rats of the Naked ex Back-Cross and Holzman strain, weighing from 200 to 250 g were kept in Makrolon cages on wire gratings during the experiments. They were not deprived of feed. Tritiated TCDD was incorporated 10–15 hr before application into the various formulations listed below.

1. A methanolic solution (50 μl) was dispersed over a skin area of about 3 cm^2 and left to dry. This section was then covered with aluminium foil and fixed by an elastic bandage.
2. A soil/water paste (75 mg corresponding to 50 mg dry soil) containing labelled TCDD was spread out on aluminium foil over an area of about 3–4 cm^2 and fixed in contact with the skin by an elastic bandage.
3. An activated carbon–water mixture (100 mg corresponding to 50 mg dry activated carbon) containing labelled TCDD was administered by the method used for the soil.
4. and 5. Vaseline, polyethylene glycol 1500 or polyethylene glycol 1500 with 15% water containing labelled TCDD was heated to about 50°C and 100 μl aliquots spread out on aluminium foil over an area of about 3–4 cm^2 and fixed in contact with the skin by an elastic bandage.

Tritium determination in the liver. At the end of the experiments animals were killed by cervical dislocation, the liver removed and disintegrated with a Polytron homogenizer (supplied by Kinematica GmbH, Lucerne). Aliquots of 150–180 mg of the homogenates were dissolved in 2 ml Soluene 100 (Packard Instrument Co., Downers Grove, IL, USA) isopropanol (1:1) at a temperature of 40–50°C. The samples were then bleached with 0·1 to 0·2 ml 30% hydrogen peroxide and left to stand for a few hours to prevent chemiluminescence. Afterwards, 0·1 ml glacial acetic acid and 10 ml Insta-Gel (Packard Instrument Co.) were added to each sample and radioactivity determined in a liquid scintillation counter (Betaszint 5000, Laboratorium Prof. Dr. Berthold, Wildbad, Federal Republic of Germany). Corrections for quenching were made using automatic external standardization. Radioactivity was calculated for the whole organ and related to the dose applied.

Rabbit ear bioassay for testing acnegenic activity. Male rabbits of the 'Russian' strain, weighing around 2·5 kg were used. In order to evaluate the influence of adjuvants on its acnegenic potency, unlabelled TCDD was administered to the rabbit ear surface in the formulations listed below.

1. Acetone solution of TCDD (containing 64 μg/ml).
2. Either soil (50 mg) or activated carbon (50 mg), spiked with various amounts of TCDD. Samples were mixed with water to give a paste suitable for easy application (soil–water, 2:1; carbon–water, 1:1·8).
3. Either vaseline or polyethylene glycol 1500 with water (15%), spiked with different amounts of TCDD.

A small piece of aluminium foil was stuck onto the centre of a piece of elastoplast, leaving a narrow uncovered adhesive edge. Each preparation was spread onto the aluminium foil over an area of about 4 cm^2 and the pad was fixed to the inner surface of the rabbit's ear. Acetone solution was applied in advance to the surface of the ear and allowed to evaporate before covering. An additional strip of elastoplast was used as a safeguard. After 24 hr the pads were removed and residues of the preparation were washed off the skin surface. The ears were examined every 2 days for signs of inflammation, hyperkeratosis and chloracne.

RESULTS

Because TCDD has a long biological half-life and is stored in tissues such as the liver and fat, the amount

Table 1. *Percentage of TCDD in the liver after oral administration of 14·7 ng TCDD (in ethanol)/rat*

No. of animals	Time after treatment, hr	Percentage of dose in the liver
2	8	33·2
7	24	36·7 ± 1·2
6	48	30·8 ± 2
2	72	22·3
2	96	17·5
2	120	17·5

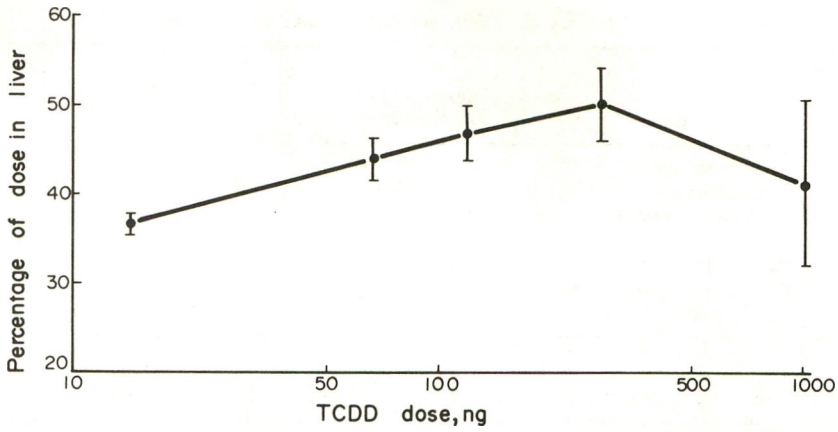

Fig. 1. Percentage of the TCDD dose found in the liver 24 hr after oral administration of labelled TCDD (2·1 μCi/rat, specific activity 46·4–0·63 Ci/mmol). Values are the mean ± SD for groups of six animals.

absorbed is reasonably well reflected by the concentration in these organs. The present determinations were carried out using the liver since it is easily removed. The results of the initial check of the method are given in Table 1. The highest levels were obtained after 24 hr (with remarkable reproducibility); so this time period was chosen for all the subsequent experiments.

The proportion of TCDD found in the liver was dependent on the dose level (Fig. 1). Increasing retention was observed up to a dose of 280 ng TCDD/rat whereas at 1070 ng/rat the value had decreased considerably. Concomitant oral administration of TCDD with soil or activated carbon greatly influenced absorption (Table 2). Activated carbon almost completely prevented intestinal TCDD uptake; only traces of radioactivity could be detected in the liver. Absorption of the dioxin from mixtures with soil was considerably lower than after administration of the ethanolic solution and was also dependent on the duration of contact of the soil with the TCDD. When the soil had been stored in contact with the dioxin for 10–15 hr before administration 24% of the dose was subsequently found in the liver compared with a value of 16% when the storage period was 8 days at 30–40°C.

Permeation of the dioxin across the epidermis was also highly dependent on the formulation. The radio-

Table 2. *Percentage of TCDD in the liver 24 hr after oral administration of 0·5 ml of various formulations containing TCDD*

Formulation	TCDD dose (ng)	No. of animals	Percentage of dose in the liver
50% Ethanol	14·7	7	36·7 ± 1·2
Aqueous suspension of soil (37%, w/w) that had been in contact with TCDD for:			
10–15 hr	12·7, 22·9	17	24·1 ± 4·8
8 days	21·2, 22·7	10	16 ± 2·2
Aqueous suspension of activated carbon (25%, w/w)	14·7	6	≤0·07

Table 3. *Percentage of TCDD in the liver after dermal administration of TCDD in various formulations*

Formulation and amount administered	TCDD dose (ng)	No. of animals	Percentage of dose in the liver
Methanol, 50 μl	26	6	14·8 ± 2·6
Vaseline, 0·1 ml	26	3	1·4 ± 0·4
Polyethylene glycol 1500, 0·1 ml	350	4	9·3 ± 3·4
Polyethylene glycol 1500 + 15% H_2O, 0·1 ml	350	4	14·1 ± 4·9
Soil/water paste, 75 mg (50 mg dry soil)	26	5	*c.* 0·05
	350	5	1·7 ± 0·5
	1300	3	2·2 ± 0·5
Activated carbon/water paste, 100 mg (50 mg dry carbon)	26	4	<0·05
	1300	4	<0·05

Table 4. *Acnegenic potency of TCDD, administered topically in various formulations*

Formulation	Amount of formulation administered/animal	Minimum dose of TCDD that induced lesions (μg/ear)
Acetone solution	10–50 μl	0·6–1·5
Soil/water paste	50 mg	2–3
Activated carbon/water paste	50 mg	*c.* 160
Vaseline	0·05 ml	0·8–1
Polyethylene glycol 1500 + 15% water	0·05 ml	0·65–1·3

activity level in the liver was highest when TCDD was applied in methanolic solution (Table 3) and amounted to roughly half of that after an oral dose. Adsorption of the compound onto activated carbon completely prevented its percutaneous uptake. When the dioxin was administered with soil, reproducible quantities of radioactivity in the liver were measurable only after administration of at least 50 μCi of TCDD/animal. Application in a lipophilic ointment (vaseline) also yielded low dioxin levels in the liver. With polyethyleneglycol 1500 formulations, incorporation of 15% water into the ointment base caused a marked increase in dioxin uptake.

The induction of typical acnegenic skin lesions by TCDD was tested using similar formulations to those used for the permeation studies in order to establish a correlation between the absorption of the compound and the appearance of chloracne. The application technique was quite satisfactory and reliable; only one animal out of 32 stripped off the elastoplast bandage. Usually, the first macroscopic signs appeared on day 5 after the start of treatment with the exception of formulations with activated carbon where minute lesions could not be observed before day 13.

The minimum dose of pure dioxin that induced skin lesions was around 1 μg/ear (Table 4). A somewhat higher dose level was necessary when the dioxin had been mixed with soil, whereas even 160 μg, adsorbed onto activated carbon, produced only very slight changes on the skin surface. Using vaseline and polyethylene glycol preparations the minimum dose that produced lesions was again 1 μg TCDD/ear, but complete removal of the ointments from the skin surface after 24 hr could not be achieved. In about half of the experiments only one ear was treated and the other served as a control; no response on the untreated skin was observed.

DISCUSSION

Since the excretion of TCDD in the urine is delayed and the blood levels are extremely low, it was not possible to follow the absorption rate by common techniques. However, in view of the results of Fries & Marrow (1975), who found 70% of the body burden of TCDD to be located in the livers of female rats and our own results, the determination of TCDD in this organ seems to provide a good method of monitoring and comparing absorption in the rat, though these values do not represent the total amount absorbed. The dioxin is present in the liver in unmetabolized form (Vinopal & Casida, 1973). The increasing percentage of the dose of TCDD retained in the liver at higher dose levels is not easily explained. It is possible that, at high concentrations, clearance of TCDD from the liver is decreased either by organ damage or by a limited excretion capacity. This might also explain the discrepancy between our findings and those of Piper *et al.* (1973), who, after 3 and 7 days found 47 and 45%, respectively, of a ^{14}C-TCDD dose in the liver, whereas we found only half of this percentage, using a 700 times lower dosage level. Differences in the rat strain used may give another explanation.

As expected, TCDD uptake is highly dependent on the formulation in which it is applied. Mixing the compound with soil or activated carbon results in adsorption to the soil particles and, therefore, reduced availability of the dioxin. Contact time of TCDD with soil influences availability, probably because the binding of the dioxin to the soil particles is strengthened with increasing contact time. This is in agreement with the results of the preliminary experiments to examine adsorption onto the soil; extractability with organic solvents dropped after prolonged storage. In this storage period 'natural' conditions were simulated to some extent in that the mixture was held at 40°C and alternately moistened and dried. It can be assumed that with even more prolonged contact time this binding is further increased. The remarkable adsorption effects of TCDD led us to the view that, repeated oral administration of activated carbon, might enhance excretion in cases of enterohepatic circulation of TCDD. Preliminary experiments, however, did not show any effect of charcoal treatment in rats, indicating that enterohepatic circulation of TCDD in rats was unlikely.

When the pure dioxin was administered topically absorption was considerably lower than that after oral intake. Although a direct comparison of these data is not possible, a lower dermal toxicity of the dioxin would be anticipated. Schwetz *et al.* (1973) observed that the acute toxicity level of the compound in the rabbit after dermal application was half that after oral administration. Activated carbon or soil both exerted a similar influence on TCDD uptake as in the oral studies. However the effects of the different formulations were not entirely parallel, for example in the formulations with soil, the reduction of the uptake was much more pronounced after dermal administration. This may be because only a minor part of the contaminated soil is in direct contact with the skin, and, after evaporation of the water, TCDD is totally immobilized on the particles. Skin penetration of the dioxin applied with ointments is

quite different. TCDD is retained in the lipophilic vaseline and, therefore, has little tendency to penetrate the skin. In contrast, the less lipophilic polyethylene glycol favours percutaneous movement of the compound. Addition of water enhances this effect, possibly by softening the epidermal layer. The effect of different formulations on the induction of chloracne on the rabbit ear was much less than would have been expected from the dermal absorption data. This may be because chloracne induction requires only contact with the superficial skin layers rather than absorption into the body. There was no response on the untreated ear of animals treated on one ear only. The minimum effective dose of pure dioxin is in the same range as that reported by Jones & Krizek (1972). Nevertheless, the appearance of acnegenic lesions after administration of TCDD in different formulations mirrors, at least qualitatively, data from percutaneous absorption.

In the general environment TCDD is adsorbed onto various materials such as soil or plant surfaces. Such material decreases the dermal or intestinal absorption of TCDD. This fact must be taken into account when evaluating the possible effects of TCDD, distributed in the environment. The extremely strong or even irreversible adsorption of TCDD onto activated carbon merits special attention. Possibly decontamination procedures might be based on these features.

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A TOXIN FROM THE PALMYRA PALM, *BORASSUS FLABELLIFER*: PARTIAL PURIFICATION AND EFFECTS IN RATS

J. B. GREIG, S. J. E. KAY and R. J. BENNETTS

MRC Toxicology Unit, Medical Research Council Laboratories, Woodmansterne Road, Carshalton, Surrey SM5 4EF, England

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Abstract—The neurotoxicity, but not the hepatotoxicity, to the rat of an edible portion of the young shoot of the palmyra palm, *Borassus flabellifer*, was confirmed. Extracts of this material were shown to contain the toxic factor which was found to be heat-stable and insoluble in organic solvents. The toxin contains a cationic functional group and has a molecular weight of *c.* 1400. A 400-fold enrichment of the toxin was achieved and its effects in the rat are described and discussed. Brine-shrimp (*Artemia salina*) larvae can be used to assay partially purified preparations of the toxin.

INTRODUCTION

The palm *Borassus flabellifer* L., commonly called the palmyra palm, is widely distributed in tropical regions of the Asian continent. In common with a similar East African species (Sim, 1909) it has considerable economic value to the local population, the timber being used for construction and the leaves as a thatching material or to make such items as ropes and baskets. In addition the contents of the unripe seed and the embryo of the germinating seed are edible, the latter being used as a vegetable that is considered to be "cooling, nutritive and diuretic" (Dymock, Warden & Hooper, 1893). The juice of the plant may be drunk fresh or following fermentation to produce a toddy (Chopra, 1933). In Sri Lanka the outer portion of the young shoot, locally called kottakilangu, is either used as a vegetable or dried and milled to provide a flour (Arseculeratne, Panabokke, Tennekoon & Bandunatha, 1971). It has been reported (Arseculeratne *et al.* 1971) that a diet of this flour is toxic to rats, the animals dying within 10 days following a period of ataxia. Hepatic lesions have been observed when the diet has been fed to rats intermittently over prolonged periods (Panabokke & Arseculeratne, 1976). Other reports (Greshoff, 1902; Watt & Breyer-Brandwijk, 1962) describe a slow-acting poison in the roots of *B. flabellifer*; however, on the basis of geographical location, the palm involved may be a separate species, *B. sundaicus*. This paper describes the partial purification of a toxic principle from kottakilangu and its effects on rats.

EXPERIMENTAL

Chemicals. AnalaR grade solvents and chemicals, as well as Amberlite ion-exchange resins, 100–200 mesh, were obtained from BDH Chemicals Ltd., Poole, Dorset. Sephadex gels and Blue Dextran were purchased from Pharmacia (Great Britain) Ltd., London. Celite 545 was supplied by Koch-Light Laboratories Ltd., Colnbrook, Bucks.

Chromatography and spectrophotometry. Thin-layer chromatography (TLC) used Macherey-Nagel Sil G-25 UV_{254} plates purchased from Camlab, Cambridge. Plates were developed in methanol/water/acetic acid (75:24:1, by vol.), spots being visualized with I_2 vapour.

High-performance liquid chromatography (HPLC) was carried out on a Model 830 chromatograph equipped with a Model 837 spectrophotometer (Dupont Instruments, Hitchin, Herts). A strong cation-exchange column (Dupont, SCX, 500 × 2·1 mm) was eluted with 0·05 M-NH_4Cl at 3·45 MPa (flow rate *c.* 0·9 ml/min). The detector was operated at 220 nm.

UV spectra or absorbances were measured using SP.800 or SP.500 instruments respectively (Unicam Instruments Ltd., Cambridge).

Test material. Dried, unmilled kottakilangu was the gift of Professor S. N. Arseculeratne* and Dr. J. N. Jeyaratnam*. It was ground in a grain mill (C. F. Casella & Co. Ltd., London) to a powder that would pass through a 1·2-mm mesh. The resultant flour was stored in jars at room temperature. No changes of colour, odour or texture were observed over periods of several years.

Preparation of extracts of kottakilangu flour. Method A: Portions of kottakilangu flour (50 g) were extracted with methanol (200 ml) in an Atomix blender (MSE, London) at full speed for four 0·5 min periods. The mixture was centrifuged (at 300 g for 10 min) and the supernatant liquid was decanted. The residue was transferred to the blender, mixed with aqueous methanol (250 ml; 1:1, v/v) and was extracted, centrifuged and decanted as above. The aqueous methanol extraction was repeated three more times and the four supernatants were combined for freeze drying.

Method B: Kottakilangu flour (400 g) and metha-

*Professor S. N. Arseculeratne, Dept. of Microbiology, Faculty of Medicine, University of Peradeniya, Sri Lanka, and Dr. J. N. Jeyaratnam, Dept. of Public Health & Preventive Medicine, Faculty of Medicine, P.O. Box 271, Kynsey Road, Columbo 8, Sri Lanka.

nol (2 litre) were placed in a 5-litre flask equipped with a mechanical stirrer and a reflux condenser. The mixture was refluxed whilst being stirred vigorously (to prevent bumping) for 1 hr and was then allowed to cool and settle. The supernatant liquid (1·6 litre) was decanted and was replaced with methanol (600 ml) and distilled water (1 litre). The mixture was refluxed with stirring for 2 hr and was then allowed to cool and settle. The supernatant liquid was decanted and the residue washed with aqueous methanol (2 × 500 ml; 1:1, v/v). The aqueous methanol extract and washings were combined, filtered through a bed of Celite 545 and the filtrate was evaporated under reduced pressure (rotary evaporator) at 60–70°C.

Details of the fractionation of the toxic extract are given in the results section.

Animals. Albino rats of the random-bred Lac:P strain from these laboratories were maintained, unless otherwise indicated, on MRC 41B diet and water *ad lib.* Weanling rats refers to animals of body weight 50–70 g and age 3–5 wk.

Feeding procedures and dosing schedules. Various feeding studies were carried out using small numbers of adult or weanling rats. Extracted or unextracted kottakilangu flour was mixed with an equal weight of powdered MRC 41B diet and was placed in jars in the rats' cages. The rats were then observed for a few days.

In additional *in vivo* assays of the toxicity of extracts or fractions the material was administered by oesophageal tube. Aqueous solutions were, unless otherwise indicated, administered on the morning and afternoon of each day until the rats had received an appropriate total dose, or until they showed signs of poisoning. Usually two animals (occasionally only one) were used to test each solution. Total doses are expressed as the weight of kottakilangu flour from which the material administered had been extracted. This assumes 100% recovery in any procedure. This assay is primarily qualitative.

Details of the individual experiments are given in the results section. Times of death or the occurrence of clinical signs in animals were measured from the start of treatment.

Brine-shrimp bioassays. Brine-shrimp (*Artemia salina*) eggs, obtained from local pet shops, were allowed to hatch at 27°C for 30 hr in brine-shrimp medium (Hacking, Dervish, Rosser & Syrett, 1977). Following harvesting the volume of the medium was adjusted so that there were *c.* 50 larvae/200 μl. Partially purified extracts of the toxin were adjusted to pH 7·4 with H_3PO_4 and to volumes such that 1 ml contained material from 16 g kottakilangu. In preliminary experiments portions (1–50 μl) of these solutions were added to the wells of Sterilin leucocyte migration plates (Arnold R. Horwell Ltd., London) and were incubated with 200-μl volumes of the suspension of larvae at 27°C for 24 hr. Later experiments used 1–20-μl portions of a ten-fold concentrate of the most toxic solution. Appropriate control wells were set up. In the later experiments, following the recording of hatch deaths, control deaths and compound-related deaths, estimates of the LC_{50} of the toxin were obtained by probit analysis (Finney, 1947) using a computer program adapted from that of Davies (1971).

RESULTS

Effects of feeding kottakilangu flour

The feeding of diets containing kottakilangu flour (50%, w/w) to four adult male rats (170–200 g) resulted in deaths and signs of poisoning similar to those described by Arseculeratne *et al.* (1971). The only difference observed was that, after 4 days, one survivor exhibited stereotypic behaviour; it had a tendency to walk backwards around the cage whilst staring fixedly forwards. Amounts consumed are shown in Table 1.

In another experiment groups of four male or female weanling rats were transferred to a kottakilangu flour/41B (1:1, w/w) diet and observed daily. No change was seen after 24 hr save that some rats had lost weight slightly. Five animals (two males, three females) had died within 52 hr and the survivors were in poor condition. Although the actual deaths were not observed, three rats were in rigor and their bodies were still warm when found. Of these one had been seen to undergo a characteristic fit approximately 90 min before death. This involved the animal balancing on splayed hind feet, the tail being erect and rigid and there being salivation, opisthotonus and spasmodic movements of the forelimbs.

Of the three animals which survived for a longer time (3–6 days) one was observed to undergo a seizure as described above and all showed a pattern of behaviour characterized by continual movement around the cage interspersed with bouts of climbing and biting the bars of the cage. This behaviour continued at intervals over 2 days and was separated by periods in which the rats were immobile, their eyes were closed and their respiration was slow; there was also piloerection. Terminally the animals were prostrate.

On the first 2 days the consumption of diet was

Table 1. *Comparison of the toxicity of extracted and unextracted kottakilangu*

Treatment of kottakilangu	No. of days on diet	Mean total consumption of treated kottakilangu (g/rat)	No. of deaths within 10 days*
None	3–8	22·4	4/4
Methanol extraction†	5–9	30·7	2/2
Aqueous methanol extraction‡	4	42	0/4

*Rats were adult males (170–200 g). Deaths included animals killed when moribund.
†Extraction by method A (see experimental) followed by air drying.
‡Further extraction with aqueous methanol by method A (see experimental) followed by air drying.

Table 2. *Stability of the toxic activity of aqueous methanolic kottakilangu extracts after various treatments*

Method of extraction*	Heat treatment	Added reagents*	Weight of kottakilangu to which total dose is equivalent (g)	No. of deaths within 5 days
A	–	NaCl	10·0	2/2
A	+	NaCl	10·0	2/2
A	+	NaOH	10·0	2/2
A	+	HCl	10·0 or 8·7	1/2†
B	–		10·0	2/2

*An aqueous solution of the kottakilangu extract was mixed with an aqueous molar solution of the reagent (0·5 vol), heated if appropriate at 100°C for 1 hr, cooled and neutralized if necessary, and was diluted to a volume such that 1 ml contained the extract from 3·33 g of kottakilangu flour. Weanling male rats were given three doses orally after 0, 7 and 24 hr.

†The surviving animal had received the lower dose.

equivalent to a mean intake of 4·1 and 1·8 g of kottakilangu/rat respectively. Thereafter survivors consumed less than 1 g/rat each day. There were no significant differences between the quantities consumed by male and female groups.

Extraction of the toxin from kottakilangu flour

Various experiments, for brevity not described here, indicated that Soxhlet extraction of the kottakilangu flour with *n*-hexane, diethyl ether and acetone did not remove the toxin. Extraction of the flour with methanol by method A (see Experimental) also failed to extract the toxic material. However, further extraction of the meal with aqueous methanol by method A did render the kottakilangu non-toxic (Table 1).

Evaporation of the aqueous methanol extract resulting from method A left a viscous, brown liquid (0·10 g/g kottakilangu) with a smell of freshly-baked bread. This was dissolved in water to give a volume such that each millilitre of solution contained material equivalent to 2·5 g of the original flour. Four doses of this solution, totalling 8 or 3 ml per rat, were administered by oesophageal intubation over two days to male rats of body weight 200–210 g (N = 2) or 70–80 g (N = 3) respectively. The two older animals were seen to undergo convulsive episodes, similar to those described above for younger animals fed on the kottakilangu/41B diet, between 48 and 72 hr after the first dose. One was killed after 3 days, the other died within 7 days. Of the three younger animals, within 48 hr two had died and the third had been killed during a period of convulsive activity.

Table 2 shows that neither extraction of kottakilangu flour by method B nor heat treatment of an aqueous methanolic extract obtained by method A destroyed the toxic activity. The same table indicates that the toxicity of extracts was apparently unaffected by heat in the presence of dilute acid or base. Therefore subsequent extracts of kottakilangu were obtained by the simpler procedure of method B.

Fractionation of toxin-containing extracts

Each procedure was carried out, unless otherwise specified, on an aqueous solution of the toxic extract containing material equivalent to 4 g kottakilangu/ml.

Extraction with either butan-1-ol (3 × 1 vol) or phenol (0·4 g/ml of extract) left the toxin in the aqueous phase. After precipitation with aqueous lead acetate trihydrate solution (20%, w/v; 0·65 ml/ml of extract) and centrifugation the active material remained in the supernatant.

The toxin was not retained on a column of anion-exchange resin (Amberlite CG-400) in the acetate form. Considerable purification could be achieved on a column of strong cation-exchange resin (Amberlite CG-120, NH_4^+ form, 34 × 4 cm). Extract (80 ml), that had been treated with butanol, phenol and lead acetate, was applied to the column and eluted with water

Table 3. *Toxicity to weanling male rats of fractions of a kottakilangu extract chromatographed on a cation-exchange resin (NH_4^+ form)*

Fraction no.	Eluant	Volume (ml)	Weight of kottakilangu to which total dose is equivalent (g)*	No. of doses†	No. of deaths within 4 days
1	H_2O and	800			
	0·01 M-NH_3	800	18	3	0/2
2	0·1 M-NH_3	150	24	4	0/2
3	0·1 M-NH_3	90	24	4	0/2
4	0·1 M-NH_3	100	24	4	0/2
5	0·1 M-NH_3	340	12	2	2/2
6	0·1 M-NH_3	300	12	2	2/2

*The fractions were evaporated (rotary evaporator, 70°C) and dissolved in water. The figures assume that any compound is present in only one of the fractions.

†See experimental section for dosing schedule.

(800 ml) and 0·01 M aq.NH_3 (800 ml) at a flow rate of 7·5 ml/min, this removed the bulk of the non-toxic material. The eluant and flow rate were then changed to 0·1 M aq. NH_3 and 3·25 ml/min respectively. Portions (10 ml) were collected; on the basis of differences in the UV spectra of these fractions they were combined as indicated in Table 3 (fractions 2–4 had peaks with λ_{max} near 270 nm, fractions 5 and 6 showed only end absorption). Only fractions 5 and 6 were toxic (Table 3). Later it was found that the toxin-containing 0·1 M-NH_3 eluate could be distinguished by the fact that on evaporation it left a gum in the flask whereas the residues from preceding and subsequent fractions were powdery solids. An 18-fold enrichment of the toxin could be achieved in this step, the active product corresponded to 0·23% of the weight of the starting kottakilangu.

Toxic material that had been eluted from a cation-exchange resin as above was applied to a column of Sephadex G-25 (medium, 50 × 3 cm) that had been swollen in water. Elution with water gave a major UV-absorbing peak having an elution volume almost identical to that of vitamin B_{12} (Fig. 1). The fractions (nos 21–27) that encompassed this peak contained the toxin. Some high-molecular-weight material was separated on this column but TLC revealed the presence of several compounds in the major peak.

Different fractions eluted from a CG-120 resin by 0·1 M-NH_3 have been submitted to HPLC (for conditions see the Experimental section). A major peak, eluting after 2·05 min, was present in the toxic fraction and was either absent or of small size in earlier and later fractions.

Alternative bioassays of kottakilangu toxin

Two systems have been tested to determine whether an assay, for the toxin, more sensitive than oral administration to the rat could be devised. Each system was tested with three solutions (A, B and C) which corresponded to consecutive fractions from the elution of an aqueous methanol extract of kottakilangu from a CG-120 (NH_4^+ form) ion-exchange column with 0·1 M-NH_3. This extract had received no preliminary precipitation or extraction treatments. When tested in the weanling male rat doses of solution A totalling the equivalent of 24 or 36 g of flour resulted in death within 48 and 72 hr respectively. Dosages of solution B equivalent to 8 or 12 g of flour, corresponding to 19·5 or 29·3 mg dry weight of material, were lethal within 24 hr to 1/2 and 2/2 rats respectively. Solution C was non-toxic.

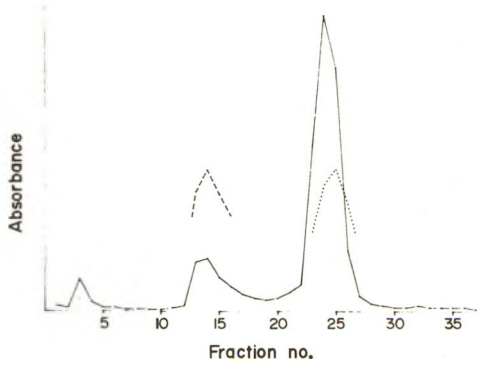

Fig. 1. Absorbance (varying scales) of fractions (10 ml) eluted by water from a Sephadex G-25 column (500 × 30 mm) after application of (a) toxin purified on ion-exchange resins (——, at 225 nm), (b) Blue Dextran, mol wt 2 × 10^6 (– – –, at 665 nm), or (c) Vitamin B_{12}, mol wt 1357 (· · · · ·, at 360 nm).

In a cytotoxicity assay using Walker rat carcinoma cells (Phillips, 1974) both solutions A and B were toxic. However, at equal dilutions in the assay medium, solution A caused a greater inhibition of cell division than did solution B. Solutions containing the toxin were mixed *in vitro* with heparinized rat blood; no haemolysis occurred.

The solutions were tested for their action on brine-shrimp larvae; in this assay solution B was clearly the most toxic and subsequent tests with a more concentrated solution provided an LC_{50} against the larvae of 1·81 mg dry weight of material/200 µl of assay medium. The 95% fiducial limits were 1·31–2·36 mg/200 µl medium. At the concentrations tested solutions A and C showed no effect.

Toxic effects of flour extracts in the rat

The assay of palmyra flour extracts has led to the observation of intoxication in weanling males following varying doses and at varying intervals after the start of dosing. It is therefore possible to make some general statements about the course of poisoning after the administration of extracts by oesophageal intubation.

No dosage has resulted in deaths or signs of toxicity within 12 hr. If a solution containing material equivalent to 8–10 g of kottakilangu is given as one dose, or the equivalent of 8–12 g as two doses, then death usually occurs within 24 hr. The first sign is a depressed appearance with piloerection, the animals may then become hyperactive, a stage which precedes the onset of violent, fatal convulsions. Only rarely has a tonic phase been seen after these convulsions. Rigor sets in very shortly after death. The administration of total dosages corresponding to 10–16 g of starting flour over a period of 2–3 days (4–6 doses) may prolong the period before the appearance of toxic effects. The strength of the convulsions is less and the intervals between them become longer. In these less violent seizures the animal usually assumes an upright posture, it balances on its splayed hind limbs and the tail is erect and rigid. There is opisthotonus with the head thrown back and the fore paws move spasmodically. There is sometimes salivation; the saliva may become brown in colour. In some animals the eyelids blink rapidly. Loss of balance may be seen at this stage and the animal falls on its back. This condition may proceed to clonic convulsions or regress to a state of hyperactivity characterized by the animal clinging to the bars of its cage or box by its teeth and making coordinated spasmodic movements of its hind limbs. Alternatively regression to a depressed, inactive condition may occur. A period of restlessness then appears to signal the onset of the next attack. Although sometimes the rats are excitable, at no stage do sudden sounds initiate convulsive behaviour. With less severe intoxication loss of weight, piloerection, and occasional brief seizures (as described above) represent the characteristic features.

DISCUSSION

The work of Arseculeratne and his colleagues has demonstrated that a diet containing either 100% (Arseculeratne *et al.* 1971) or 25% (Panabokke & Arseculeratne, 1976) of kottakilangu flour is toxic to adult male rats. We have confirmed this effect in the Lac:P rat using a diet containing 50% kottakilangu. The observation that this diet is as toxic to weanling rats of either sex as to the adult male, on the basis of the weight of flour consumed relative to body weight, has led to the use of weanling male rats in the majority of subsequent assays.

The toxic material was not extracted from the kottakilangu by methanol or less polar solvents, however it was removed by aqueous methanol (Table 1). It is probable that water alone would have extracted the active principle but its use resulted in the formation of intractable slurries. Because methanol removes some material from the flour, all extractions have incorporated a preliminary methanol treatment to remove unwanted compounds. The toxin is stable in aqueous solutions at 100°C whether at neutral pH or in the presence of dilute acid or base (Table 2). It has been postulated (Arseculeratne *et al.* 1971) that kottakilangu contains two toxic factors, one of which disturbs the metabolism of hepatic mitochondria and the other of which is lethal. The lethality of the first factor is undetermined. The Sri Lankan workers showed that the compound responsible for the mitochondrial effects is heat stable and extracted by aqueous solutions, however it is apparently not present consistently in all batches of kottakilangu. It would therefore appear that the material which is soluble in aqueous methanol corresponds to their lethal factor since, from many batches of palmyra flour, our procedures have consistently provided extracts and fractions causing toxicity in the rat. The lack of solubility in methanol would seem to preclude our material from being the equivalent of the ethanol-soluble alkaloidal compounds that have been extracted from kottakilangu (Panabokke & Arseculeratne, 1976).

The method of extraction indicates the hydrophilic nature of the toxin and this is confirmed by its failure to partition into butan-1-ol or phenol. The lack of a precipitate with Pb^{2+} ion and the absence of any retention on an anion-exchange column exclude its containing acidic groups of many types. The retention on cation-exchange columns suggests that the toxin possesses an ionized nitrogen atom; since, in some experiments (not described), extraction at alkaline pH has failed to remove the toxicity from the aqueous phase, the nitrogen is probably present in a quaternary ammonium group.

The behaviour of the toxicant on Sephadex G-25, in particular the similarity of its elution volume to that of vitamin B_{12} (Fig. 1), indicate that the molecular weight of the toxin is about 1400; the assumption made is that there is no structure-related interaction with the gel to delay the elution. This, coupled with some of the chemical and physical properties of the toxin, excludes a protein structure. A glycoside-containing structure is compatible with the absence of peaks in the UV spectrum at wavelengths above 200 nm, however the absence of any haemolytic effect and the failure to partition into butan-1-ol eliminates many simple saponins (Verdcourt & Trump, 1969).

The cytotoxicity to Walker cells of some kottakilangu fractions did not correlate well with their toxicity *in vivo*. The toxicity of these fractions to the rat is better correlated with their lethality to brine-shrimp larvae. At the present degree of purification the LC_{50} of the toxicant in the larvae is greater than that of many mycotoxins (Hacking *et al.* 1977), however this is still a more sensitive bioassay system than the rat.

The separations achievable with HPLC and the use of the brine-shrimp assay will facilitate the isolation of the kottakilangu toxin; further work is now being directed to this end.

The similarity of the signs of intoxication following either incorporation of the palmyra flour in the diet or administration of extracts of the flour by oral intubation suggests that the only difference in the action of the toxin administered by these two regimes is quantitative. Since we have never seen hepatic changes in our rats it is probable that the compound is a neurotoxin and not acting indirectly *via* disturbance of hepatic metabolism. The characteristics of the seizures following acute poisoning are consistent with this conclusion. The delay in onset of the signs is possibly due to a necessity for the toxin to undergo metabolic transformation either before it can be absorbed from the intestine or before it can enter the nervous system.

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INHIBITORY ACTION OF CITRININ ON CULTURED HEPATOMA CELLS

G. LORKOWSKI

**Institut für Mikrobiologie, Universität Münster, Tibusstrasse 7–15, D-4400 Münster, Bundesrepublik Deutschland,*

E.-E. CREPPY, G. BECK and G. DIRHEIMER

Laboratoire de Toxicologie et de Biologie Moléculaires, Faculté de Pharmacie et I.B.M.C. du C.N.R.S., 15 rue René Descartes, Strasbourg, France

and

R. RÖSCHENTHALER*

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Abstract—Citrinin at concentrations of up to 25 μM was cytostatic to hepatoma tissue culture cells. At concentrations between 50 and 200 μM it was cytotoxic. At a concentration of 100 μM, citrinin inhibited RNA, protein and DNA syntheses by the cells but the onset of inhibition of the three macromolecular syntheses occurred at different intervals after the addition of citrinin: 5–7 min for RNA synthesis, 12 min for protein synthesis and 2 hr for DNA synthesis. DNA synthesis was not completely blocked 12 hr after the addition of citrinin, while RNA synthesis ceased first (after 5–6 hr) followed by protein synthesis (after 8 hr).

INTRODUCTION

Citrinin (4,6-dihydro-8-hydroxy-3,4,5-trimethyl-6-oxo-3*H*-2-benzopyran-7-carboxylic acid) is a mycotoxin that is produced by strains of *Penicillium citrinum* and some other species of Penicillia and Aspergilli (Saito, Enomoto & Tatsuno, 1971). After its discovery by Hetherington & Raistrick (1931) its toxic action on gram-positive and gram-negative bacteria was investigated (Smith, 1949). Lorkowski & Röschenthaler (1979) have shown that citrinin inhibits RNA and DNA synthesis and to a lesser extent protein synthesis in *Escherichia coli*.

Citrinin has been found as a natural contaminant of rice in Japan (Saito *et al.* 1971), and also of wheat, rye, barley and oats in Canada (Scott, van Walbeek, Kennedy & Anyeti, 1972) and of barley and oats in Denmark (Krogh, Hald & Pederson, 1973). It has been shown to be toxic to many animal species including mice, rats, rabbits, swine and poultry (Phillips & Hayes, 1978). The target organ is the kidney in which it induces pathological changes of the tubuli (Carlton & Tuite, 1977; Kitchen, Carlton & Hinsman, 1977).

Little data exists on the mode of action of this mycotoxin. In an isolated publication, Terao & Ueno (1978) reported that citrinin inhibits *E. coli* RNA polymerase and preferentially inhibits eukaryotic RNA polymerase I in comparison with RNA polymerase II. In this paper we describe the action of citrinin on macromolecular syntheses in cultured hepatoma cells.

EXPERIMENTAL

Citrinin was prepared and purified by the method of Jackson & Ciegler (1978), and the purity was verified by thin-layer chromatography and nuclear magnetic resonance spectroscopy. The citrinin was dissolved in ethanol at a concentration of 22·5 mg/ml.

Hepatoma tissue culture (HTC) cells, derived from a malignant tumour of rat liver parenchymal cells (Morris hepatoma 7288c) and adapted to growth in suspension cultures (Thompson, Tomkins & Curran, 1966), were used throughout the experiments. The cells (initial concentration c. 3×10^5 cells/ml) were grown in Swim's medium S77 supplemented with 10% calf serum (Lab. Gibco; Flobio, Courbevoie, France) at 37°C. Citrinin was added to the cultures to give a final concentration of 0, 10, 25, 50, 100 or 200 μM. The number of living cells was determined by trypan blue exclusion in a Neubauer microcytometer as previously described (Creppy, Lugnier, Beck, Röschenthaler & Dirheimer, 1979). It was assumed that a certain dose of citrinin had a cytostatic effect when the number of living cells remained constant, and a cytotoxic effect when this number decreased.

For the determinations of protein, RNA and DNA synthesis citrinin was added to the cultures (*c.* 3×10^5 cells/ml) to give a final concentration of 100 μM and the rates of synthesis (rates of incorporation of radioactive precursors into the macromolecules) were measured as described previously (Creppy *et al.* 1979). All assays were done in triplicate and only the average values are reported.

RESULTS

At all of the concentrations of citrinin used the growth of the cell cultures ceased. At concentrations of 10 μM and 25 μM a small decrease in the number of living cells occurred soon after the addition of citrinin and in the following incubation period the fraction of

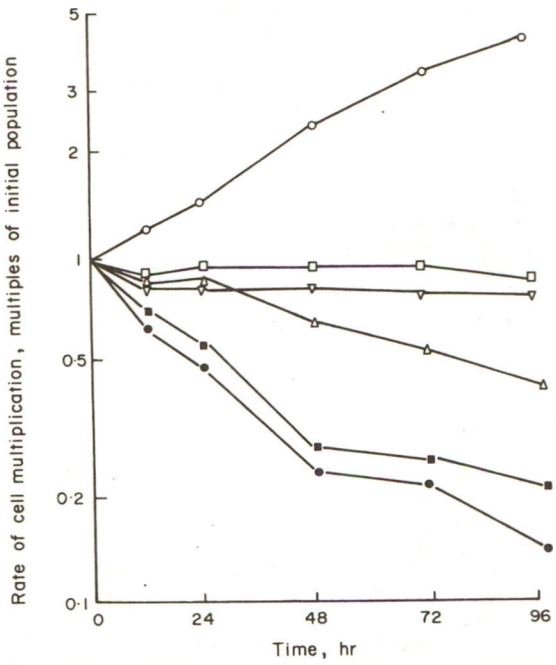

Fig. 1. Rate of hepatoma tissue culture cell multiplication in the presence of different concentrations of citrinin: control (○); 10 μM (□); 25 μM (▽); 50 μM (△); 100 μM (■); 200 μM (●).

living cells remained constant (Fig. 1). At concentrations of citrinin from 50 μM up to 200 μM there was a distinct cytotoxic effect.

At the rather high concentration of citrinin tested (100 μM) the cells initially showed synthetic activity (Fig. 2). The onset of inhibition of the synthesis of the three macromolecules occurred at different times. The insets of Figs 2a & 2b show that the inhibition of RNA synthesis begins to decrease 5–7 min after the addition of citrinin while under the same conditions the rate of protein synthesis begins to decrease about 12 min after the addition of the mycotoxin. The more rapid onset of inhibition of RNA than of protein synthesis is also demonstrated by the times that are required to completely block these processes: 5–6 hr for RNA synthesis but about 8 hr for protein synthesis. However, the amount of labelled material incorporated into protein when the plateau was reached was somewhat lower than that incorporated into RNA, the levels being, for protein and RNA respectively, 30 and 42% after 6 hr and 24·6 and 32% after 8 hr.

DNA synthesis was unaffected for 2 hr after the addition of citrinin at 100 μM and for 1 hr when the citrinin concentration was increased to 150 μM (Fig. 2c). After these times the rate of DNA synthesis fell considerably but then remained constant for at least 8 hr (and for 12 hr in an experiment the results of which are not shown). This contrasted with the effects of citrinin on protein synthesis which progressively decreased until it stopped.

DISCUSSION

The major effect of citrinin in animals is reported to be its nephrotoxicity (Ambrose & DeEds, 1946; Kit-

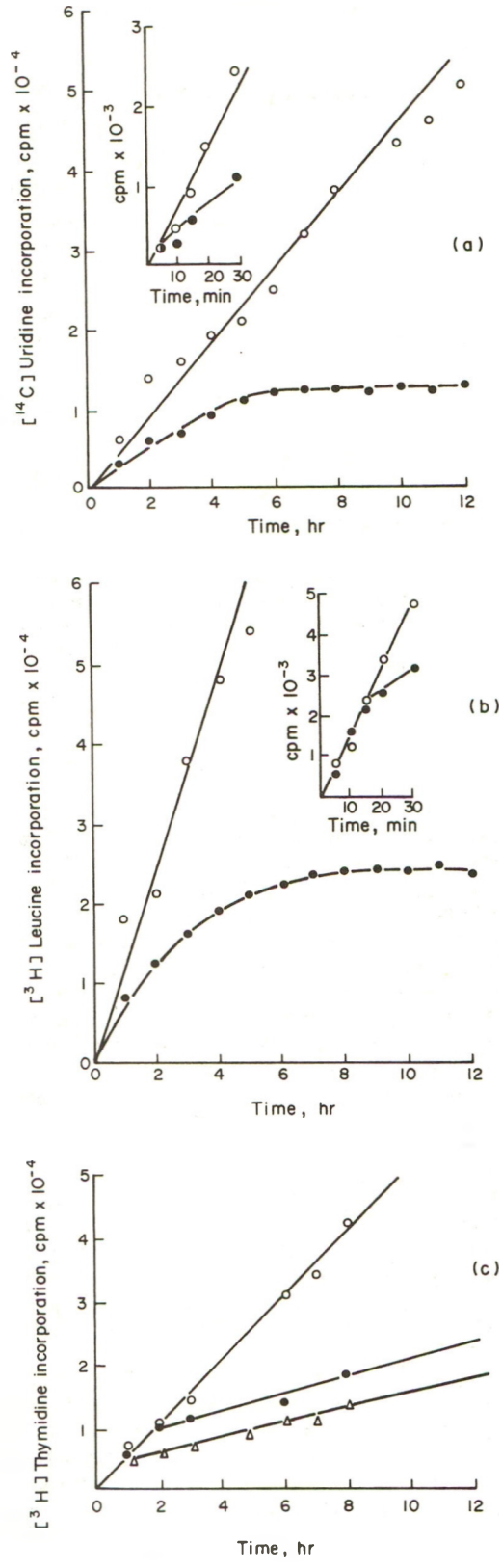

Fig. 2. Synthesis, from radioactivity-labelled precursors, of (a) RNA, (b) protein and (c) DNA by hepatoma tissue culture cells in cultures containing citrinin at concentrations of 0 (○), 100 (●) or 150 (△) μM. The citrinin and the radioactivity-labelled precursors were added at 0 hr.

chen, Carlton & Tuite, 1977; Pier, 1973). However, hepatotoxic action has also been reported (Carlton & Tuite, 1977; Ramadoss & Shanmugasundaram, 1973).

From the data obtained with HTC cells, it can be concluded that citrinin affects RNA synthesis more rapidly than protein synthesis. This effect may be explained by the results of a study of Terao & Ueno (1978). These workers found that in isolated nuclei, 100 μg citrinin/ml inhibited RNA polymerase I by 60% but inhibited RNA polymerase II by only 24%. Since RNA polymerase I is reponsible for rRNA synthesis (Roeder, 1976) its inhibition should show up quickly as a reduction of total RNA synthesis. In contrast a decrease in the activity of RNA polymerase II, which is responsible for mRNA formation, would not be expected to have such a rapid effect on the rate of protein synthesis because of the long lifetime of eukaryotic mRNAs.

The effect of citrinin on DNA synthesis is not understood. The delayed onset of inhibition of DNA synthesis shows that citrinin does not directly affect DNA synthesis in the experiments described here. However, Phillips & Hayes (1978) have shown that the hepatic DNA content, determined by the method of Burton (1956), decreased significantly after ip injection of citrinin into mice, and that this decrease was detectable 1 hr after injection. In experiments with citrinin-treated bacteria, DNA synthesis was less inhibited than RNA synthesis if it was measured by ^{3}H-thymidine incorporation but was more inhibited than RNA synthesis if it was estimated by colorimetric measurements of changes in cellular DNA content. Since citrinin is known to have a mutagenic effect on bacteria (Lorkowski & Röschenthaler, 1979; Ueno & Kubota, 1976), these results were interpreted as evidence for the existence of DNA repair syntheses in citrinin-treated bacteria. However, more data must be obtained before these results can be interpreted conclusively and further experiments on this problem are in progress.

It has also been reported that citrinin is a potent inhibitor of sterol synthesis in yeast and in rat liver (Tanzawa, Kuroda & Endo, 1977). However, since in eukaryotic cells and in bacteria similar effects of citrinin on RNA synthesis are observed, and since bacteria do not synthesize sterols, this inhibition may be a secondary effect that is limited to eukaryotes.

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EVIDENCE FOR *IN VITRO* AND *IN VIVO* INTERACTION BETWEEN OCHRATOXIN A AND THREE ACIDIC DRUGS

P. GALTIER and R. CAMGUILHEM

Laboratoire de Pharmacologie-Toxicologie, INRA, 180 chemin de Tournefeuille, 31300 Toulouse

and

G. BODIN

Ecole Nationale Vétérinaire, chemin des Capelles, 31300 Toulouse, France

(*Received 20 December 1979*)

Abstract—Phenylbutazone, ethyl biscoumacetate and sulphamethoxypyridazine each competitively inhibited the *in vitro* binding of ochratoxin A to porcine serum albumin, indicating that these drugs and the toxin probably bind at the same site on the protein. Adult male rats given oral doses of 4 mg ochratoxin A/kg/day for up to 10 days showed the same signs of intoxication whether given the toxin alone or together with one of the three drugs in doses of 10, 20 or 50 mg/kg/day. Calculation of LD_{50} values and histological investigation showed an increase in ochratoxin A toxicity when the toxin was administered with ethyl biscoumacetate or phenylbutazone. This finding may reflect a displacement of the toxin from binding sites on the plasma proteins *in vivo*, although some additive or synergistic action of the toxin and the associated drug is another possibility. In contrast, sulphamethoxypyridazine decreased the ochratoxin A toxicity, probably by counteracting an enteritis-producing effect of the ochratoxin on the intestinal microflora.

INTRODUCTION

Ochratoxin A (7-carboxy-5-chloro-8-hydroxy-3,4-dihydro-3-methylisocoumarin linked through the 7-carboxy group to L-phenylalanine by an amide bond) is a nephrotoxic mycotoxin produced by both Aspergillus and Penicillium fungi (Scott, 1977). This toxin has been demonstrated *in vitro* (Chu, 1971) and *in vivo* (Chang & Chu, 1977; Galtier, 1974b) to bind to plasma albumins.

This work was designed to elucidate *in vitro* the displacement of this protein binding by three acidic drugs, sulphamethoxypyridazine, phenylbutazone and ethyl biscoumacetate, and then to determine *in vivo* the toxic interactions that occurred when each of these competing substances was administered with ochratoxin A to rats.

EXPERIMENTAL

Materials. Ochratoxin A was produced by *Aspergillus ochraceus* as previously described (Galtier, 1974a); its purity was verified by TLC, spectrophotometric and spectrofluorimetric assays. Purified porcine albumin was obtained from Sigma Chemical Co., St. Louis, MO, USA (fraction V powder, Catino A 1885). The purity of this protein was confirmed by electrophoresis. For the *in vitro* study, samples of pure phenylbutazone, ethyl biscoumacetate (Tromexan) and sulphamethoxypyridazine (Sultirène) were used. These chemicals were generously provided by Ciba-Geigy AG, Basel, Switzerland, and Spieca, Paris. For the *in vivo* studies, commercial forms of these drugs were used.

In vitro *measurement of ochratoxin displacement from plasma protein.* Equilibrium dialysis was performed at 38°C, using dialysis bags made from Visking Tubing (SICL, London) boiled, washed and equilibrated in phosphate buffer (pH 7·4). Inside the sac was 1 ml of buffered albumin solution ($57{\cdot}9 \times 10^{-6}$ M) and this was dialysed in 10 ml screw-cap vials against 4 ml ochratoxin A ($6{\cdot}70 \times 10^{-5}$ M) plus 1 ml of phosphate buffer containing one of the inhibitors (10^{-4} M), for 8 hr with stirring. Four replicates of each test were carried out. The free form of the mycotoxin in the outer solution was measured spectrophotometrically (λ = 380 nm) in a Unicam SP 1800 apparatus after centrifugation. Results were corrected for the absorbance of a blank assay obtained from dialysis of ochratoxin A against phosphate buffer without albumin. The binding parameters were calculated according to the Scatchard method (1949).

Determination of the toxic effect of in vivo *interaction*

Animals and diet. Male Wistar rats from our own colony and weighing around 250 g were randomly divided into 16 groups each of nine animals. They were housed at 25–27°C in cages of four or five, and given UAR food (Epinay, France) and water *ad lib.*

Dosage. Ochratoxin A in bicarbonate solution was administered by oesophageal intubation and, at the same time, phenylbutazone, sulphamethoxypyridine or ethyl biscoumacetate was administered in aqueous solution or a viscid suspension. In all cases the dosage volume was 2 ml/kg body weight. The test animals were given 4 mg mycotoxin/kg/day plus one of the drugs in doses of 10, 20 or 50 mg/kg/day for 10 days

and any survivors were observed for a further 10 days. Control groups received a vehicle or ochratoxin A or one of the competing drugs alone.

Calculation of lethal doses and examinations. The 10-day LD_{50} values were computered according to the classic probit method. The algorithm used was that of Ross (1970). Following the LD_{50} calculations, the parallelism between log dose-probit curves was tested by means of a chi-square test; when it was verified the significant differences between intercepts with the ordinate corresponded to significant differences between LD_{50} values.

Each surviving animal was weighed every morning throughout the 10-day treatment period and 10-day observation period. Animals that died were autopsied and any organs that appeared abnormal were subjected to further investigation. In addition, half of the treated animals were examined histologically, sections being prepared from the liver, kidney, lungs, adrenal glands, stomach and intestine from each rat. Tissue sections were stained with haemalum–eosin.

RESULTS

In vitro *measurement of ochratoxin displacement from plasma protein*

At a concentration of 10^{-4} M, each of the three acidic drugs (sulphamethoxypyridazine, phenylbutazone and ethyl biscoumacetate) competitively inhibited the binding of ochratoxin A to porcine serum albumin. There was a linear relationship between the reciprocal of the concentration of unbound ochratoxin A and the reciprocal of the moles of ochratoxin A bound per mole of porcine albumin, as indicated by the Lineweaver plots of these interactions (Fig. 1). The differences in the degrees of binding were also demonstrated by the values of the binding parameters. The number of binding sites (n) was unchanged whereas the intrinsic association constant (K_a) decreased when a drug was added, being 68,900 M^{-1} for ochratoxin A alone and 37,200, 34,300 and 22,300 M^{-1} for interaction with phenylbutazone, sulphamethoxypyridazine and ethyl biscoumacetate, respectively.

Determination of the toxic effect of in vivo *interaction*

General observations. Ochratoxin A had similar effects whether given alone or in conjunction with a drug. The main effects were reduction in spontaneous activity, hypothermia, cachexy and huddling, while tremors and diarrhoea also occurred frequently. The body weight of the treated animals decreased from day 3 of treatment (Fig. 2). Survival times varied according to the drug and were dose-related. Compared with those given ochratoxin A alone, rats treated with the toxin and phenylbutazone or ethyl biscoumacetate survived for a shorter period while those given the toxin and sulphamethoxypyridazine survived longer.

Lethal doses. The cumulative LD_{50} calculated for rats receiving ochratoxin A alone was 33·43 ± 2·69 (SEM) mg/kg. The LD_{50} values for the animals receiving the toxin and a drug are indicated in Table 1; in all cases the parallelism between the log dose-probit curves was verified and the significant differences ($P < 0{\cdot}01$) in origin ordinates demonstrated the significant effect of drug associations on the cumulative LD_{50} of ochratoxin A.

When the rats were given either of the acidic drugs alone there were neither deaths nor decreases in the body weight up to a daily dose of 50 mg/kg.

Post-mortem and histological examinations

Gross autopsy findings in animals treated with ochratoxin A alone or together with one of the three drugs were similar to those previously reported after prolonged oral administration of the mycotoxin (Galtier, Bodin & Moré, 1975). These consisted of hyperaemia of the duodenum and jejunum with possible

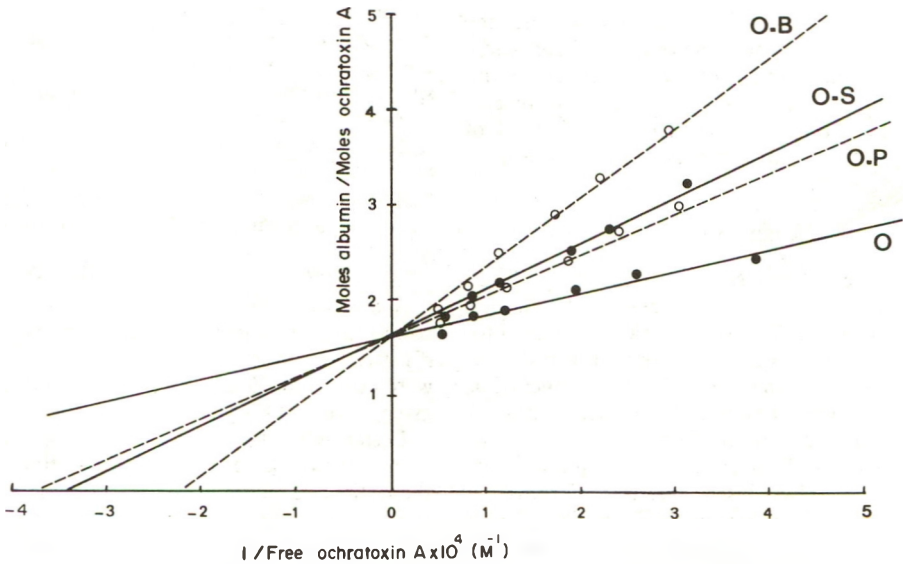

Fig. 1. The effects of ethyl biscoumacetate (O-B), sulphamethoxypyridazine (O-S) and phenylbutazone (O-P) on the binding of ochratoxin A (O) to porcine serum albumin. The concentration of inhibitor was 10^{-4} M in all experiments.

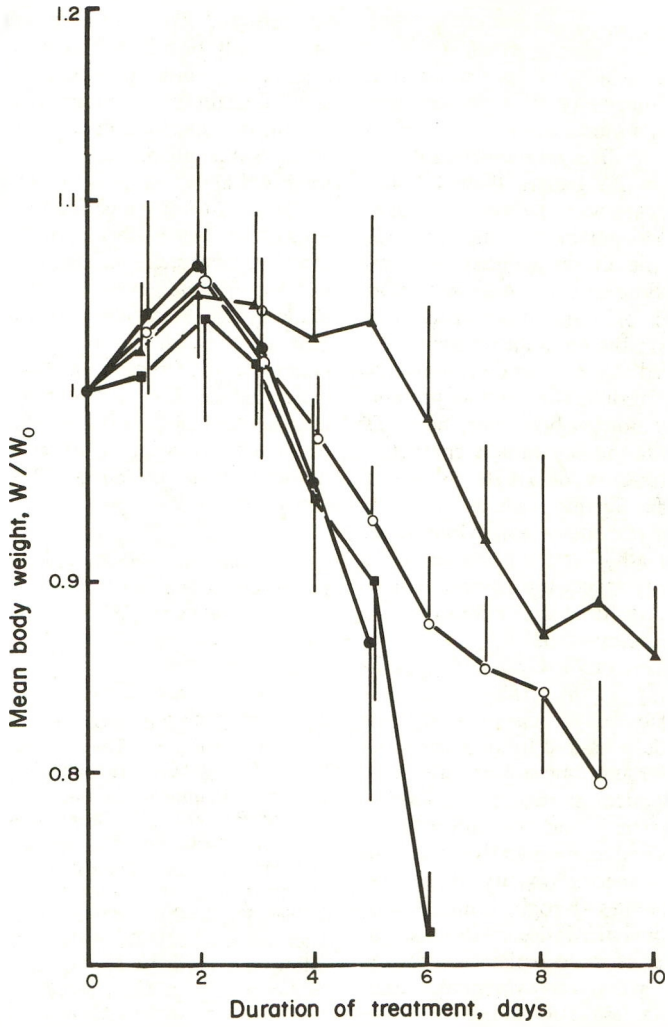

Fig. 2. Changes in the body weights of rats given ochratoxin A (4 mg/kg/day) alone (○) or associated with sulphamethoxypyridazine (▲), phenylbutazone (■) or ethyl biscoumacetate (●) each in doses of 50 mg/kg/day. Mean weights for each day (W) are expressed in terms of the mean weight on day 1 of treatment (W_0) for groups consisting initially of nine rats.

haemorrhagic gastritis, hypertrophy and paleness of the kidneys and, frequently, haemorrhage of the thymus and adrenals. Histological examination of rats receiving ochratoxin A alone revealed congestion and thickening of the inter-alveolar walls and possible haemorrhagic areas in the lungs, epithelial degenerative nephritis with hyaline casts in the kidneys, centrilobular congestion in the liver and ulcerating lesions in the stomach. When the toxin was given with phenylbutazone or sulphamethoxypyridazine the kidneys also showed glomerular congestion, while the gastric necrotic areas were more marked and were invaded by neutrophils in the rats given the mycotoxin with phenylbutazone or ethyl biscoumacetate.

Table 1. *Cumulative LD_{50} values for adult male rats given daily oral doses of ochratoxin A and acidic drugs for up to 10 days*

Dose of acid drug (mg/kg/day)	LD_{50} (mg/kg) of ochratoxin A* administered with		
	Ethyl biscoumacetate	Phenylbutazone	Sulphamethoxypyridazine
10	31·3 ± 3·5	29·6 ± 2·5	32·3 ± 2·6
20	24·2 ± 2·6	25·6 ± 2·5	35·2 ± 6·0
50	21·1 ± 2·4	21·1 ± 2·2	55·4 ± 4·0

*Values are means ± SEM for groups of nine rats each given daily doses of 4 mg ochratoxin A/kg for up to 10 days. Calculations are based on the numbers of deaths within 20 days of the start of treatment. The cumulative LD_{50} of ochratoxin A administered alone to a comparable group of rats was 33·4 ± 2·7 mg/kg.

DISCUSSION

The binding of ochratoxin A to porcine serum albumin at 38°C is a strong association. It has been shown that the binding of a model drug to the plasma proteins has an appreciable effect on the drug's distribution if the drug has a K_a greater than $10^4\ M^{-1}$ (Martin, 1965), as is the case with the anti-inflammatory drug, coumarin anticoagulant and sulphonamide used in this study. Since these three acidic drugs competitively inhibit the binding of ochratoxin A to porcine serum albumin, the toxin and these drugs may bind to a common site on the albumin molecule. All the compounds are ionized at pH 7·4 and are presumably associated with a cationic site on the protein. Although such sites have not yet been identified, this region could correspond to the one already characterized by Sudlow, Birkett & Wade (1976) as site I, which can accept specific ligands such as warfarin, acenocoumarin, sulphinpyrazone or phenylbutazone. Moreover, the fact that ethyl biscoumacetate is the most potent inhibitor may reflect the structural likeness between this coumarin drug and ochratoxin A, which is an isocoumarin compound.

The LD_{50} values indicated in Table 1 clearly demonstrate an increase in mycotoxin toxicity as a result of association with either ethyl biscoumacetate or phenylbutazone. This could be due to an *in vivo* displacement of the serum albumin-bound ochratoxin A concentration. The subsequent increase in the level of free toxin in the plasma may lead to a more rapid excretion *via* the urine and consequently to an increase in the mycotoxin's specific toxicity to the cells of the proximal renal tubules (Krogh, Elling, Gyrd-Hansen, Hald, Larsen, Lillehøj, Madsen, Mortensen & Ravnskov, 1976). Another possible explanation for the potentiation of toxicity is that the two foreign compounds have an additive or synergistic effect. Since ethyl biscoumacetate is a well-known coumarin anticoagulant and ochratoxin A has been reported to possess an indirect antivitamin-K effect in rats (Galtier, Boneu, Charpenteau, Moré & Alvinerie, 1979), the simultaneous administration of these two compounds could lead to a synergistic haemorrhagic action; however, the rats in this study showed no intense haemorrhagic syndrome but only the usual hyperaemia and slight gastro-intestinal haemorrhage associated with experimental ochratoxicosis (Galtier *et al.* 1975). Since phenylbutazone is known to induce gastric ulcers and renal failure, additive or synergistic toxic effects could explain the more marked gastric ulcerating lesions and the glomerular congestion in the kidneys of rats given this drug together with ochratoxin A.

In contrast to these two drugs, sulphamethoxypyridazine administered with ochratoxin A led to a dose-related decrease in the toxicity of the mycotoxin (Table 1). In this case, the sulphonamide may have had a therapeutic effect on a possible enteritis resulting from an ochratoxin-induced disturbance of the intestinal microflora (Galtier, Moré & Bodin, 1974). This hypothesis was supported by the decrease in ochratoxin A toxicity observed by us (unpublished data, 1979) when the mycotoxin was simultaneously administered with sulphaguanidine, a sulphonamide that is only poorly absorbed in the gastro-intestinal tract and is used particularly for the treatment of local intestinal infections. Under the experimental conditions described in this paper, administration of 50 mg sulphaguanidine/kg/day increased the ochratoxin A LD_{50} from 33·4 to 42·8 mg/kg.

The *in vitro* evidence for the ability of three acidic drugs to displace ochratoxin A from binding sites on the plasma proteins offers at least a partial explanation for the changes in ochratoxin A toxicity demonstrated when the mycotoxin is associated with one or other of these drugs *in vivo*. Other pharmacological factors may interfere with this particular mechanism. Nevertheless, this work demonstrates the possibility that a toxicologically significant interaction may occur between a fungal toxin that is known to occur naturally in foods (Scott, 1977) and drugs that are widely used in therapeutics.

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CHROMOSOME ABERRATIONS IN MAMMALIAN CELLS EXPOSED TO VITAMIN C AND MULTIPLE VITAMIN PILLS

H. F. STICH, L. WEI and R. F. WHITING

Environmental Carcinogenesis Unit, British Columbia Cancer Research Centre, 601 West 10th Avenue, Vancouver, B.C., Canada V5Z 1L3

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Abstract—Three multiple vitamin tablets with different ascorbic acid contents, three vitamin C pills with a synthetic or natural source of ascorbic acid, seven iron-fortified multiple vitamin tablets, and vitamin C pills plus iron supplement were tested for their capacity to induce chromosome aberrations in cultured Chinese hamster ovary cells. All of these commercially available vitamin pills induced chromosome breaks and exchanges. The active dilution range and the extent of chromosome damage induced varied among the pills examined. The observed frequencies of metaphase plates with chromosome aberrations ranged up to 42%.

INTRODUCTION

Ascorbic acid has been shown to exert anticarcinogenic and antimutagenic activity; it prevents nitrosation thus inhibiting the formation of carcinogenic nitroso-compounds (Greenblatt, 1973; Lo & Stich, 1978; Mirvish, 1975; Mirvish, Wallcave, Eagen & Shubik, 1972), reduces conversion to ultimate carcinogens (Floyd, Soong & Culver, 1976), suppresses UV-induced skin carcinogenesis (Black & Chan, 1975; Chan & Black, 1977) and inhibits mutagenesis induced by a variety of compounds (Guttenplan, 1977, 1978; Marquardt, Rufino & Weisburger, 1977; Rosin & Stich, 1979; Shamberger, Corlett, Beaman & Kasten, 1979). Moreover, ascorbate may have other beneficial effects (Cameron, Pauling & Leibovitz, 1979; Pauling, 1970). Relatively large daily intakes ranging from 0·5 to 10 g have been recommended (Cameron *et al.* 1979; Pauling, 1970).

Adverse effects of ascorbate have also been demonstrated. Ascorbate induces chromosome aberrations (Stich, Karim, Koropatnick & Lo, 1976; Stich, Wei & Whiting, 1979), sister chromatid exchanges (Galloway & Painter, 1979) and mutations (Rosin, San & Stich, 1979) in cultured mammalian cells. Moreover, ascorbate enhances methyl cholanthrene induced neoplastic transformation of 10T½ cells (M. P. Rosin, personal communication 1979). The potential health hazard of these undesirable side effects poses a problem that requires careful examination.

Commercially available pills containing vitamin C, multiple vitamins, iron-fortified multiple vitamins or iron supplement, or a combination of vitamin C pills and iron supplement were examined for their capacities to induce chromosome aberrations in Chinese hamster ovary (CHO) cells following short-term exposures. The effects of various dilutions of the tablets on the frequency of cells with at least one chromosome aberration and on the average number of chromosome exchanges per cell were determined.

EXPERIMENTAL

Various commercially available vitamin tablets were purchased over the counter. To each ground tablet was added 20 ml of Eagle's minimum essential medium (MEM, buffered at pH 7·4) supplemented with 2·5% foetal calf serum (FCS). This mixture was suction filtered through a membrane filter (0·8 μm) and neutralized with NaOH to pH 7·4. CHO cells (140,000 cells/dish) were seeded 2 days in advance on 22 mm^2 coverslips in 3·5 cm plastic dishes (Falcon, Cockeysville, MD) and kept in MEM supplemented with 15% FCS. To each dish of CHO cells was added 1 ml of one of the serially diluted vitamin solutions (in MEM supplemented with 2·5% FCS). The cells were incubated with the vitamins for 3 hr at 37°C in a water-saturated, 5% CO_2 incubator. The cells were then rinsed with MEM, and incubated in 2 ml of MEM (supplemented with 15% FCS) for an additional 16 hr. Colchicine (10 μg/ml) was added to the cells, which were then incubated for 4 more hr. Cells were harvested by hypotonic treatment (1% sodium citrate for 15 min), fixed in acetic acid–methanol (3:1, v/v), air dried and stained with 2% orcein. Fifty to two hundred metaphase plates were scored for chromosome- and chromatid-type breaks and exchanges. Only definite chromosome breaks with dislocated distal fragments and single or multiple exchanges were included in the counts of chromosome aberrations.

RESULTS

The results summarized in Table 1 clearly demonstrate the capacity of various vitamin pills to induce chromosome aberrations. The extent of these aberrations is relatively severe. Similar levels are only induced by such potent mutagens and carcinogens as aflatoxin B_1, 1-methyl-3-nitro-1-nitrosoguanidine and 4-nitroquinoline-1-oxide (San, Stich & Stich, 1977; Stich & Laishes, 1975; Stich & San, 1970). To permit a better assessment of the results, the amounts of ascorbate, iron and other ingredients in each pill are given in Table 2.

Data in Table 1 indicate that the toxic and clastogenic effects of the vitamin preparations are correlated primarily with their contents of ascorbic acid

Table 1. *Induction of chromosome aberrations in CHO cells by vitamin C and multiple vitamin pills*

Specimen no.	Dilution factor, i.e. vol. of medium/pill (ml)...	Frequency of metaphase figures having at least one aberration (%) and, in parentheses, average number of exchanges per metaphase plate										
		20	40	70	100	150	200	300	400	500	700	1000
Vitamin C												
1		T	T	T	T	MI	42·1 (0·90)	28·3 (0·90)		12·5 (0·42)		0·9 (0·01)
2		T	T	MI	24·2 (0·70)	3·0 (0·04)	1·8 (0·02)	2·3 (0·03)		0·8 (0·01)		0·5 (0·00)
3		T	T	T	T	T	T	30·8 (0·77)		26·8 (0·98)		14·8 (0·64)
Multiple vitamins												
4		T	T	T	T	MI	36·0 (1·04)		13·1 (0·33)		5·4 (0·09)	1·2 (0·01)
5		T	T	T	MI	MI	23·2 (0·77)		10·1 (0·19)		3·8 (0·04)	1·1 (0·02)
6		T	T	MI	7·4 (0·07)		2·4 (0·03)		0·8 (0·00)		0·8 (0·00)	0·5 (0·00)
Multiple vitamins fortified with iron												
7		T	T	MI	28·8 (0·66)	20·8 (0·61)	4·4 (0·07)		0·8 (0·00)		0·5 (0·00)	0·5 (0·00)
8		MI	25·0 (0·50)	18·3 (0·45)	4·9 (0·18)	2·4 (0·04)	1·8 (0·02)		0·5 (0·00)		0·5 (0·00)	0·0 (0·00)
9		MI	14·3 (0·53)	10·8 (0·46)	15·1 (0·56)	0·8 (0·00)	0·5 (0·00)		0·5 (0·00)		0·8 (0·00)	0·0 (0·00)
10		T	T	MI	30·0 (0·70)	11·8 (0·19)	8·3 (0·25)		3·1 (0·12)		0·9 (0·01)	0·9 (0·00)
11		T	T	T	T	MI	11·9 (0·21)		8·0 (0·24)			4·4 (0·12)
12		T	20·0 (0·30)	8·2 (0·24)	1·9 (0·02)	0·5 (0·00)	0·8 (0·00)		0·5 (0·00)		0·8 (0·00)	0·8 (0·00)
13		MI	13·6 (0·17)	1·5 (0·01)	0·5 (0·00)		0·5 (0·00)		0·0 (0·00)			0·0 (0·00)
Iron supplement												
14		T	MI	7·7 (0·10)	0·5 (0·00)		0·5 (0·00)		0·8 (0·00)		0·5 (0·00)	0·0 (0·00)
Vitamin C pills and iron supplement												
1 (plus 0·3 mg/ml of iron (no. 14))		10·8 (0·31)	7·9 (0·17)	2·8 (0·07)	0·9 (0·01)	1·5 (0·02)	0·8 (0·01)	0·7 (0·01)		0·7 (0·00)		0·00 (0·00)
2 (plus 0·3 mg/ml of iron (no. 14))		MI	11·2 (0·07)	6·5 (0·06)	2·0 (0·03)	2·0 (0·00)	0·5 (0·00)	0·5 (0·00)		0·0 (0·00)		0·0 (0·00)

T = Toxic, no detectable mitosis and variable cell loss MI = Mitotic inhibition, less than one metaphase among 5000 cells

In the control, non-treated CHO cells the frequency of metaphase figures having at least one aberration was 0·5% and there were no exchanges.

Table 2. *Contents of vitamin C and multiple vitamin pills*

Compound	Specimen no....	1	2*	3*	4	5	6	7	8	9	10	11*†	12	13*‡	14
Vitamins															
C (mg)		100	100	100	60	50	40	60	50	40	150	100	75	50	
A (I.U)					5000	5000	5000	5000	5000	500	5000	10000	6000		
B_1 (mg)					1·5	2	3	13	2	3	3	4	3		
B_2 (mg)					1·7	3	2·5	1·5	3	2·5	5	4	5		
B_6 (mg)					2	1	1	2	1	1	1	4	1		
B_{12} (mcg)					6	3	3	6	3	3	5	5	6	10	
D (I.U.)					400	400	400	400	400	400	400	400	400		
E (I.U.)					10	10		10	10		10	10	10		
Niacinamide (mg)					20	20	20	20	20	20	25	20	25		
Minerals															
Iron (mg)§								10	4	4	6	15	10	60	300
Copper (mg)												1	1	0·5	
Manganese (mg)												1		2	
Calcium (mg)												125	160		
Magnesium (mg)												50	50		
Phosphorus (mg)													125		
Iodine (mg)												0·1	0·5		
Others															
Folic acid (mg)					0·1			0·1				0·1	0·1	0·1	
Biotin (mg)												10	50		
Bioflavonoids			25	12·5								35		25	

*Ingredients derived from natural sources.
†Tablets also contain zinc (1 mg), pantothenic acid (5 mg), choline (15 mg), inositol (15 mg), lysine (20 mg), methionine (20 mg), *p*-aminobenzoic acid (15 mg), betaine (10 mg).
‡Tablets also contain rosehip powder (50 mg) and desiccated liver powder (200 mg).
§Iron as ferrous fumarate except specimen 8 (sulphate) and specimen 14 (gluconate).

and iron. Peak frequencies of aberrations produced by the vitamin C pills were high (24–42%) and occurred at concentrations (0·3–1 mg/ml) similar to those observed with pure ascorbate (Stich *et al.* 1976; Stich, Wei & Whiting, 1979). At comparable concentrations supplementation of vitamin C pills with iron (0·3 mg/ml) resulted in reduced toxicity and decreased frequency of chromosome aberrations. This effect of iron was also observed with purified ascorbate and iron (Stich *et al.* 1979). Reduced toxicity was also observed in multiple vitamin tablets fortified with iron (compare specimen no. 4 with 7 and 5 with 8 in Table 1). Vitamin C tablets prepared with 'natural' ingredients produced both higher (no. 3) and lower (no. 2) responses than the 'synthetic' ones (no. 1) at comparable ascorbate concentrations. This discrepancy is probably due to unspecified ingredients.

DISCUSSION

Ascorbic acid can induce a genotoxic effect in a variety of test organisms, including DNA-phages (Morgan, Cone & Elgert, 1976; Murata, Oyadomari, Ohashi & Kitagawa, 1975), RNA-phages (Wong, Morgan & Paranchych, 1974), bacteria (Omura, Shinohara, Maeda, Nonaka & Murakami, 1978), and cultured rodent or human cells (Galloway & Painter, 1979; Stich *et al.* 1976; Stich *et al.* 1979). Considering the wide range of test systems used, there seems to be little doubt that ascorbic acid must be classified as a mutagenic agent. It is therefore not surprising that tablets containing vitamin C are inducers of chromosome aberrations in mammalian cells.

All the tests for mutagenic, chromosome-breaking and DNA-damaging activity were performed *in vitro*. The question of the capability of these tests to predict a mutagenic hazard for man is unresolved. A relatively good correlation has been observed between mutagenicity *in vitro* and carcinogenicity in whole animals (Ames & Hooper, 1978; Poirier & de Serres, 1979; Purchase, Longstaff, Ashby, Styles, Anderson, Lefevre & Westwood, 1976; Stich, San, Lam, Koropatnick & Lo, 1977; Sugimura, Sato, Nagao, Yahagi, Matsushima, Seino, Takeuchi & Kawachi, 1976). However, the metabolic changes of a compound which take place *in vitro* may differ profoundly from those occurring *in vivo*. This difference may be particularly marked in the case of compounds that are an integral part of cellular metabolism. Moreover, it may be argued that organisms must have evolved protection mechanisms against all the chemicals that are essential for their survival. They may lack such protection against compounds to which they have not previously been exposed or against nonphysiological concentrations of otherwise non-toxic chemicals.

The present study demonstrates chromosome-damaging capacity of tablets containing vitamin C in one *in vitro* test system. It does not provide any answers to the question of the possible mutagenic or clastogenic action of ascorbic acid in mammals, including man.

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COMPARATIVE METABOLISM OF PHENOBARBITONE IN THE RAT (CFE) AND MOUSE (CF1)

J. V. CRAYFORD and D. H. HUTSON

Shell Research Limited, Shell Toxicology Laboratory (Tunstall), Sittingbourne Research Centre, Sittingbourne, Kent, ME9 8AG, England

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Abstract—The fate of orally administered [^{14}C]phenobarbitone (40 mg/kg) was studied comparatively in CFE rats and CF1 mice. Metabolism and elimination were rapid. Hydroxylation was more complete in rats than in mice but the major metabolite in both species was *p*-hydroxyphenobarbitone, which was excreted mostly in the urine in both free and conjugated form. Unmetabolized phenobarbitone was excreted in the urine of both rat and mouse. Two minor metabolites appeared to be common to both species. The effect of pretreatment with non-radioactive phenobarbitone on the metabolism of [^{14}C]-phenobarbitone was minimal in the rat but afforded a twofold increase in *p*-hydroxylation in the mouse (*in vivo*). Despite the good yield of *p*-hydroxyphenobarbitone in the intact rat, [^{14}C]phenobarbitone was not metabolized by liver-microsomal preparations from either untreated or phenobarbitone-induced rats.

INTRODUCTION

Phenobarbitone, like DDT, β-BHC, γ-BHC and dieldrin, causes a marked increase in the incidence of hepatic tumours when incorporated into the diet of CF1 mice. Specifically, when male and female mice were fed diet containing 500 ppm phenobarbitone, tumours were noted at wk 68, and at wk 110 the percentage incidence was 83% in males and 78% in females (Thorpe & Walker, 1973). Similar results have been reported by Ponomarkov & Tomatis (1976). None of these five compounds elicit this response in rats.

A comparative study of the effects of dieldrin and phenobarbitone on the subcellular structure and function of mammalian liver cells revealed that both compounds elicited very similar responses. The response in the rat (and dog) was that expected of a classical microsomal mono-oxygenase inducer, i.e. a proliferation of the smooth endoplasmic reticulum of the liver parenchymal cells associated with enhanced activity of the cytochrome *P*-450-dependent oxidizing enzymes (aniline hydroxylase and chlorfenvinphos dealkylase) (Wright, Potter, Wooder, Donninger & Greenland, 1972). Measurement of the DNA content of rat livers indicated that the response was a hypertrophy (due to cell enlargement). The response of the mouse in terms of liver enlargement and induction of mono-oxygenation was similar to that in the rat, but increases in liver DNA indicated that hyperplasia was an important element in the increase in liver size. This difference between rat and mouse may be an important clue to the mechanism of tumour formation in mice.

It is now widely recognized that the rates and/or routes of biotransformation are important in the carcinogenic action of many organic compounds. In view of this, and the opportunity offered by the rat/mouse species difference, it was appropriate to investigate the metabolism of dieldrin and phenobarbitone in rat and mouse. Such studies may provide the identity of reactive metabolites which initiate the response; furthermore, if a species difference in biotransformation can be shown to be linked to the response, metabolism studies offer a route to some information on the likely response in man. Our studies on the metabolism of [^{14}C]dieldrin in rats and mice (Hutson, 1976), however, failed to reveal any distinguishing features that could account for dieldrin-dependent tumour formation in the CF1 mouse and not in the CFE rat.

There is surprisingly little quantitative information on the metabolic fate of phenobarbitone in mammals. The compound is excreted unchanged and as *p*-hydroxyphenobarbitone by the dog (Butler, 1954 & 1956), by man (Algeri & McBay, 1956; Butler, 1956; Curry, 1955a,b) and by the horse (Nicholson, 1968). *p*-Hydroxyphenobarbitone was found to be partly free and partly conjugated (almost totally conjugated with glucuronic acid in the dog and horse but only partially conjugated in human urine). None of these studies utilized radiolabelled phenobarbitone and so *p*-hydroxyphenobarbitone was considered to be the major metabolite because no other metabolites were identified. The first study using [^{14}C]phenobarbitone was reported by Glasson & Benakis (1961). This study in rats revealed that urinary *p*-hydroxyphenobarbitone, free (19%) and as its glucuronide conjugate (27%), was indeed the major metabolite. Phenobarbitone (27%) was excreted unchanged; the remaining 27% of the 0–24-hr urinary metabolites consisted of four unidentified compounds. A study of the biliary elimination of [^{14}C]phenobarbitone (Klaasen, 1971a) showed that 18% of an iv 75-mg/kg dose given to rats was eliminated in the bile as polar metabolites in 6 hr. The non-acidic metabolites of phenobarbitone in the rat and guinea-pig have been analysed by methylation/silylation followed by gas chromatography–mass spectrometry (Harvey, Glazener, Stratton, Nowlin, Hill & Horning, 1972). As well as *p*-hydroxyphenobarbitone, very small amounts of *m*-hydroxyphenobarbitone, of a 3,4-dihydrodiol derivative and of a 3,4-catechol derivative were detected. The *N*-gluco-

side has recently been reported as a urinary metabolite of phenobarbitone in man (Tang, Karlow & Grey, 1979).

We now report comparative studies of the elimination of [^{14}C]phenobarbitone and its major metabolites in normal and phenobarbitone-pretreated CFE rats and CF1 mice.

EXPERIMENTAL

Materials. [^{14}C]Phenobarbitone (5-ethyl-5-phenylbarbituric-2-^{14}C acid) with a specific radioactivity of 3·34 mCi/mmol (14·4 μCi/mg) was purchased from New England Nuclear (Boston, MA, USA). Thin-layer chromatography (TLC) on silica gel in chloroform–acetone (9:1, v/v) (solvent A) revealed two impurities at R_F 0·53 (19%) and R_F 0·93 (1%). These were removed by preparative TLC in the same solvent to afford [^{14}C]phenobarbitone (R_F 0·31) apparently free from radioactive impurities and from impurities that were ultraviolet absorbing. [^{14}C]Phenobarbitone (75·8 μCi; 5·265 mg) was diluted with 109 mg of non-radioactive phenobarbitone (BDH Ltd., Poole, Dorset) in distilled water to give an aqueous solution of [^{14}C]phenobarbitone (10·9 mg/ml; 7·23 μCi/ml; specific radioactivity 0·66 μCi/mg). Rats were each dosed with 1 ml of this solution. Part of the solution (1·4 ml) was made up to 3 ml with distilled water to afford the solution (1·025 mg/0·2 ml; 0·68 μCi/0·2 ml) for dosing mice (0·2 ml per animal). Limpet β-glucuronidase (type L1) and bovine liver β-glucuronidase (type B1) were purchased from Sigma Chemical Co. Ltd. (Poole, Dorset).

Animal experiments. Male CFE rats and male CF1 mice were bred in our laboratory and maintained under specified pathogen-free conditions prior to the experiment. The normal (non-pretreated) groups were fed '86' powdered diet (Scientific Products Farm Ltd., Ash, Kent) when aged 10 wk (±4 days) for 1 wk, and were then treated with [^{14}C]phenobarbitone by stomach tube. Rats received 1·0 ml of solution containing 10·9 mg (7·23 μCi), a dose approximately equivalent to 40 mg/kg body weight. Mice received 0·2 ml of solution containing 1·025 mg (0·68 μCi), a dose again of approximately 40 mg/kg body weight. The animals were maintained on powdered diet and water in metabolism cages (Jencons Ltd., Hemel Hempstead, Herts.) for 3 or 4 days, after which they were killed. Urine and faeces were collected daily. Animals pretreated with non-radioactive phenobarbitone commenced the treatment when aged 8 wk (±4 days), rats and mice receiving diets containing 1000 and 500 ppm, respectively. After 2 wk on these diets, the animals were maintained for 36 hr on normal powdered diet and then treated with [^{14}C]phenobarbitone as described above for the normal groups.

Extra mice were required for the isolation of the major metabolite of phenobarbitone. Six animals were maintained in one cage on diet containing 500 ppm phenobarbitone for 28 days. The excreta (combined urine and faeces) and sawdust bedding were collected twice a week and stored at −15 ± 5°C prior to extraction.

Radiochemical methods. Liquid samples were assayed by scintillation counting using the toluene–methanol–phenylethylamine-based mixture described by Dobbs (1963). Faeces were analysed by combustion to $^{14}CO_2$ which was absorbed into the scintillation mixture. A Packard Tricarb Liquid Scintillation Spectrometer, Model 3003, was used for counting and the efficiency was measured by the Channels ratio method.

Thin-layer and paper chromatography. TLC was carried out on Merck Kieselgel F_{254} plates (Merck AG, Darmstadt, Federal Republic of Germany). The following solvents were used: solvent A, chloroform–acetone (9:1, v/v); solvent B, ethyl acetate; solvent C, ethyl acetate–dichloromethane (1:1, v/v). Radioactivity was located using a Berthold thin-layer chromatogram scanner, Model LB 2723 and/or by autoradiography with Kodirex X-ray film (Kodak Ltd., London). Radioactivity on chromatograms was measured by integrating the profiles derived from the scanner. Paper chromatograms, using Whatman No. 1 paper, were developed (descending) in solvent D (butan-1-ol–ammonia (sp. gr. 0·88)–water, 86:1:13, by vol.) or in solvent E (butanol–acetic acid–water, 11:5:4, by vol.) and were analysed for radioactivity on a Packard Radiochromatogram Scanner, Model 7201.

Analysis of urine and faeces. Urine was radioassayed by scintillation counting and then stored at −15 ± 5°C prior to further analysis. Daily composites for each treatment group were prepared and analysed by TLC (solvent B). Faeces were stored at −15 ± 5°C prior to analysis. Daily composite samples of faeces were prepared for each treatment group and extracted with methanol. The residues were radioassayed *via* combustion. The extracts were radioassayed by scintillation counting and analysed by TLC (solvent B). The relative quantities of unchanged phenobarbitone, of hydroxyphenobarbitone and of conjugates in urine and faecal extracts were assessed by integration of the radioscans of the TLC plates.

Isolation of the major metabolite of phenobarbitone from rat urine. Day 1 and 2 urine samples from [^{14}C]phenobarbitone-treated normal (not pretreated) rats were combined and evaporated to dryness. The syrup was taken up in methanol and filtered. The filtrate was evaporated to dryness and the product was partitioned between water and ethyl acetate. Analysis by TLC (solvent B) indicated that the organic layer contained phenobarbitone (R_F 0·62) and a slightly more polar metabolite (R_F 0·5). The aqueous phase contained mostly very polar metabolites (R_F 0). The organic layer was concentrated and the major radioactive component was isolated by preparative TLC (solvent B) and purified by further TLC in the same solvent. It was finally purified by preparative paper chromatography in solvent D to afford 3·4 mg of metabolite (expressed as equivalent weight of phenobarbitone). Attempts to methylate the metabolite with diazomethane resulted in a mixture of at least three peaks (TLC, dichloromethane), probably *via* sequential methylation of the N-H or OH groups of the barbiturate ring. Therefore, the metabolite was analysed directly by mass spectrometry (AEI-MS 30) and by nmr spectrometry (Varian Associates HA 100).

Isolation of a metabolite of phenobarbitone from mouse excreta. The combined urine, faeces and sawdust bedding from the mice receiving non-labelled phenobarbitone in the diet (see above) were extracted

with warm methanol and evaporated to dryness. The material was rendered aqueous with 500 ml distilled water and was then extracted four times with 500-ml portions of ethyl acetate. The extracts were combined, evaporated to low volume and loaded onto a column of 100–200 mesh silica gel (2·8 cm × 30 cm). The column was eluted with 100-ml portions of toluene (× 1), dichloromethane (× 2), dichloromethane–ethyl acetate (1:1, v/v; × 4), ethyl acetate (× 2) and acetone (× 2). Fractions 6–10 were combined, evaporated and chromatographed (TLC, solvent C). The ultraviolet-absorbing zone migrating with the same R_F value (0·48) as a sample of *p*-hydroxy[^{14}C]phenobarbitone isolated from rat urine was eluted and re-purified in the same system. It was finally purified by high-performance liquid chromatography on a 20 cm × 0·95 cm column of 10 μm Lichrosorb-NH_2 (BDH Ltd., Poole, Dorset) eluted with dichloromethane (18 ml), dichloromethane–methanol (9:1, v/v; 14 ml) and dichloromethane–methanol (1:1, v/v). The eluant was monitored at 260 nm with a Cecil Instruments flow monitor. The metabolite was eluted in the third solvent, from which it was recovered by evaporation and then analysed by mass spectrometry (Finnigan 3200) and nmr spectrometry (Brücker 360 MHz instrument, Shell Biosciences Laboratory).

Microsomal mono-oxygenation of phenobarbitone in vitro. Homogenates of liver were prepared in 0·1 M-sodium phosphate buffer, pH 7·4. Microsomes were resuspended in this buffer. The NADPH-generating system used was glucose-6-phosphate and glucose-6-phosphate dehydrogenase. The materials, incubation conditions and the [^{14}C]chlorfenvinphos positive control reaction were as described by Hutson (1976) for the microsomal mono-oxygenation of dieldrin.

RESULTS

Excretion of [^{14}C]phenobarbitone and its metabolites

Elimination of radioactivity. Radioactivity eliminated in the urine and faeces of normal and phenobarbitone-pretreated rats and mice following single doses (*c.* 40 mg/kg body weight) of [^{14}C]phenobarbitone is shown in Table 1. The urine was the main route of elimination for both species, accounting for about 60% of the dose in normal and pretreated rats and for 70–90% of the dose in mice. Faecal elimination was correspondingly lower in mice than in rats. The effect of phenobarbitone pretreatment was to increase the *rate* of urinary elimination of radioactivity in both species. The rates of faecal elimination were not significantly affected but the amount of faecal radioactivity was altered by pretreatment and, furthermore, the alteration was in the opposite sense for rats and mice. Thus, the substantial faecal elimination by rats (29% over 3 days) was enhanced by pretreatment (to 44%) and the relatively low faecal elimination by mice (11%) was reduced by pretreatment (to 2·5%). The total recoveries shown in Table 1 indicate that most of the [^{14}C]phenobarbitone was eliminated within 3 days of dosing, but it is noteworthy that recoveries from the normal groups were lower than those for the pretreated groups. This again suggests that pretreatment enhanced the rate of elimination of phenobarbitone and/or its metabolites.

Urinary metabolites. Composite daily urine samples for each group were analysed by TLC (solvent B). This revealed three main radioactive zones: unchanged phenobarbitone (I; R_F 0·62), hydroxyphenobarbitone (II; R_F 0·50), a trace of material (< 1%) at R_F 0·3, a streak (< 1%) at R_F 0·05–0·2, end conjugates (III; R_F 0). Metabolite II was assumed to be a hydroxyphenobarbitone by analogy with earlier work (cited in the Introduction) and because on methylation with diazomethane its R_F changed from 0·50 to 0·70. The proportion of free hydroxyphenobarbitone relative to phenobarbitone increased in both species between 24 and 48 hr after dosing. At the period of maximum excretion (0–24 hr) hydroxyphenobarbitone predominated in the normal rat, whereas phenobarbitone predominated in the normal mouse. These data are summarized in Table 2. The effect of phenobarbitone pretreatment on the composition of the rat urinary metabolites was mainly to increase the proportion of conjugates relative to free hydroxyphenobarbitone. The amount of unchanged phenobarbitone

Table 1. *Radioactivity in the urine and faeces of untreated and phenobarbitone-pretreated male rats and mice after a single dose of [^{14}C]phenobarbitone* (c. *40 mg/kg*)

Species/strain	Treatment group	No. in group	Route of excretion	Radioactivity (% of dose ± SEM) excreted on day – 1	2	3	Excretion in 3 days (% of dose)	Total excretion: urine + faeces (% of dose)
Rat/CFE	Normal	4*	Urine	40·4 ± 2·24	20·3 ± 1·44	2·3 ± 0·09	63·0	
		4*	Faeces	15·0	13·7	0·7	29·4	
								92·4
	Pretreated	5	Urine	47·0 ± 2·46	12·0 ± 0·66	1·1 ± 0·13	60·1	
		5	Faeces	28·6	14·9	0·8	44·3	
								104·4
Mouse/CF1	Normal	4*	Urine	53·9 ± 4·81	16·8 ± 2·34	1·6 ± 0·42	72·3	
		4*	Faeces	8·8	1·7	0·8	11·3	
								83·6
	Pretreated	5	Urine	74·7 ± 8·27	16·4 ± 2·24	2·5 ± 1·62	93·6	
		5	Faeces	1·8	0·4	0·3	2·5	
								96·1

*All values for one animal have been omitted because the day 1 yield of radioactivity was apparently abnormally low, whereas values for day 2 and 3 samples were normal with respect to the rest of the group.

Table 2. *Proportions of phenobarbitone, 4-hydroxyphenobarbitone and conjugates eliminated in the urine of male rats and mice*

Species	Treatment group	Period (hr)	Total urinary excretion (% of dose)	Percentage of dose excreted as		
				Phenobarbitone (I)	Hydroxy-phenobarbitone (II)	Conjugates (III)
Rat	Normal	0–24	40	10·6	20·2	9·2
		24–48	20	3·1	10·4	6·5
		0–48	60	13·7	30·6	15·7
	Pretreated	0–24	47	11·3	10·8	24·9
		24–48	12	2·2	3·9	5·9
		0–48	59	13·5	14·7	30·8
Mouse	Normal	0–24	54	24·6	11·0	18·4
		24–48	17	3·3	7·4	6·3
		0–48	71	27·9	18·4	24·7
	Pretreated	0–24	75	13·2	47·0	14·8
		24–48	16	3·0	10·6	2·3
		0–48	91	16·2	57·6	17·1

was very similar in the normal and the pretreated rats. In the mouse, however, pretreatment increased the amount of free hydroxyphenobarbitone about threefold.

The conjugate fractions (III) from normal rats and normal mice (0–48-hr urine) were isolated by TLC (solvent B) and each was separated by paper chromatography (solvent D) into five zones (III/1–5) present in the following proportions (% dose in parentheses):

	III/1	III/2	III/3	III/4	III/5
Rat	<1 (0)	57 (9)	18 (3)	25 (4)	Trace
Mouse	8 (2)	40 (10)	17 (4)	35 (9)	Trace

Metabolite III/2, the major conjugate in both species, was isolated from rat urine and was totally hydrolysed by bovine β-glucuronidase to a compound indistinguishable from the urinary hydroxyphenobarbitone (solvent B). This metabolite has been tentatively identified as the glucuronide conjugate of II. Metabolites III/3 and III/4 were not hydrolysed by this enzyme but were partially hydrolysed by a limpet preparation containing β-glucuronidase and sulphatase. They may, therefore, be sulphate conjugates.

The identification of metabolite II from rat and mouse. Metabolite II was isolated from the urine of normal rats dosed with [^{14}C]phenobarbitone. Its mass spectrum contained a parent ion M^+ at *m/e* 248, indicating that hydroxylation of the phenobarbitone had occurred. Loss of 29 (Et) gave the major fragment at *m/e* 219 and demonstrated that the ethyl group was unmodified. The nmr spectrum of II (in deuteroacetone) revealed CH_3 (triplet at δ 0·9), CH_2 (multiplet at δ 2·3) and 4 aromatic protons (symmetrical quadruplet at δ 6·8–7·2). The latter feature, in particular, confirms the structure of II as *p*-hydroxyphenobarbitone. There was no evidence from the aromatic proton region for the presence of other isomers. The metabolite was also isolated from the excreta of mice treated only with non-radioactive phenobarbitone in their diet, and therefore the isolated metabolite is representative of the product from pretreated animals. Both the mass spectrum and the nmr spectrum (illustrated in Fig. 1) confirmed the structure as *p*-hydroxyphenobarbitone.

Faecal metabolites. Methanol extracts of faeces from the normal rats were analysed by TLC (solvent B). The 0–24-hr sample contained phenobarbitone, hydroxyphenobarbitone and conjugates in the ratio 18:74:8. The 24–48- and 48–72-hr samples contained mainly hydroxyphenobarbitone. Data from the three chromatograms were computed to show that, of the 20% of the dose in the faecal extract, 80% was present as hydroxyphenobarbitone. Soxhlet extracts of the residues followed by TLC showed a similar picture to that of the first extracts but with a higher proportion of conjugates. Overall, approximately 75% of the faecal radioactivity was present as hydroxyphenobarbitone. Mouse faeces were not analysed because they contained relatively little radioactivity.

The metabolism of [^{14}C]phenobarbitone in vitro

[^{14}C]Phenobarbitone was incubated at 37°C in a concentration of $4·3 \times 10^{-5}$ M for a variety of times up to 3 hr with a variety of liver preparations. The mixtures were analysed by TLC (solvent B) either directly or after precipitation of the protein with ethanol and evaporation. Radioscanning revealed no metabolism in the following liver fractions: control male rat liver 10% homogenate (fortified with 1 mg/ml NADP), rabbit liver 10% homogenate and rabbit liver 10,000 g supernatant (prepared from a different rabbit from that used for the preparation of the homogenate). During these experiments it was found that liver fractions for phenobarbitone studies should not be prepared in sucrose. [^{14}C]Phenobarbitone and sucrose appeared to form an adduct which migrated at R_F 0 (solvent B) and could not be broken by ethanol treatment. Liver fractions were therefore prepared in phosphate buffer. Finally liver microsomes were prepared from a male rat which had received 2000 ppm phenobarbitone in its diet for 14 days. These were incubated under standard conditions (cofactors for mono-oxygenation) with [^{14}C]phenobarbitone at concentrations of $5·4 \times 10^{-4}$ M and $2·7 \times 10^{-3}$ M. Glucose-6-phosphate dehydrogenase was omitted from a control reaction (to prevent mono-oxygenation). A positive control reaction containing [^{14}C]-chlorfenvinphos (Donninger, Hutson & Pickering, 1972) was included in the set. Samples were analysed by TLC (solvent B) at 20 and 40 min. No metabolism

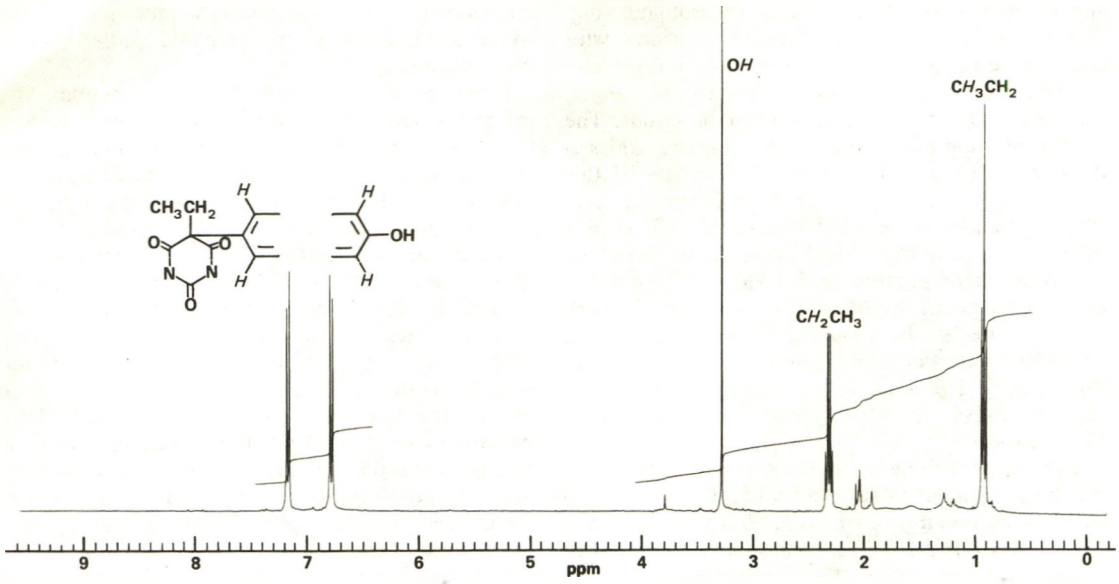

Fig. 1. The 360 MHz proton nmr spectrum of *p*-hydroxyphenobarbitone isolated from mouse excreta.

of phenobarbitone to hydroxyphenobarbitone could be detected either by radioscanning the plates or by autoradiography under conditions which would have revealed 0·1% metabolism. Under the conditions used, chlorfenvinphos was oxidized at a rate of 0·5 nmol/min/mg protein (50% conversion), a typical value for microsomes from a phenobarbitone-induced rat. No further experiments were performed

DISCUSSION

Phenobarbitone (c. 40 mg/kg) is rapidly metabolized in both rats and mice. Hydroxylation is more complete (>85%) in rats than in mice. The major metabolite in both species is *p*-hydroxyphenobarbitone which is excreted mostly in the urine in free and conjugated form. However, appreciable biliary/faecal elimination (*c.* 30%) also occurs in the rat. Unmetabolized phenobarbitone is excreted in the urine of both species: 14% in 0–48-hr rat urine and 28% in 0–48-hr mouse urine. The major urinary conjugate in both species (9–10% of the dose) is probably *p*-hydroxyphenobarbitone glucuronide. No other glucuronides are apparently present, but two of the other metabolites (3 and 4% of the dose in rats and 4 and 9% of the dose in mice) may be sulphate conjugates.

The effects of pretreatment of the animals with phenobarbitone were different in the two species. Pretreatment was stopped 36 hr before dosing with [^{14}C]phenobarbitone in an attempt to minimize perturbation of metabolism caused by the presence of varying amounts of non-labelled phenobarbitone (i.e. it was hoped that any effect on the metabolism of [^{14}C]phenobarbitone would be due only to the induction of microsomal mono-oxygenase). Pretreatment of rats had virtually no effect on the amount of phenobarbitone eliminated unchanged in the urine but it enhanced the *rate* of elimination of *p*-hydroxyphenobarbitone and it enhanced the amount of radioactivity and its rate of elimination in the faeces. This was probably due to a combination of (i) slightly increased hydroxylation and (ii) the increased rate of bile flow known to occur in phenobarbitone-treated rats (Klaasen, 1971b). The effect of pretreatment on the metabolism of [^{14}C]phenobarbitone in the mouse was more obvious, affording an approximately two-fold increase in the amount of *p*-hydroxylation (free and conjugated) and a lower yield of unmetabolized phenobarbitone. The urinary elimination of *p*-hydroxyphenobarbitone and its conjugate compared with biliary/faecal elimination was more efficient in normal mice than in normal rats and the pretreatment of mice increased this efficiency to a point where only 2–3% of the radioactivity was eliminated in the faeces. If account is taken of the yield of *p*-hydroxyphenobarbitone in the faeces of rats, it becomes apparent that the pretreatment of mice induced the hydroxylation of phenobarbitone to an extent similar to that found in the normal and pretreated male rats. The well-known sex-difference in the hepatic microsomal mono-oxygenase activity in the rat may well be relevant. It has been noted previously (Hutson, 1976) that the enzyme activity in male rats tends to be several times higher than that in female rats and in male and female mice. The percentage increase in enzyme activity over the basal levels in the latter three groups is probably higher than that for male rats.

The minor metabolite at R_F 0·3 (solvent B) was not identified; its chromatographic properties suggest that it may be either the 3,4-dihydrodiol or the 3,4-catechol reported by Harvey *et al.* (1972). The discovery of the dihydrodiol was the first experimental indication that a 3,4-arene oxide is involved in the metabolism of phenobarbitone. This intermediate possesses properties that would allow its covalent interaction with DNA, RNA and proteins and it could be an ultimate carcinogen. However, *p*-hydroxylation *via* arene oxide formation, also affording varying amounts of *m*-hydroxylation and dihydrodiol formation, is a very common reaction of drugs, pesticides

and other compounds containing an aromatic ring. The reaction is by no means always associated with carcinogenesis, particularly when the intermediate suffers a rapid rearrangement mainly to one isomer, e.g. the *p*-hydroxy derivative of phenobarbitone. The main protection against the action of arene oxides is the enzyme epoxide hydratase. The activity of this enzyme is higher in rat liver than in mouse liver (Walker, Bentley & Oesch, 1978) and this difference is often cited as one that would make the mouse sensitive to the action of arene oxides. However, the difference is only about twofold and virtually all the work done to date has been on the microsomal enzyme. The recent report of Mumby, Gill & Hammock (1978) that epoxide hydratase activity is higher in mouse- than in rat-liver *cytosol* may lead to a reappraisal of this situation.

The polar conjugates (III/1–5) were chromatographically similar in rats and mice, although differences in the amounts of III/1 and III/4 were observed. Clearly a detailed analysis of these minor differences must be made before their significance can be assessed. It may also be necessary to carry out a comparative study of the covalent binding of [^{14}C]phenobarbitone to components of rat and mouse liver before making a final judgement on the role of biotransformation in the response of mouse liver.

However, the available data presented here indicate that the metabolism of phenobarbitone in rats and mice is similar in its main features; the major difference is that less hydroxylation occurs in the mouse, but after induction with phenobarbitone (the condition pertaining during the dietary intake of the compound), this difference is eliminated.

An attempt to study the *p*-hydroxylation step *in vitro* was abandoned following our failure to observe any hydroxylation by rat (and rabbit) liver fractions containing active microsomal mono-oxygenase. The result was in accord with that of Peters, Shorthouse, Thorne, Ward & Huskisson, (1973) who also failed to achieve metabolism using rat-liver microsomes. This is a striking example of a failure to mimic the *in vivo* situation *in vitro*. The reason for the failure is unknown but obvious explanations are (i) that phenobarbitone hydroxylation occurs *in vivo* in an organ other than the liver, (ii) that the reaction occurs in another subcellular organelle, or (iii) that the reaction is catalysed by a microsomal enzyme, conditions for which were not optimal. Non-metabolism of phenobarbitone *in vitro* is of interest in relation to the negative result for this compound reported in bacterial mutagenicity tests by Ames and coworkers (McCann, Choi, Yamasaki & Ames, 1975). The rat-liver post-mitochondrial supernatant included in this test system probably failed to metabolize the phenobarbitone in the expected way.

The physical characteristics of phenobarbitone with respect to microsomes are in accord with its being a very poor substrate for the microsomal mono-oxygenase. For example, its oil/water partition coefficient is 1·03, its maximum type I spectral change is only 0·003 (*cf.* hexobarbitone 0·045) and the binding constant is 0·16 mM (*cf.* hexobarbitone 0·08 mM) (Jansson, Orrenius, Ernster & Schenckman, 1972). However, it does stimulate NADPH oxidation reasonably well. These characteristics, and its rapid clearance from the body, are consistent with its action as a rather poor mono-oxygenase inducer (when compared with the more biostable inducers).

It may be significant that the five compounds cited in the Introduction as causing liver tumours in mice are all inducers of hepatic microsomal mono-oxygenase. The mechanism of initiation of this response is unknown but the diversity of structure of the inducers (several hundred are known) has led to the hypothesis that induction is mediated through a common endogenous inducer (Marshall & McLean, 1971). Singlet oxygen and (the possibly related) lipid peroxides have been postulated as candidates for a common denominator in the process (Paine, 1978). Inducers may cause a leak of singlet oxygen from cytochrome *P*-450 because they can bind with it and stimulate NADPH oxidation, yet, for chemical reasons, they may be poorly hydroxylated (Ullrich & Diehl, 1971). The evidence relating tumour formation in the mouse with the inducing action of phenobarbitone and the other compounds mentioned is purely circumstantial but the explanation for the sensitivity of this species may lie in the combined effect of hyperplasia and a rise in the concentration of an endogenous inducing agent. An explanation for the mechanism of the mouse tumour formation is important in relation to the validity of the mouse as a test species in carcinogenesis studies.

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LYSIS OF RABBIT POLYMORPHONUCLEAR LEUCOCYTE GRANULES BY SURFACTANTS OF DIFFERING STRUCTURE AND IRRITANCY

W. T. GIBSON

Environmental Safety Division, Unilever Research, Colworth Laboratory, Colworth House, Sharnbrook, Bedford, MK44 1LQ

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Abstract—Pure and commercial surfactants were tested for their ability to cause granule lysis. Lytic ability increased with alkyl chain length in homologous series of alkyl isethionates, sulphates and carboxylates. Using pure surfactants of the same chain length (C_{12}), but different head groups, it was found that lytic ability of anionic surfactants increased with the polarity of the head group, and also that the inclusion of a bulky hydrophilic group such as a triethoxy moiety increased the potency of the parent surfactants. For the nonionic alcohol ethoxylates tested, lytic ability increased with the number of ethoxy units present. The significance of these results and those obtained for the commercial detergents is discussed in the light of their known *in vivo* skin irritancy.

INTRODUCTION

The latency of lysosomal enzymes, due to the exclusion of substrates by the lysosomal membrane, has been recognized ever since their discovery (de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955) and detergents such as triton X-100 are commonly used as lytic agents to allow full expression of lysosomal enzyme activity. The release of lysosomal enzymes *in vivo* is widely believed to be a contributory factor to inflammation (Hirschhorn, 1974) and thus it might be expected that primary cutaneous inflammation caused by surfactants involves lysosomal membrane damage. It has already been suggested (Lazarus, Hatcher & Levine, 1975) that epidermal insult may result in damaged cells from which lysosomal hydrolases would leak out, causing tissue necrosis and chemotaxis of leucocytes. In order to test the importance of lysosomal damage in skin irritation, this study was undertaken to examine the major structural factors influencing lysosomal lysis by surfactants and also to assess whether the *in vitro* potency of a surfactant in such a test is related to its *in vivo* irritancy towards skin.

The large number of surfactants (both pure and commercial samples) to be tested necessitated a large quantity of lysosomes and a rapid means of measuring their rupture. The use of lysosomes from epidermal tissue would have been most relevant, but these are very difficult to isolate in high yield (Dicken & Decker, 1966), probably because of the drastic procedures required to homogenize epidermis (Gray & Yardley, 1975). The granules of rabbit heterophil polymorphonuclear leucocytes (PMNL) were chosen as an alternative because they are simple to isolate in high yield (Cohn & Hirsch, 1960) and form a turbid suspension which provides a rapid and convenient means of following lysis (Lowe & Turner, 1973; Weissman, Becher & Thomas, 1964). The granule population comprises three types of particle, of which two resemble the primary lysosomes of other tissues while the third type contains degradative enzymes but no acid hydrolases (Baggiolini, Hirsch & de Duve, 1970). This was therefore considered to be a very suitable model system with which to assess the effect of different surfactants on lysosomal integrity.

EXPERIMENTAL

Isolation and characterization of rabbit heterophil PMNL. Glycogen-induced peritoneal exudate PMNL were obtained from male New Zealand White rabbits by the method of Cohn & Hirsch (1960). A total white-blood-cell count was made, and the average yield was found to be about 5×10^8 cells per rabbit. Differential counts, after staining with uranin or Leishmann's stain, showed that over 95% of the cells were heterophil PMNL.

Isolation of granule-rich suspension. The peritoneal exudate was centrifuged at 600 g for 10 min at 4°C, and the supernatant discarded. The cell pellet was washed by resuspension in isotonic saline (0·9% w/v NaCl), containing 50 i.u. heparin/ml, and recentrifugation under the same conditions. The washed cell pellet was then resuspended in ice-cold 0·34 M sucrose to give a concentration of 1×10^8 cells/ml. The cells were homogenized by forcing the suspension through a Millipore filter grid ('swinnex' type) 10–15 times and the homogenate was diluted with ice-cold 0·34 M sucrose (1 in 2 or 1 in 3) and centrifuged at 600 g for 10 min at 4°C. The opaque supernatant (granule-rich suspension) was decanted, and the pellet taken up in a small volume of ice-cold 0·34 M sucrose and rehomogenized. This suspension was diluted and centrifuged as described above. The supernatant from this step was combined with that obtained from the first centrifugation, and this pooled fraction constituted the granule-rich suspension used in the lysis test. It was stored at −20°C and only thawed out immediately before use; once thawed, it was not refrozen.

Measurement of granule lysis by turbidimetry. Lysis of granules by surfactants was measured by the method of Lowe & Turner (1973), except that optical density (OD) changes were recorded at 400 nm, not

515 nm. The concentration of the granule-rich suspension was adjusted with 0·34 M sucrose to give an OD_{400} of about 0·5. Thereafter, 0·5 ml of the suspension was incubated in a semi-micro glass cuvette in a Unicam SP 800 (Pye-Unicam, Cambridge) spectrophotometer, and the OD_{400} recorded for about 1 min. The appropriate concentration of surfactant solution (0·01–0·05 ml) was added and mixed, and the decrease in OD_{400} over a period of 20 sec was measured. Each determination was made in duplicate or triplicate. The maximum obtainable decrease in OD_{400} was measured at the start of each experiment, using the detergent triton X-100, to allow comparison of one batch of granules with another. Results are expressed as a percentage of this maximum decrease in OD_{400}, and are the mean ± standard deviation from a number of determinations (usually three).

Preparation of pure surfactants. The pure surfactants used in this study were made available by courtesy of Mr. C. T. James and Mr. T. F. M. Ferguson. Their preparation and structure have been described previously (Ferguson & Prottey, 1976; Prottey & Ferguson, 1976). Mr. C. T. James checked the purity of the alkyl ethoxylates and ethoxy sulphates by gas–liquid and thin-layer chromatography, and by infrared spectroscopy. No evidence of degradation products was found.

Specification of commercial detergents. The commercial detergents used were as follows (the EO number refers to the number of moles of ethylene oxide reacted with each mole of alcohol).

Anionic. A1. Dodecyl benzene sulphonate (commercial), A2. sodium lauryl sulphate (pure C_{12}), A3. dodecyl ethoxy sulphate (commercial; 3 EO), A4. sulphated N2.
Cationic. C1. Cetyl trimethyl ammonium bromide (pure C_{16}), C2. didecyl dimethyl ammonium chloride (95% C_{10}), C3. alkyl dimethyl benzyl ammonium chloride (commercial).
Nonionic. N1. Alcohol ethoxylate (C_{16}, C_{18}, C_{20}; 10 EO). N2. alcohol ethoxylate (C_{12}–C_{15}; 11 EO).

Irritation testing of commercial detergents. The skin irritancy of the commercial detergents used in this study had previously been assessed in this laboratory. Because they were not all tested at the same time, comparisons were made with respect to appropriate standards included in each test (usually A1, A2 or C1). Briefly, irritation tests were performed as follows. In application tests, standards and test samples, at 0·5% or 1% active detergent (AD), were applied to the clipped dorsal skin of Colworth Wistar albino rats (not more than 27 days old) once or twice daily for 4 days. On day 5 the animals were killed. Irritation reactions were assessed and graded macroscopically (erythema, oedema, cracking and scaling) throughout the course of treatment and also microscopically at the end of the test. From these results, overall numerical reaction scores were obtained and examined for significant differences between pairs of treatment groups using non-parametrical statistical tests (the Sign test and the Wilcoxon Matched Pairs Signed Ranks test). Treatment groups could then be ranked in order of their increasing irritant effects.

Intradermal injection tests were designed to compare the full irritation potential of substances independent of their ability to penetrate the stratum corneum. Samples (including appropriate standards) at concentrations of 0·02, 0·05, 0·1 and 0·5% AD were injected at random sites on the clipped dorsal skin of New Zealand White rabbits (9–12 wk old). The size and appearance of reactions were assessed at 24 and 48 hr after injection, and the mean total reaction sizes for each test solution were plotted to establish dose-response relationships. In addition, pairs of reactions to two treatment solutions were compared directly on each animal and the Sign test carried out to determine the significance of the differences.

RESULTS

Effect of various pure surfactants on PMNL granules

A number of surfactants differing in alkyl chain length and in the nature of their head groups were used to test the relationship between the chemical structure of a surfactant and its ability to cause granule lysis. The ranking of surfactants did not change significantly with concentration, as illustrated in Fig. 1 for five C_{12} surfactants, and thus, for comparative purposes, only the results obtained at 0·5 mM final concentration are presented (Figs 2 and 3). In Fig. 2 the effect of alkyl chain length on the extent of granule lysis is illustrated by the results obtained for homologous series of alkyl carboxylates, alkyl isethionates and alkyl sulphates (all sodium salts). Lytic ability was greatest at a chain length of C_{14} in the

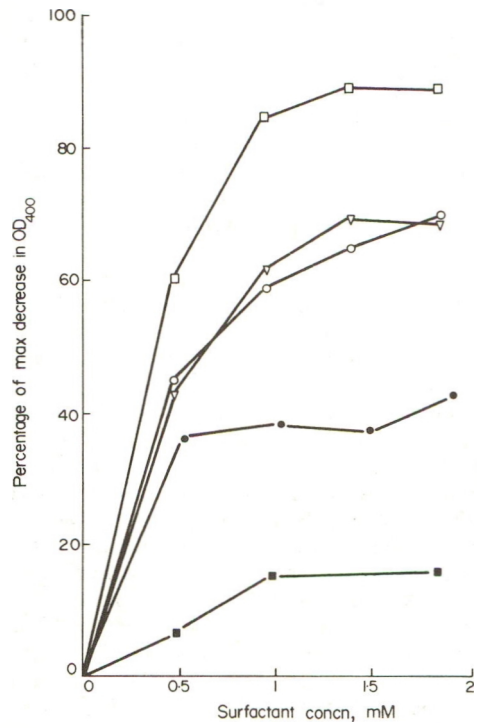

Fig. 1. The effect of concentration on granule lysis by pure C_{12} surfactants. Results are shown for sodium lauryl triethoxy sulphate (□——□), sodium lauryl monoethoxy sulphate (▽——▽), sodium lauryl sulphate (○——○), lauryl hexaethoxylate (●——●) and lauryl triethoxylate (■——■). (The maximum obtainable decrease in OD_{400} was that produced by triton X-100.)

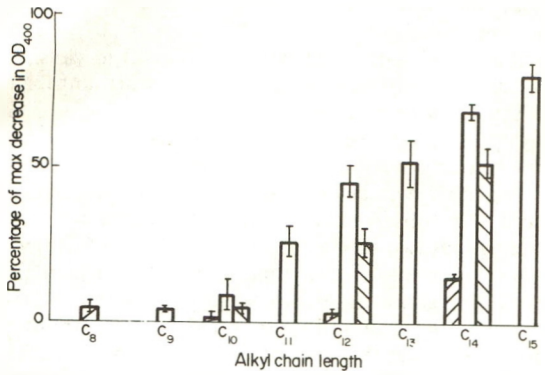

Fig. 2. The effect of alkyl chain length on granule lysis by pure surfactants. Results for homologous series of carboxylates (▨), isethionates (▧), and sulphates (□) are shown. In each case, the final concentration of surfactant was 0·5 mM. (The maximum obtainable decrease in OD_{400} was that produced by triton X-100.)

carboxylate and isethionate series, and at C_{15} in the sulphate series; that is, it increased with alkyl chain length up to that of the longest chain homologue tested in each series. Below a chain length of C_{11} or C_{12}, surfactants showed little or no effect. Alkyl carboxylates were notably ineffective compared with the more polar isethionates and sulphates of the same chain length (see also below).

The effect of altering the nature of the head group while keeping the alkyl chain length constant was investigated with a group of seven surfactants (five anionic, two nonionic) based on a C_{12} chain. The results are shown in Fig. 3 in the form of a histogram, and it is apparent from this diagram that the nature of the head group, given a constant chain length, greatly influenced the extent of granule lysis. Sodium laurate (alkyl carboxylate) was the least effective surfactant in the group, but the potency of this class of surfactant (i.e. anionic) was greatly enhanced if the polarity of the head group was increased by replacing the carboxylate group with sulphate or isethionate groups. The fact that sodium lauryl triethoxy sulphate was more potent than sodium lauryl sulphate showed that the effectiveness of a strongly anionic surfactant could be further increased by the inclusion of a bulky, hydrophilic triethoxy unit in the head group. Lytic ability of the nonionic ethoxylated surfactants tested was also found to depend on the number of ethoxy units in the head group (hexaethoxylate was more potent than triethoxylate).

Effect of commercial detergents of differing irritancy on PMNL granules

Nine commercial detergents were tested, including three irritation test standards (A1, A2, C1), two nonionic detergents (N1, N2), two cationic detergents (C2, C3) and two anionic detergents (A3, A4). The specifications of these detergents are listed in the experimental section. The extent of granule lysis was measured at concentrations of 0·02, 0·04 and 0·08% AD, and the results are shown in Fig. 4. It is clear from these histograms that the order of potency changed with concentration, the differences between detergents becoming less marked as their concentration increased. Certain trends were consistently observed, however, and on the basis of these it is possible to classify the detergents to some extent. Firstly, both nonionic detergents, N1 and N2, produced little or no lysis at any concentration. Secondly, a group of three anionic detergents (A1, A2 and A3) were similar in effectiveness over the range of concentrations, being consistently high in the order of potency. Thirdly, the effect of A4 tended to be greater than those of the other anionic surfactants, but the differences were not all significant at each concentration. Finally the position of the cationic detergents (C1, C2, C3) in the order of potency changed markedly (from low to high) as their concentration increased. For example, C2 and C3 showed no activity at 0·02%, were about half as effective as A1 at 0·04%, but were as effective as the latter at 0·08%.

Relative irritancies of commercial detergents

Data from two different types of test (topical application and intradermal injection) were analysed for significant differences in irritancy relative to known standards and the rankings obtained are as follows. From repeated open application tests on rat skin the relative irritancies were: C3 > C1, A1, A2 > N1, N2. From intradermal injection tests on rabbit skin the relative irritancies were C2, C3 > A1, A2, A3, A4 > N1, N2. For some detergents, data were only available from one type of test, but the overall pattern is fairly clear: two cationic detergents (C2 and C3) were more irritant than the anionic detergents (A1–A4) which were in turn generally more irritant than the nonionic detergents (N1 and N2). The exception to this general pattern of cationic > anionic > nonionic was C1, which was similar in irritancy to A1 and A2 in a topical application test.

DISCUSSION

The major factors influencing the ability of a surfactant to induce granule lysis were alkyl chain length (Fig. 2) and the polarity of the head group (Fig. 3). In

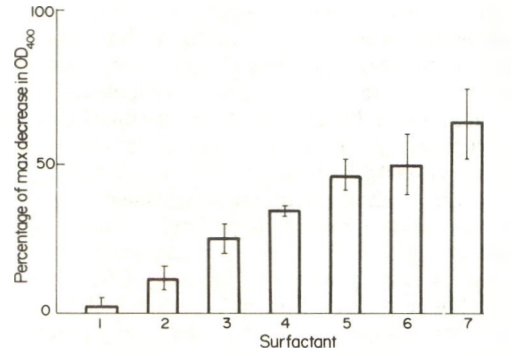

Fig. 3. The effect of head group on granule lysis by pure surfactants. The surfactants studied are coded as follows: 1. sodium laurate; 2. lauryl triethoxylate; 3. sodium lauroyl isethionate; 4. lauryl hexaethoxylate; 5. sodium lauryl sulphate; 6. sodium lauryl monoethoxy sulphate and 7. sodium lauryl triethoxy sulphate. In each case the final concentration of surfactant was 0·5 mM. (The maximum obtainable decrease in OD_{400} was that produced by triton X-100.)

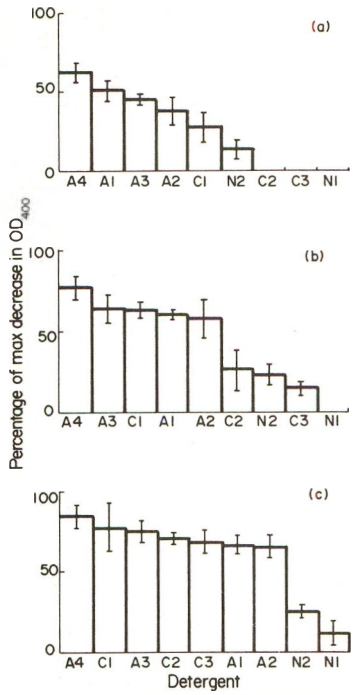

Fig. 4. Granule lysis by commercial detergents at final concentrations of: (a) 0·02%, (b) 0·04% and (c) 0·08% AD. The specifications of the detergents are given in the experimental section. (The maximum obtainable decrease in OD_{400} was that produced by triton X-100.)

the studies with homologous series of surfactants, activity was found to increase with alkyl chain length up to the maximum tested (C_{14} for alkyl carboxylates and isethionates; C_{15} for alkyl sulphates). Increasing the polarity of the head group increased the potency of a surfactant of given chain length, and this could be enhanced still further by the incorporation of a bulky hydrophilic moiety (e.g. triethoxy group) into the head group. The effectiveness of the nonionic surfactants tested, although generally less than anionic surfactants, also seemed to depend on the size of the hydrophilic head group, with lauryl hexaethoxylate being noticeably more potent than lauryl triethoxylate. In many respects these observations are similar to those made by Prottey & Ferguson (1976) who investigated surfactant-induced histamine release from mast cells. However, in their experiments, for a given chain length, nonionic ethoxylated surfactants were of similar potency to strongly anionic surfactants and a slightly lower optimum chain length of around C_{12}–C_{14} was found. These differences are probably related to differences between the composition of cellular and subcellular membranes that would affect the binding and penetration of surfactants into the lipid bilayer.

Several laboratory tests on homologous series of anionic surfactants have revealed a maximum effectiveness at a chain length of around C_{12}, and this correlates well with the relative irritancies of these compounds (reviewed by Prottey, 1978). Some positive correlations between *in vitro* and *in vivo* potency have also emerged from the present study, especially from the survey of commercial detergents. Both nonionic detergents (N1 and N2) were relatively less irritant than sodium lauryl sulphate (A2) in causing granule lysis and were also relatively less irritant than this standard towards rat skin. Also, there was a group of anionic detergents (A1, A2 and A3) which had similar lytic abilities and which also have similar irritancies. Nevertheless, some important non-correlations were observed; of particular concern was the finding that two cationic detergents (C2 and C3) were similar to or less effective than sodium lauryl sulphate in causing granule lysis, whereas they were more irritant than this standard when tested on skin. Another discrepancy was that sodium laurate was lowest in the *in vitro* order of potency for pure C_{12} surfactants, whereas it was capable of causing a marked response, second only to that of sodium lauryl sulphate, when applied to rat skin (Prottey & Ferguson, 1975). The effect of sodium lauroyl isethionate on granule integrity was more marked, in absolute terms, than might have been predicted for this very mild surfactant from *in vivo* skin tests (Prottey & Ferguson, 1975), although its position relative to sodium lauryl sulphate and lauryl triethoxylate (Fig. 3) agrees with the *in vivo* order of potency for these surfactants (Prottey & Ferguson, 1975).

Although it is clear from this study that lysosomes are susceptible to damage by surfactants *in vitro*, the results do not permit any positive conclusions about their importance in the mechanisms of irritancy. The absence of a total correlation between the *in vitro* and *in vivo* orders of potency implies that lysosomal rupture is not always of primary importance in skin irritation, and that other qualifying factors must be considered. Damage to lysosomal membranes *in vivo* can only occur if the intracellular concentration of free surfactant is high enough, and this will depend on the rate of penetration of the surfactant through the stratum corneum and its ability to lyse or penetrate the plasma membrane of living epidermal cells. It is interesting in this context that nonionic surfactants generally penetrate the stratum corneum much more rapidly than anionic surfactants of the same chain length (Black & Howes, 1979) and are equally. or in some cases more effective in causing plasma membrane lysis (Ferguson & Prottey, 1976), yet are generally only mild irritants in comparision with strongly anionic surfactants. This may reflect their weaker effect on lysosomal membranes, as shown in this study, while the poor penetration of strongly anionic surfactants may be outweighed by their ability to cause both cellular and subcellular membrane damage at low concentrations. The high irritancy of cationic detergents is more difficult to account for since their penetration through intact skin is likely to be very slow (D. Howes, personal communication 1978) and they are less active against lysosomal membranes than anionic detergents, at least at low concentrations. However, they do bind strongly to the stratum corneum, and in repeated application tests this may lead to impaired barrier function and increased penetration (D. Howes, personal communication 1978) followed by an irritant response. Further studies are required to clarify the relative importance of lysosomal damage in skin irritation by different detergents.

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EFFECTS OF HOUSING CONDITIONS ON FOOD INTAKE, BODY WEIGHT AND SPONTANEOUS LESIONS IN MICE. A REVIEW OF THE LITERATURE AND RESULTS OF AN 18-MONTH STUDY

M. Chvédoff, M. R. Clarke, E. Irisarri, J. M. Faccini and A. M. Monro

Centre de Recherche, Laboratoires Pfizer, 37400 Amboise, France

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Abstract—Mice were housed in groups of one, two, four or eight/cage for 18 months. The more densely housed groups showed markedly reduced food consumption, slightly decreased mean body weights, and a smaller variance of body weights. Tumour incidence was not affected, but gastritis was more prevalent in the more densely housed groups.

INTRODUCTION

The laboratory rodent which is used as a test system to evaluate the toxicity of chemicals is unreliable for a variety of reasons. Although the major deficiency is the fundamental biological problem of interspecies differences in response to a toxic stimulus, progress is also impeded by results that are frequently irreproducible between laboratories, or even within a laboratory. This latter problem can be addressed, in part, by controlling the environment of the animals (Golberg, 1974; Magee, 1970; Roe, 1965). This is particularly important in chronic toxicity studies, where compound-related changes must be distinguished from the background of changes that occur 'spontaneously' in ageing animals.

The 18-month study reported here was conducted to evaluate the effects of the numbers of mice per cage on various physiological parameters and on spontaneously occurring histological lesions and tumours.* It is already well known that certain toxicity parameters, including the incidence of tumours, can be affected by the number of mice/cage (Wiberg, Airth & Grice, 1966; Wiberg & Grice, 1965).

EXPERIMENTAL

Weanling mice from Charles River, France (Crl, CD-1, 1CR, BR) were distributed by stratified randomization of weight into four groups of 40/sex/group. Within groups the sexes were housed separately but in the same room at densities of one, two, four or eight/cage respectively. All cages were the same size (27 × 21 × 14 cm) and were constructed of makrolon. They had solid bottoms and sides and stainless-steel grid tops and contained a sterilized sawdust bedding which was changed weekly. The atmosphere was maintained at 22 ± 1°C and at a relative humidity of 60 ± 10%. A 12-hr lighting cycle operated. Animals had free access to water and to a standard laboratory diet (UAR No. AO4, Usine d'Alimentation Rationnelle, Villemoisson-sur-Orge). The animals were observed daily. Individual body weights were measured weekly for the first 2 months, and thereafter monthly. Food and water consumption was measured monthly in five cages/group.

Blood samples were withdrawn from the orbital sinus under light ether anaesthesia at the end of the study. Determinations were made by standard methods of Hb, RBC, WBC and differentials, and of plasma concentrations of Na, K, glucose, urea, proteins, glutamate–oxalacetate transferase and alkaline phosphatase. Animals were killed (after 18 months) by asphyxiation by CO_2 and given a full autopsy. The weights of the liver, kidneys and testes were recorded and histopathology was carried out on about 25 tissues. Moribund animals and animals found dead were autopsied, their organs were weighed as above, and they were subjected to histopathological examination as far as autolysis allowed.

Statistical analysis was complicated† by the hierarchical design of the study—group within study, cage within group and animal within cage. In addition, mortality modified the numbers of mice/cage in a random manner the potential effect being most serious when a mouse of the two/cage group died, leaving a single survivor in the cage. However, such deaths generally occurred late in the study and the surviving animals were treated statistically as still belonging to their original group.

Analysis of body weights used a hierarchical classification analysis of variance on the two, four and eight/cage groups (group within study, cage within group, animal within cage). Comparisons with the one/cage group were carried out separately because of the clearly greater variance within this group. In the analysis of food and water consumption it was assumed that the between cages variance component was negligible compared with that between individuals. This enabled the between individuals variance

*Some of the results were previously presented as a poster at the 21st Congress of the European Society of Toxicology, Dresden, June 1979.

†Full details of the analyses are not presented in this paper but may be obtained from one of the authors (M.R.C.).

component to be estimated from the between cages mean squares in the analysis of variance. Certain clinical chemistry results (urea, alkaline phosphatase and GOT) were logarithmically transformed prior to analysis of variance and Student's *t*-test. Organ weights were analysed by the Mann–Whitney U-test, using mean weights of right and left kidneys and testes. Histopathological lesions and tumours were analysed first by an overall chi-square test, and where significant ($P = 0{\cdot}05$) inter-group comparisons were carried out using Fisher's exact test (two-tailed). For each test the sample size was the number of animals examined microscopically, less the number of samples of the particular tissue not available e.g. due to autolysis.

RESULTS

There were no obvious differences between groups in the clinical signs observed. The more densely crowded animals showed a higher level of physical activity, and some minor fighting incidents amongst them led to inconsequential scratches and wounds. The percentages of animals surviving after 18 months were for males and females respectively as follows: one/cage (80, 73); two/cage (75, 83); four/cage (75, 75); eight/cage (80, 80). The percentage survival rates were apparently unrelated to the caging conditions.

Differences in weight gain between the groups were evident after about 2 months and increased gradually during the study (Fig. 1). In both sexes the weights were inversely related to the numbers of animals/cage. In males the mean weight of the one/cage group was significantly different from that of the eight/cage group at 6 months, from the four and eight/cage groups at 12 months and from the two, four and eight/cage groups at 18 months. In females, the only significant difference was between the one/cage group and the eight/cage group at 12 months, although intergroup differences may have been masked by the greater variance in females than in males. Variance was inversely related to the number animals/cage in both sexes, with the singly housed group being markedly different from the other groups, and the two/cage group tending to show more variance than the four and eight/cage groups. This effect was most evident after 12 months (see Fig. 2)—random mortality thereafter tending to obscure the differences.

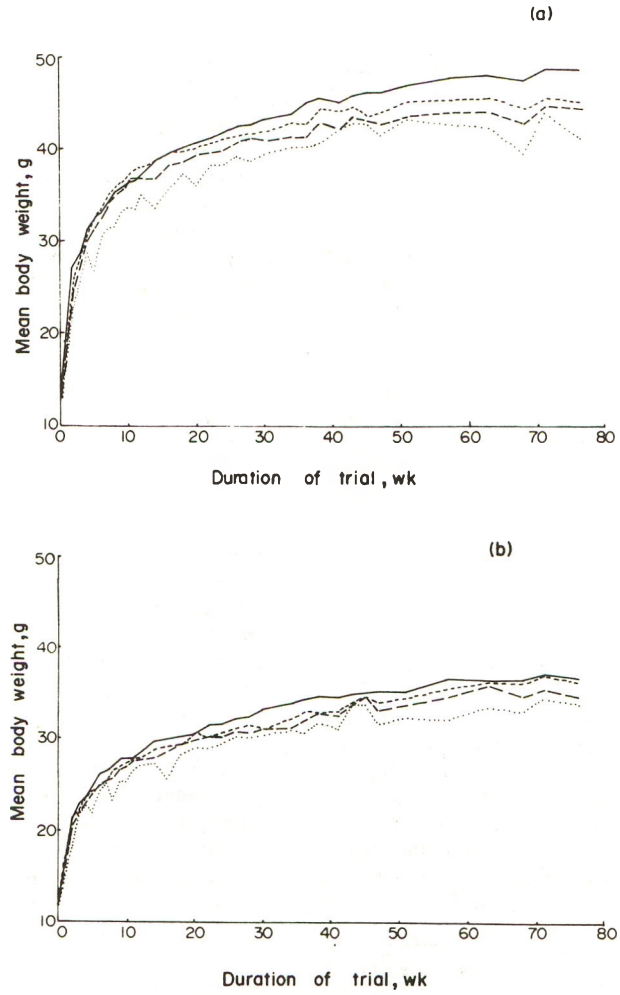

Fig. 1. Mean body weights of (a) males and (b) females. Animals were caged in groups of one/cage (——), two/cage (– – – –), four/cage (— —) or eight/cage (· · · · ·).

Food and water consumption (Tables 1 and 2) were generally less in the groups of higher cage density. The greatest intergroup difference (between 'consecutive' groups) in food consumption was between the one/cage and two/cage groups, while the overall difference between the one/cage and eight/cage groups was approximately two-fold. There were no noteworthy intergroup differences in clinical chemistry or haematological parameters.

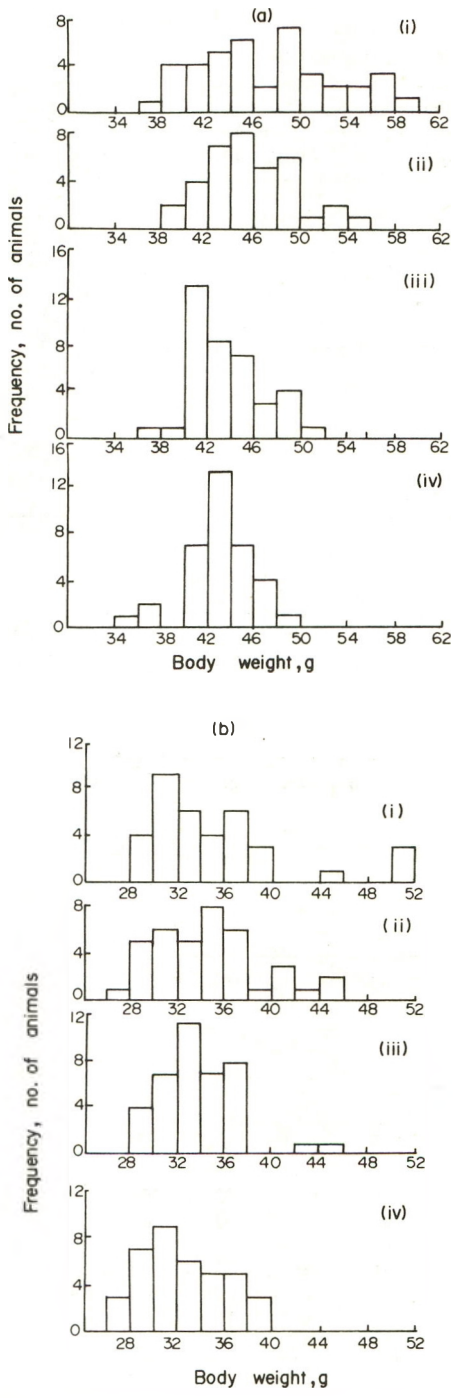

Fig. 2. Distribution of body weights at month 12 in (a) males and (b) females for groups of (i) one/cage (ii) two/cage (iii) four/cage and (iv) eight/cage.

Autopsy after 18 months (mice were killed during weeks 78 and 79) revealed only those lesions commonly observed in mice of this strain and age. In males the mean liver weights, relative to body weights, increased with the number of animals per cage, this effect apparently deriving from the relatively low body weights rather than the increased liver weights. Absolute mean testis and kidney weights were also less in the more densely caged groups (Table 3).

The only histologically detectable lesions that were apparently related to the caging are shown in Table 4. The clearest relationship was the incidence in both sexes of chronic diffuse gastritis, in which the incidence was at least four-fold higher in the eight/cage group than in the one/cage group. This lesion was characterized by proliferative inflammation of the mucosa and sub-mucosa, thickening of the mucous membrane with glandular hyperplasia and dilatation, submucosal oedema and foci of chronic inflammatory cells. By contrast, the incidence of ovarian cysts and of endometrial cystic hyperplasia was lower in the eight/cage than in the other groups. The decreased incidence of extramedullary haematopoiesis with crowding apparent in the females was not reflected in the males. Hyperplasia of the adrenal capsule showed a parabolic incidence, being less in the one/cage and eight/cage groups than in the other two.

There were no statistically significant differences in tumour incidence, either overall or in specific tissues, between the various groups. However, the incidence of tumour-bearing animals, of pulmonary tumours in males and of uterine tumours was lowest in the eight/cage group.

DISCUSSION

There were three striking effects which correlated with increased cage density: decreased variance of body weights, decreased food consumption relative to body weight and increased incidence of gastritis.

The smaller variance in body weights amongst the multiply-housed animals is probably a result of all the animals within a cage being constrained towards a similar level of physical activity (and hence of caloric utilization). By contrast singly-caged animals may range from highly active to almost inactive animals. A lesser mean body weight amongst aggregated mice has been reported previously (De Feudis, 1974).

It is interesting that although food consumption declined with increased cage density, especially in females (that for the eight/cage group being about half that for the one/cage group), this was hardly reflected in body weights (differences 7–15%). A similar phenomenon was previously reported (Albert, Medras & Gorska, 1962) in a multiple-housing experiment. *A priori*, the most reasonable explanation may relate to loss of body heat: several animals in one cage tend to sleep huddled together and by so doing reduce overall heat loss. In contradiction to this was the clinical observation that the more densely housed animals exercised more, although the critical period of darkness was not observed. There was no evidence from plasma biochemistry results to suggest any significant differences in intermediary metabolism. Nevertheless, the slightly increased relative liver weights in the

Table 1. *Food consumption*

Duration of experiment (months)	No. of mice/cage	Food consumption (g/mouse/day)							
		Males				Females			
		1	2	4	8	1	2	4	8
6		7·6	6·2	4·9	4·2	6·8	5·9	4·6	3·6
12		7·0	6·3	5·3	5·0	7·8	5·7	5·0	4·9
18		7·2	4·8	4·1	4·2	7·6	5·3	4·1	3·2

Table 2. *Water consumption*

Duration of experiment (months)	No. of mice/cage	Water consumption (g/mouse/day)							
		Males				Females			
		1	2	4	8	1	2	4	8
6		7·4	8·3	4·9	4·7	8·5	7·1	5·1	4·1
12		6·7	10·3	6·4	6·5	10·1	5·6	5·1	5·8
18		7·6	9·5	4·8	5·7	8·8	6·0	5·5	4·1

Table 3. *Organ weights*

Parameter	No. of mice/cage	Males				Females			
		1	2	4	8	1	2	4	8
Kidney weight (g)		0·43	0·44	0·41	0·40*	0·27	0·25*	0·25*	0·23*
Testis weight (g)		0·150	0·145	0·140*	0·138**	/	/	/	/
Liver weight (g)		2·03	2·12	2·08	2·13	1·70	1·56	1·70	1·64
Relative liver weight (% body weight)		4·33	4·66*	4·92**	5·05***	4·78	4·46*	5·04	5·06

Values are medians; those marked with asterisks differ significantly from the one/cage group (*$P < 0·05$; **$P < 0·01$; ***$P < 0·001$).

Table 4. *Histopathology*

Type of lesion	Sex	No. of mice/cage	Percentage incidence of lesion				P*
			1	2	4	8	
Extramedullary haematopoiesis	M		2	10	3	0	NS
	F		23	7	7	3	0·05
Chronic diffuse gastritis	M		2	8	20	29	0·01
	F		11	13	15	46	0·001
Ovarian cysts	F		53	64	72	28	0·001
Endometrial cystic hyperplasia	F		72	70	59	45	NS
Adrenal capsular hyperplasia	M		7	8	18	0	NS
	F		10	50	32	28	0·01
Pulmonary adenoma/carcinoma	M		20	29	34	12	NS
	F		8	12	15	8	NS
Uterine tumours	F		5	8	8	0	NS
All tumours	M		45	45	40	28	NS
	F		40	40	45	23	NS

NS = Not significant (at the $P > 0·05$ level)

*These values are derived from the overall chi-square test.

more densely caged groups may be indicative of modified metabolic activity related to the housing conditions. A similar suggestion about hepatic metabolism of rats in relation to density of caging has been made by other workers (Thiebot, Soubrie, Chermat, Simon & Boissier, 1977).

The increase in gastritis associated with crowding is presumably a reflection of the well-known susceptibility of the stomach to stress. The induction of stress in laboratory animals using a variety of methods (restraint, cold, noise, etc.) is well-known to result in gastric inflammation and ulceration (Selye, 1936; Brodie & Valitski, 1963). Such a raised incidence of gastritis would offer several disadvantages to the conduct of a toxicity study: a high background incidence in control animals would make chemically-induced effects harder to detect and assess, and it might also modify the gastro-intestinal absorption of orally-administered compounds and so distort the linear relationship with dose of the bioavailability of a test compound in a multiple-dose experiment.

A possible effect of the caging on tumour incidence was only apparent in the lower proportion of tumour-bearing animals and of pulmonary and uterine tumours in the eight/cage groups than in the other groups. The effect may, of course, be real despite the fact that it did not achieve statistical significance, and that comparison with our in-house data accumulated during the past 5 yr indicated that in no group was the tumour incidence significantly different from the expected value based on our one mouse/cage data.

An effect on tumour incidence would not have been unexpected, since it has previously been reported that the incidence of spontaneous or induced tumours in mice depends on the numbers of mice/cage, although the pattern differs according to the site of the tumour (Albert *et al.* 1962; Andervont, 1944; Chouroulinkov, Guillon & Guérin, 1969; Fare, 1965; Muhlbock, 1951; Peraino, Fry & Staffeldt, 1973; Riley, 1975). Furthermore, it is also known that reduced weight gain or food consumption in rodents leads to a reduced incidence of tumours (Roe & Tucker, 1974; Rusch, 1944; Tannenbaum & Silverstone, 1953; Tucker, 1979; Visscher, Ball, Barnes & Sivertsen, 1942; Waxler, Tabar & Melcher, 1953). In this experiment we carried out an animal-by-animal analysis of tumour incidence versus body weight and found no suggestion of a correlation in any of the groups. It was interesting to note that the eight/cage group, in addition to containing no uterine tumours, also contained the lowest incidence of uterine and ovarian cysts.

The pattern of adrenal capsular hyperplasia among the groups may indicate that certain parameters e.g. pituitary stimulation, were at a maximum at the intermediate densities of two or four/cage. Literature reports (Dechambre & Gosse, 1973; Hatch, Wiberg, Zawidzka, Cann, Airth & Grice, 1965; Thiebot *et al.* 1977) indicate that adrenal activity in mice or rats is related to the housing conditions of the experiment, although again it is clear that isolated animals do not necessarily possess a higher adrenal activity than grouped mice—the particular conditions of the experiment are as critical. Interestingly, in one study the adrenal weights of mice housed at different cage densities were found to follow a similar parabolic pattern (Christian, 1955). As discussed by Riley (1975) it seems that both 'isolation stress' and 'crowding stress' can be important.

It is not clear what role stress played in the present experiment. Increased stress in the more densely housed animals would be consistent with the competition for food and water, their increased physical activity, their decreased growth (Doyle, Kelley & Siegel, 1977; Harrison, 1958) and the higher incidence of gastro-intestinal lesions. On the other hand, the more efficient caloric utilization (food consumption/weight gain) of this group suggests that they may have been less stressed. The lower incidence of tumours in the eight/cage group would be consistent with less stress (Riley, 1975).

It is tempting to speculate on a possible role for prolactin in explaining the effects on the adrenals, uterus and ovary. It is a stress-sensitive hormone, originating in the pituitary, well known for its effects on the organs in question (Horrobin, 1976). Furthermore it has a bell-shaped dose-response curve which would be compatible with the findings of this study.

Opinion is divided about which caging conditions for rodents lead to stress. There is probably not a simple answer as the number of animals/cage must also be related to the density (i.e. cage size) of housing. It is interesting to note that one group of workers (Riley & Spackman, 1977), using cage densities from 1 to 20 animals/cage, failed to repeat earlier results in mice in which tumour incidence was found to be related to cage density. They concluded that cage crowding *per se* is not stressful provided that the overall housing environment is not stressful.

We conclude that under our particular laboratory conditions there are two major and contradictory considerations to weigh when deciding whether to house mice singly or in groups. Firstly, a point in favour of groups would seem to be the likelihood of obtaining a greater homogeneity of animal weights (and presumably of other physiological parameters). However, although experimental groups should be as nearly identical as possible, they should perhaps not be homogeneous, a heterogeneous group being more likely to reflect the diversity of human response to toxic stimuli. Secondly, the crowding of the present experiment led to a clear increase in gastritis. This, in our view, represents a strong argument against the multiple housing conditions of this experiment. We conclude that we should continue to house mice one/cage in chronic toxicity studies in our laboratory.

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SHORT PAPER

MUTAGENICITY OF *p*-NITROSOPHENOL

P. GILBERT and J. RONDELET
Laboratory of Analytical Chemistry

and

F. PONCELET and M. MERCIER
Laboratory of Biotoxicology, University of Louvain, School of Pharmacy, UCL-73.69, B-1200 Brussels, Belgium

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Abstract—*p*-Nitrosophenol was not mutagenic towards *Salmonella typhimurium* strains TA1530 and TA1535, which are sensitive to *N*-nitroso compounds, but had a direct mutagenic effect on strain TA1538. This was accompanied by a significant cytotoxic effect which again was not seen in strains TA1530 and TA1535.

Introduction

N-Nitrosamines are compounds of considerable interest because they include some of the most potent carcinogenic agents known (Druckrey, Preussmann, Ivankovic, Schmähl, Afkham, Blum, Mennel, Müller, Petropoulos & Schneider, 1967; Magee & Barnes, 1967). Some have also been shown to be mutagenic in several test systems (Montesano & Bartsch, 1976) but only after they have been metabolically activated. The presence of these compounds in certain foods (Lijinsky & Epstein, 1970) and their formation in the stomach following ingestion of nitrite with various amino compounds (Wogan & Tannenbaum, 1975) may be potential health hazards. However, at present, there are no conclusive data demonstrating that human cancers could be associated with the presence of low concentrations of some of the *N*-nitrosamines that occur in our environment or that are formed *in vivo*.

Phenolic materials, which are present in large quantities in the contents of the human stomach (Long, 1961) and are major dietary constituents (Challis, 1973; Challis & Bartlett, 1975; Knowles, Gilbert & McWeeny, 1961) react more readily with nitrite than do most amino compounds and should therefore act as competitive inhibitors of *N*-nitrosamine formation (Bogovski, Castegnaro, Pignatelli & Walker, 1972; Challis, 1973). However several recent studies have shown that the C-nitroso derivatives of some phenols may act as catalysts for *N*-nitrosamine formation from nitrite and secondary amines at gastric pH (Davies & McWeeny, 1977; Pignatelli, Castegnaro & Walker, 1976; Walker, Pignatelli & Castegnaro, 1979). Therefore, phenolic materials present in the stomach contents or in foodstuffs may increase human exposure to *N*-nitrosamines by catalysing the formation of these carcinogenic compounds in the stomach.

This paper reports on the mutagenic activity of *p*-nitrosophenol to which man may frequently be exposed and which has been shown to exert a catalytic effect on the formation of *N*-nitrosodiethylamine (Walker *et al.* 1979) and *N*-nitrosopyrrolidine (Davies & McWeeny, 1977).

Experimental

Materials. *p*-Nitrosophenol was obtained from Aldrich Europe (Beerse). It was purified by chromatography on a column (3 × 20 cm) of Kieselgel 60 (Merck AG, Darmstadt, Federal Republic of Germany) 70–230 mesh ASTM, eluted with benzene–acetone, 95:5 (v/v). The purity of *p*-nitrosophenol was controlled by thin-layer chromatography on plates (20 × 20 × 0·025 cm) of Polygram sil. G/UV254 (Macherey-Nagel, Düren, Federal Republic of Germany), using benzene–acetone–methanol, 45:45:10 (by vol.); R_F value 0·56. The developer was that described for nitroso compounds after photoreaction (Feigl, 1960). The purified *p*-nitrosophenol was stored in the dark at −18°C under gaseous nitrogen to avoid oxidation. Aqueous solutions and dilutions were prepared as required.

Mutagenicity assays. *Salmonella typhimurium* strains TA1530, TA1535 and TA1538 were kindly provided by Professor B. N. Ames, Berkeley, CA, USA. Plate tests were performed by the classical procedure (Ames, McCann & Yamasaki, 1975) substrate dilutions (0·1 ml/plate) being mixed with bacteria (0·4 × 2 × 10^7/plate from an overnight culture in nutrient (Difco) broth) in histidine–biotin (0·05 mM)-supplemented top agar (2 ml/plate), layered on minimal glucose agar (Vogel Bonner E medium) in Petri dishes. The plates were incubated for 48 hr at 37°C in the dark and the numbers of *his*$^+$ revertant colonies were calculated. The toxicity of the substrate was evaluated by determining the bacterial survival with a lower

bacterial inoculum (10^4- and 10^3-fold dilutions) on plates of nutrient agar (Difco).

Tests were validated according to the recommendations of de Serres & Shelby (1979). The histidine prototrophy of the colonies was checked by a further incubation (72 hr), to permit enlargement of the revertant colonies, and was confirmed by thinning out these colonies on plated agar without histidine, on which their growth was observed.

Results

No mutagenic effect was detected after incubation of *p*-nitrosophenol with the *S. typhimurium* strains TA1530 and TA1535, which have been shown to be sensitive to *N*-nitroso compounds (Table 1). Moreover, no cytotoxic effect was observed in the dose range used.

With strain TA1538, a slight but dose-related increase in the reversion rate was obtained in the same range of concentrations (Table 1). In this case *p*-nitrosophenol had a markedly cytotoxic effect, and the decrease in bacterial survival was dose related.

When assayed under the same conditions, phenol showed neither mutagenic activity nor cytotoxicity in any of the tested strains of Salmonella (Table 1).

Discussion

In a recent report devoted to the evaluation of results of bacterial mutagenicity tests, de Serres & Shelby (1979) consider that an experimental result may be taken as positive when there is a reproducible increase in the number of *his*$^+$ revertants and this increase is related to the dose of substrate. Such an increase was observed with *S. typhimurium* strain TA1538 for doses of *p*-nitrosophenol ranging from 0 to 50 μg/plate. Although reproducible, the enhancement remained very low, but the concomitant cytotoxic effect on TA1538 made the assay of higher doses of substrate impossible; the mutagenic effects would then have been obscured by the lethal action on the bacteria.

Since *p*-nitrosophenol was cytotoxic only to TA1538 and not to TA1530 or TA1535 and since phenol had no effect on either TA1535 or TA1538, one can speculate on the existence of a relationship between the mutagenic activity of *p*-nitrosophenol towards TA1538 and a consecutive cytotoxic effect. If that is the case, the true mutagenic activity of *p*-nitrosophenol is likely to be much more pronounced than it appears.

Furthermore, since the mutagenic activity was only detected with TA1538, the strain sensitive to frameshift mutagens, and not with strains TA1530 and TA1535, which are commonly reverted by *N*-nitroso compounds, it seems realistic to consider that *p*-nitrosophenol may exert its potential genotoxic effect by mechanisms different from those described for *N*-nitroso compounds.

Thus, besides its catalytic effect on the *N*-nitrosation of various amines, *p*-nitrosophenol seems to be weakly mutagenic itself towards an *S. typhimurium* strain sensitive to frameshift mutagens. Because of the high frequency of human exposure to phenols and of the presence of these compounds together with nitrite, amines and/or nitrosamines, in the organism, more attention should be drawn not only to the possible influence of phenols on the formation of *N*-nitrosamines but also to their ability to form mutagenic *C*-nitrosophenols.

Table 1. *Effects of* p-*nitrosophenol and phenol on* Salmonella typhimurium

Concn of test compound (μg/plate)	Mutagenic and cytotoxic effects* on *S. typhimurium* strain					
	TA1530		TA1535		TA1538	
	His$^+$ revertants (no./plate)	Survival (10^6/plate)	*His*$^+$ revertants (no./plate)	Survival (10^6/plate)	*His*$^+$ revertants (no./plate)	Survival (10^6/plate)
			***p*-Nitrosophenol**			
0	33	17·9	9·7 ± 1·4	6·86 ± 1·28	16·0 ± 2·0	3·1 ± 0·3
10	37	15·4	11·5 ± 1·2	7·58 ± 1·65	21·4 ± 1·7	2·4 ± 0·6
20	—	—	6·9 ± 1·0	10·75 ± 1·69	20·3 ± 2·3	1·85 ± 0·22
30	—	—	13·5 ± 2·2	7·8 ± 2·02	28·0 ± 2·0	1·3 ± 0·1
40	—	—	10·3 ± 2·0	9·5 ± 1·43	27·7 ± 1·5	0·4 ± 0·07
50	32	17·8	10·7 ± 2·0	10·1 ± 3·91	41·0 ± 3·0	0·7 ± 0·16
75	44	15·1	—	—	—	—
100	25	12·1	—	—	—	—
			Phenol			
0			7·5	5·6	15·5	10·4
10			—	—	16·5	12·5
20			7·0	5·4	12·0	12·8
30			—	—	10·0	9·7
40			11·5	6·2	12·5	15·0
50			—	—	12·5	10·8
60			9·5	7·6	—	—
80			8·0	8·9	—	—
100			6·5	9·2	—	—

*Values are means of 3–6 assays in duplicate, except those for TA1530, which are derived from duplicate counts of single assays.

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Review Section

REPEATABILITY AND REPRODUCIBILITY OF MEASUREMENTS OF VINYL CHLORIDE CONCENTRATIONS IN MATERIALS AND ARTICLES MADE OF POLYVINYL CHLORIDE

L. Rossi

Communautés Européennes, Directorat Général III, Bruxelles, Belge

and

J. Waibel and C. G. vom Bruck

NATEC, Institut für naturwissenschaftlich-technische Dienste GmbH, Hamburg, Bundesrepublik Deutschland

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Summary—In the autumn of 1978 and the spring of 1979 two collaborative experiments were carried out with the aim of determining the variability of measurements of the vinyl chloride (VC) content of PVC films. The methods used were both based on the headspace gas-chromatographic technique, and the VC content ranged approximately from 0·2 to 3 mg/kg. Optimum working conditions yielded a reproducibility (as defined in ISO/DIS 5725, 20 October 1977, p. 2) of 0·4 mg/kg and repeatabilities (again as in ISO/DIS 5725) of 0·2 mg/kg at VC concentrations of 0·6 mg/kg.

Introduction

In order to ensure that the consumer is adequately protected, the Council of Ministers of the European Communities has issued a Directive (*Off. J. Europ. Commun.* 1978, **L44**, 15) on vinyl chloride (VC). Article 2.1 and Annex 1 of this Directive state that materials and articles made of PVC may not contain more than 1 mg VC/kg.

A method suitable for detecting these amounts of VC had to be selected, and an official method based on the technique of headspace gas chromatography was proposed. Collaborative studies were carried out in a number of experienced laboratories, under the supervision of the Plastics Working Group of the EC Commission, with L. Rossi as Chairman, to determine the reliability of the proposed method, and to permit the participating laboratories to discover any difficulties in its operation and propose necessary improvements. A few PVC-film samples were investigated with respect to their VC content to find out the deviation of results within each laboratory as well as the deviation between different laboratories.

The former deviation is expressed as repeatability (r), defined (ISO/DIS 5725, 20 October 1977, p. 2) as "the value below which the absolute difference between two single test results obtained with the same method on identical test material, under the same conditions (same operator, same apparatus, same laboratory, and a short interval of time), may be expected to lie with a specified probability; in the absence of other indications, the probability is 95%". The latter is expressed as reproducibility (R), defined (ISO/DIS 5725) as "the value below which the absolute difference between two single test results obtained with the same method on identical test material, under different conditions (different operators, different apparatus, different laboratories and/or different time), may be expected to lie with a specified probability; in the absence of other indications, the probability is 95%". Thus, reproducibility allows a judgement to be made on the deviations to be expected in results on the same sample from different laboratories and on the unavoidable differences that will occur when the method is applied in practice. Determination of repeatability provides some means for a laboratory to control its own ability to apply the official method. The accuracy of the method can, to a certain extent, be estimated from the reproducibility of the results of the different laboratories, provided the data are checked against pure VC or, even better, against a standard PVC sample of known VC content. Some provision is made from controlling the selectivity of the method, by the use either of detectors specific for VC or of columns of different polarities.

In the autumn of 1978, therefore, a collaborative experiment was carried out with 27 participants from seven EC countries (Appendix I) to determine the variability of measurements of the VC content of PVC films. The operative procedure was based on the EC working document III/354/78-EN (Rev. 1). Since the variations in the results among the participants were considered too large (see below), a second collaborative experiment was carried out in the spring of 1979. Again 27 laboratories from seven EC countries participated (Appendix I) and the operative procedure described in working document III/127/79-EN was used. This method differs from that used in the earlier study in that the addition procedure is used only to verify the linearity of the instrument. Once the instrument has been proved to operate satisfactorily, unknown VC levels in PVC can be calculated directly from the response of the instrument by the appli-

cation of correction factors. This procedure is possible because there is no matrix effect of the PVC on the response to VC under the conditions of this method (Puschmann, *Angew. Makromol. Chem.* 1975, **14**, 29).

A brief summary of the results of the first collaborative study by the EC Plastics Working Group on the determination of the VC content of PVC films is followed in this paper by a full description of the evaluation of the second collaborative study.

Statistical evaluation of the results of the first (1978) collaborative test

Principle

The basis of this procedure is to add known amounts of VC to the unknown PVC sample, to draw a straight line through the plot of the GC response over added VC concentrations, and to read the unknown VC concentration from the intercept of this line with the abscissa.

Procedure

Not less than 200 mg of the material or article, sampled from the product under investigation and reduced to small pieces, was weighed accurately into each of a set of at least six phials. An equal quantity was weighed, as far as possible, into each and the phial was closed immediately. To each phial were then added *N*,*N*-dimethylacetamide (DMA; 10 ml/g sample), containing an internal standard (diethyl ether or 2-*cis*-butene) if considered necessary, and a standard VC solution in quantities to give concentrations of 0, 0·06 and 0·12 mg added VC/litre DMA. The phials were sealed, suspended in a waterbath (e.g. at 60°C) and agitated until a homogeneous solution was obtained. The sealed vials had to be kept at constant temperature long enough (at least 2 hr at 60°C) for the samples to attain a state of equilibrium before the headspace sampling operation was begun.

The area (or height) of the GC peaks relating to VC, and to the internal standard if used, were measured and a graph was constructed in which the ordinate values showed either the areas (or heights) of the VC peaks or the ratios of the areas (or heights) of the VC peaks to those of the internal standard peaks, and the abscissa values were the quantities of VC (in mg/litre) added. The intersection of the extrapolation of the graph with the negative abscissa axis showed the unknown concentration (Fig. 1).

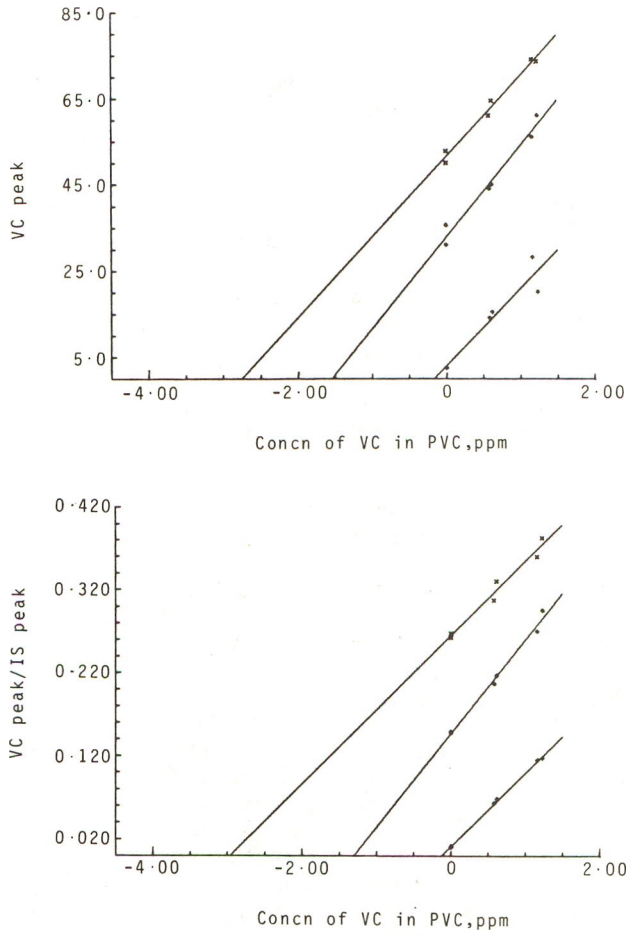

Fig. 1. First collaborative study; evaluation method. The straight line that best fits the experimental points is extrapolated to the abscissa, where the result can be read.

Table 1. *Results of first (1978) collaborative study on the determination of VC in PVC films*

Sample no.	No. of laboratories		Estimated VC content (mg/kg)	Repeatability† (r; mg/kg)	Reproducibility† (R; mg/kg)
	Included	Rejected*			
			Without internal standard		
1	21	4	2·57	0·76	1·79
2	22	3	1·55	0·54	1·23
3	20	5	0·17	0·17	0·34
			With internal standard		
1	12	3	2·67	1·06	1·43
2	14	1	1·57	0·48	0·87
3	13	2	0·15	0·14	0·25

*Because of 'outlying' results.
†As defined in ISO/DIS 5725, 20 October 1977, p. 2, and on p. 527 of this paper.

Results

In the summary of results presented in Table 1, the results of the participants submitting statistically 'outlying' results (VC content or repeatability) have been discarded. The wide variation in results was due, at least in part, to the use of the addition method, which involves an extrapolation procedure in the calculation of the VC content of the PVC sample (Fig. 1).

Second (1979) EC collaborative study of the determination of VC in PVC

Summary

In the second collaborative study for measuring VC in PVC, 27 laboratories again participated. The PVC film contained about 0·6 mg VC/kg PVC. The method, originally described in working document III/127/79–EN, was based on the headspace gas-chromatographic technique of Puschmann (*loc. cit.*) but involved the use of correction factors rather than the addition of known amounts of VC. Details of the determination procedure are given in Appendix II and the results were calculated as described below. The findings of the study are summarized in Table 2.

Check of linearity

The check of linearity of the response to VC concentration involved, first, a regression analysis, for each participant of (a) VC peak on VC content and (b) VC peak over internal standard peak on VC content over internal standard content. Inspection of the plots of the regresssion lines with and without an internal standard (Fig. 2) showed no indication of a curvilinear response except in the case of participant no. 20.

Regression lines of participant 20 (Fig. 3) were therefore tested further. The residual sum of squares about the regression (SQ_R) was estimated, the number of degrees of freedom being ν_R. The sum of squares due to repeated measurements (SQ_ϵ, with ν_ϵ degrees of freedom) was calculated ($SQ_\epsilon = \frac{1}{2}\Sigma(X_{i1} - X_{i2})^2$; ν_ϵ = number of pairs of measurements). The sum of squares due to the deviation from the straight line (SQ_D) equalled $SQ_R - SQ_\epsilon$ and $F = [SQ_D/(\nu_R - \nu_\epsilon)]/[SQ_\epsilon/\nu_\epsilon]$. A value for F in excess of the tabulated F values (*CRC Handbook of Tables for Probability and Statistics*; 2nd ed., p. 304; Chemical Rubber Co., Cleveland, OH, 1968) constitutes strong evidence that a response is not linear with the VC content of the samples. The line measured *without an internal standard* had an F value of 32; the one measured *with an internal standard* had an F value of 73. This means that the response of this participant's detector was almost certainly not linear.

As stated in the working document no. III/127/79-EN (Commission of the European Communities, 1979), the regression lines have to be linear within 10%, i.e. the standard deviation about the regression line over the mean value of the ordinate should be less than 10%. Deviation from this requirement need not necessarily lead to wrong results (Table 3). Table 4 shows that all participants were within this limit except nos 12 and 27 for the lines without the internal standard and nos 9 and 12 for the lines with the internal standard

Table 2. *Summary of findings in the second (1979) collaborative study on the determination of VC in PVC film*

Correction factor*	Doubtful results	Repeatability† (r; mg/kg)	Reproducibility† (R; mg/kg)
fa	Discarded	0·17	0·21
fi	Discarded	0·056	0·17
fa	Included	0·21	0·57
fi	Included	0·19	0·40

*Without (fa) and with (fi) internal standards.
†As defined in ISO/DIS 5725, 20 October 1977, p. 2, and on p. 527 of this paper.

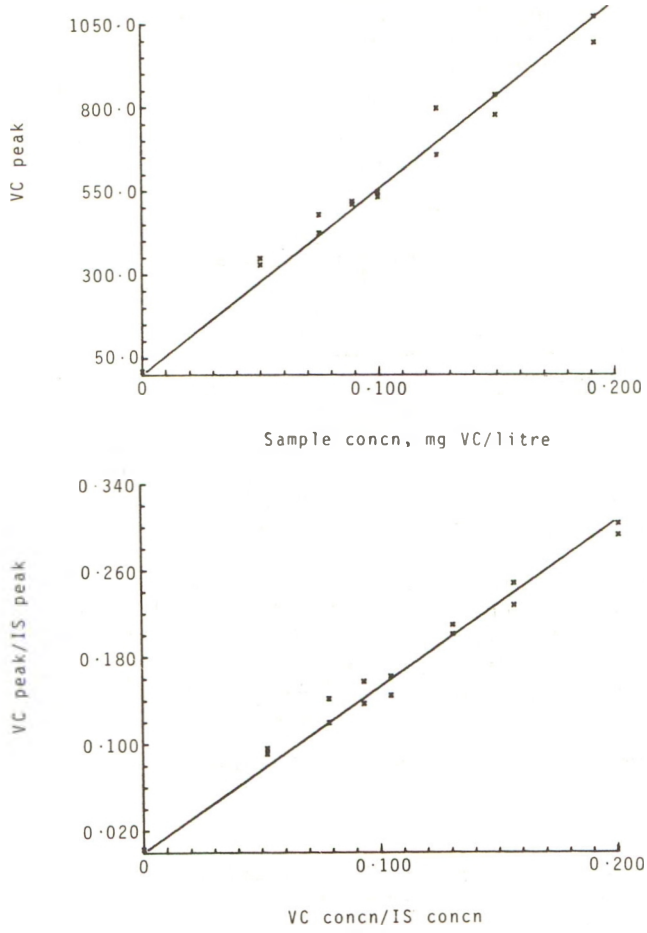

Fig. 2. Example of the estimation of linear response.

Blank values

Most participants had blank values that could be neglected, but two (nos 10 and 19) had to take their blank values into account.

Results of sample measurements

Each participant carried out—with and/or without an internal standard—one blank test, two calibration tests with VC solution A3 (containing about 0·1 mg VC/litre DMA or internal standard solution) and five sample tests of the PVC film (c. 0·6 mg VC/kg PVC). The VC concentrations obtained are summarized in Table 3, in which the doubtful results are marked with an asterisk, and displayed in Fig. 4.

The first step in calculating the concentrations was the calculation of the two correction factors, fa and fi:

$$\text{fa} = (\text{VC peak})/(\text{VC concn of solution A3})$$

$$\text{fi} = \frac{\text{VC peak}}{\text{IS peak}} \cdot \frac{\text{IS concn}}{\text{VC concn}} \text{ (of solution A3)}$$

The mean of the correction factors was taken for all calculations of VC levels. Then, the concentration of VC in PVC was calculated, respectively, from:

$$C_a = \frac{\text{VC peak}}{\text{fa}} \cdot \frac{\text{quantity of solution}}{\text{quantity of dissolved PVC}}$$

$$C_i = \frac{\text{VC peak/IS peak}}{\text{fi/IS concn}} \cdot \frac{\text{quantity of solution}}{\text{quantity of dissolved PVC}}$$

Repeatability and reproducibility

Repeatability and reproducibility, as defined earlier, were calculated from Table 3. Individual repeatabilities of the laboratories were found by multiplying individual standard deviations by 3·8, as each participant supplied only five results. (The usual factor of 2·8 would apply only if the numbers of measurements had been considerably higher.) The results are summarized in Table 2.

Consequences of the results of the second collaborative test

Because the individual standard deviations could be calculated and, to a certain extent, controlled by each laboratory, it was decided to eliminate the results of participants with standard deviations of $\geqslant 0{\cdot}1$. This seemed justified because most participants had smaller standard deviations. On the other hand, careful compliance with the prescribed procedure

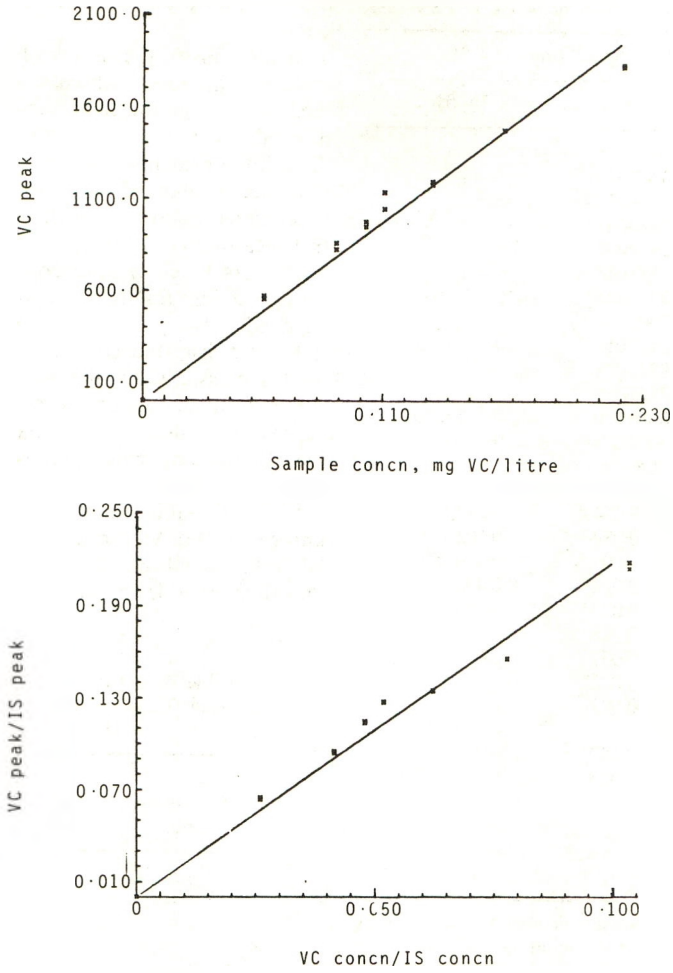

Fig. 3. Linearity check for participant 20.

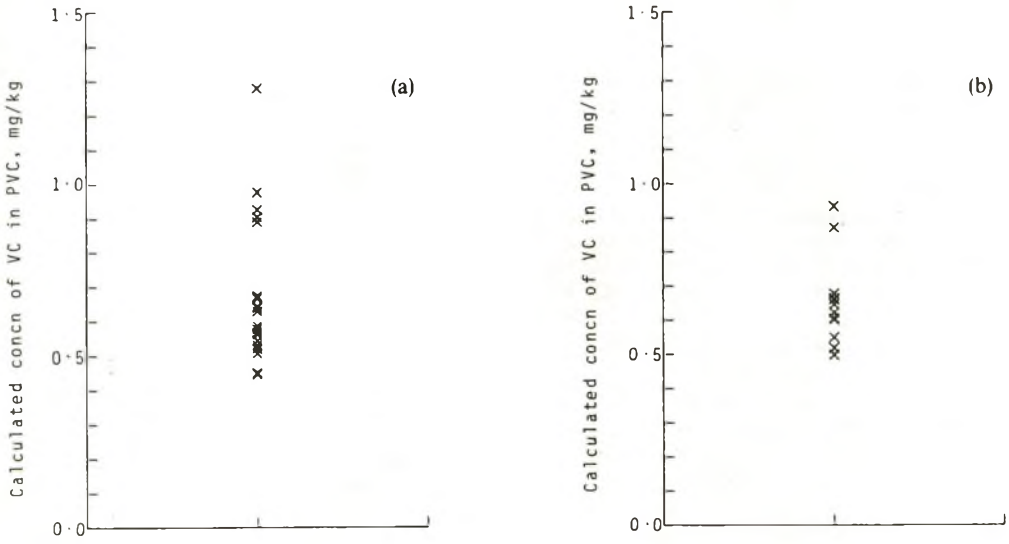

Fig. 4. Results of the second collaborative study for measurements (a) without and (b) with an internal standard.

Table 3. *Calculated concentrations of VC in PVC*

Participant no.	VC concn* (mg/kg)/1 SD	
	By fa†	By fi†
1	0·670/0·048	
2	0·507/0·023	
3	0·530/0·031	
4‡	*1·28/0·006	
5	0·629/0·008	
6	0·586/0·018	0·601/0·010
7	0·669/0·085	*0·872/0·053
8	0·450/0·0	
9	0·561/0·017	0·518/0·022
10§	*0·892/0·025	
11	*0·978/0·036	
12	*0·906/0·176	*0·934/0·202
13	0·520/(0·21)‖	0·621/0·011
14	*0·928/0·098	
15	0·644/0·047	0·677/0·041
16	0·667/0·014	0·647/0·011
17	0·568/0·077	0·605/0·014
18	0·675/0·007	0·660/0·005
19	0·446/0·022	0·498/0·014
20	0·577/0·029	0·549/0·027
21	0·544/0·013	
22	0·449/0·037	
23	0·639/0·022	
24	0·543/0·028	0·663/0·029
25	–¶	0·565/0·008
26	*0·994/0·157	*0·920/0·134
27	0·623/0·065	0·535/0·013
Mean–*values excluded...	0·575	0·595
–*values included...	0·672	0·658

*Placed before a number, an asterisk indicates a doubtful result. Means were calculated including and excluding these results.
†Without (fa) and with (fi) internal standard.
‡Amount of dimethylacetamide (DMA) not stated.
§Small peak in VC area in DMA.
‖One VC count, but also the internal standard count, deviated appreciably.
¶VC content determined without an internal standard was not stated.

should enable a laboratory to achieve, with practice, an acceptable repeatability. The Working Group's discussion of the results revealed that participants 10, 11 and 12 did not keep to the prescribed procedure and therefore had to be considered as 'outliers'.

After the results of participants 10, 11, 12 and 26 had been rejected for these reasons, participants 4, 7 and 14 (no. 7 only with the internal standard) became 'statistical outliers' because of the results of their VC-concentration determinations, althouth the repeatability of no. 4 was very good. It can thus be seen that a low repeatability does not necessarily imply an equally good accuracy of results. After elimination of these participants, marked with an asterisk in Table 3 and comprising six out of 26 laboratories for the method without an internal standard and three out of 15 using an internal standard, the reproducibility (Table 2) was calculated. There is, however, no justification in principle for treating the results obtained with an internal standard as if no such standard were used (see, for example the results of participant 13 in Table 3).

In the light of the results of this collaborative test and the subsequent discussion, modifications were incorporated in the final version of the EC official method for the determination of VC in PVC, although the principles of the determination procedure remained unchanged. The full text of this final version is presented in Appendix II. In particular, some control measurements were introduced to enable individual users to check and control their performance. The procedure for the calculation of results was also changed.

Table 5 lists the columns and oven temperatures used successfully by the participants in the study. Which of these columns is most suitable in a particular case depends on the internal standard used and on the volatile impurities present in the PVC sample under investigation.

It is advisable that PVC standards, containing known levels of VC, should be made available, so that laboratories will be able to test the level of accuracy that they are able to attain.

Table 4. *Linearity check: measure of linearity as defined in document III/127/79–EN*

Participant no.	Linearity* (%)	
	By fa†	By fi†
1	10	—
2	2	—
3	2	—
4	1·3	—
5	1·5	—
6	9	5
7	6	8
8	3	—
9	7	14
10	5	—
11	4	—
12	13	12
13‡	1·8	3
14	10	—
15	10	9
16	1·4	2
17	10	5
18	4	2
19	10	5
(30)§	10	7
20	8	8
21	6	—
22	3	—
23	6	—
24	4	2
25	7	3
26	9	7
27	15	3

*Standard deviation about regression line over mean value of ordinate.
†Without (fa) and with (fi) internal standards.
‡One obviously outlying result of this participant was discarded
§A second set of results supplied by participant no. 19.

Table 5. *GLC columns found satisfactory in the collaborative test*

Column			Stationary phase and loading	Support*	Oven temp (°C)
Material	Length (m)	Diameter (mm)			
—	1·5	3	25% TTP	Chromosorb G (80–100)	60
SS	3	3	25% DIOP	Chromosorb W–AW (60–80)	45–60
SS	3	2·16	25% DIOP + 0·5% ATPET 80	HMDS-treated DIATOMITE C (60–70)	60–85
SS	0·9	3·2	—	Chromosorb 102 (100–120)	110
SS	1–2	2·16–3·2	—	PORAPAK Q–S (80–100)	110–140
SS	3	3	20% Carbowax 1540 + 0·5% ATPET 80	Chromosorb W (60–85)	40
SS	1·5	3·2	0·2% Carbowax 1500	Carbopack C (80–100)	90
Glass	1·7	4·8	8% Carbowax 20 M	Chromosorb	70
SS	3 + 3	4	3 m with 5% PPG 3000 DOW; 3 m with 5% PEG B11/700	Chromosorb P–NAW (80–100)	40
—	6	6·35	10% WALLCOMID M18 10% PEG 400	Chromosorb W–AW/ DMCS (60–80)	50
Cr/Ni	3 + 5	2	TCP, 20:100	Chromosorb P–AW (60–80)	45
SS	2–6	2–3·2	15% UCON LB 550–x	Chromosorb W–AW (100–120)	50–70
SS	3	4	Silicone Grease DC 111 15%	Chromosorb W–AW/ DMCS (60–85)	60
—	6	2	15% SE 30	Chromosorb	25

SS = Stainless steel TTP = Tritolyl phosphate DIOP = Diisodecyl phthalate PPG = Polypropylene glycol
TCP = 1,2,3-Tris-(2-cyanoethoxy)propane

*Figures in brackets indicate mesh size.

Acknowledgements—The authors would like to thank Dr. J. Puschmann, Gendorf, Germany, for providing the PVC samples for these collaborative tests, all participants for kindly contributing their results, and the members of the EC Working Group for discussions on the results and on the method.

APPENDIX I

List of institutions and companies participating in the tests

A/S Haustrups Fabriker, Odense, Denmark
BP Chemicals Ltd., Glamorgan, UK.
British Industrial Plastics Ltd., Durham, UK.
Cadbury-Typhoo Ltd., Birmingham, UK.
Centraal Instituut voor Voedingsonderzoek, Zeist, The Netherlands
Chem. Landesuntersuchungsamt, Nordrhein-Westfalen, Münster, Germany
Chem. und Lebensmittelchem. Untersuchungsanstalt Hamburg, Hamburg, Germany
County Public Health Laboratory, Institute of Preventive Medicine, Glamorgan, UK.
DSM, Lokatie Kunststoffen, Beek, The Netherlands
Hoechst AG, Gendorf, Germany
ICI Plastics Division, Welwyn Garden City, UK.
Ijdsk Teknologisk Institut, Århus C, Denmark
Institut für Lebensmitteltechnologie und Verpackung e.V., München, Germany
Istituto Superiore di Sanita, Roma, Italy
Keuringsdienst van Waren, Utrecht, The Netherlands (two separate laboratories)
Laboratoire Central d'Analyses et de Recherches, Ministère de l'Agriculture, Massy, France
Laboratory of the Government Chemist, London, UK.
Metal Box Ltd., London, UK.
Ministerie van Volksgezondheid en van het Gezin, Instituut voor Hygiene en Epidemiologie, Bruxelles, Belgium
Ministry of Agriculture, Fisheries and Food, Food Laboratories, Norwich, UK.
Montedison, Milano, Italy
National Food Institute, Soeborg, Denmark
Odense Kommunes Laboratorium, Odense, Denmark
Shell Nederland Chemie BV, Rotterdam, The Netherlands
Solvay et Cie, Bruxelles, Belgium
Unilever Forschungsgesellschaft mbH, Hamburg, Germany

APPENDIX II

Determination of the vinyl chloride monomer level in materials and articles*

1. *Scope and field of application*
The method determines the vinyl chloride monomer level in materials and articles.
2. *Principle*
The level of vinyl chloride monomer level (VC) in materials or articles is determined by means of gas-chromatography using the "headspace" method after dissolution or suspension of the sample in *N*,*N*-dimethylacetamide.

Official Journal of the European Communities 1980, **23** (213), 42.

3. *Reagents*

3.1 Vinyl chloride (VC), of purity greater than 99·5% (v/v).

3.2 *N,N*-Dimethylacetamide (DMA), free from any impurity with the same retention time as VC or as the internal standard (3.3) under the conditions of the test.

3.3 Diethyl ether or cis-2-butene, in DMA (3.2) as the internal standard solution. These internal standards must not contain any impurity with the same retention time as VC, under the conditions of the test.

4. *Apparatus*

N.B. An instrument or piece of apparatus is mentioned only if it is special or made to particular specifications. Usual laboratory apparatus is assumed to be available.

4.1 Gas-chromatograph fitted with automatic headspace sampler or with facilities for manual sample injection.

4.2 Flame ionization detector or other detectors mentioned in point 7.

4.3 Gas-chromatographic column.

The column must permit the separation of the peaks of air, of VC and of the internal standard, if used. Furthermore, the combined 4.2 and 4.3 system must allow the signal obtained with a solution containing 0·02 mg VC/litre DMA or 0·02 mg VC/kg DMA to be equal to at least five times the background noise.

4.4 Sample phials of flasks fitted with silicone or butyl rubber septa.

When using manual sampling techniques the taking of a sample from the headspace with a syringe may cause a partial vacuum to form inside the phial or flask. Hence, for manual techniques where the phials are not pressurized before the sample is taken, the use of large phials is recommended.

4.5 Micro-syringes.

4.6 Gas-tight syringes for manual headspace sampling.

4.7 Analytical balance accurate to 0·1 mg.

5. *Procedure*

CAUTION: VC is a hazardous substance and a gas at ambient temperature, therefore the preparation of solutions should be carried out in a well-ventilated fume cupboard.

—Take all the necessary precautions to ensure that no VC or DMA is lost;

—When employing manual sampling techniques an internal standard (3.3) should be used;

—When using an internal standard, the same solution must be utilised throughout the procedure.

5.1 *Preparation of concentrated standard VC solution at approximately 2000 mg/kg*

Accurately weigh to the nearest 0·1 mg a suitable glass vessel and place in it a quantity (e.g. 50 ml) of DMA (3.2). Re-weigh. Add to the DMA a quantity (e.g. 0·1 g) of VC (3.1) in liquid or gas form, injecting it slowly on to the DMA. The VC may also be added by bubbling it into the DMA, provided that a device is used which will prevent loss of DMA. Re-weigh to the nearest 0·1 mg. Wait two hours to allow equilibrium to be attained. Keep the standard solution in a refrigerator.

5.2 *Preparation of dilute standard VC solution*

Take a weighed amount of concentrated standard solution of VC (5.1) and dilute, to a known volume or a known weight, with DMA (3.2) or with internal standard solution (3.3). The concentration of the resultant dilute standard solution is expressed as mg/l or mg/kg respectively.

5.3 *Preparation of the calibration curve*

N.B.—the curve must comprise at least 7 pairs of points;

—the repeatability of the responses (see recommendation ISO/DIS 5725: 1977) must be lower than 0·02 mg VC/l or kg of DMA;

—the curve must be calculated from these points by the least squares technique, i.e. the regression line must be calculated using the following equation

$$y = a_1 x + a_0$$

Where:

$$a_1 = \frac{n\Sigma x\, y - (\Sigma x).(\Sigma y)}{n\Sigma x^2 - (\Sigma x)^2}$$

and:

$$a_0 = \frac{(\Sigma y).(\Sigma x^2) - (\Sigma x).(\Sigma xy)}{n.\Sigma x^2 - (\Sigma x)^2}$$

where:

y = the height or area of peaks in any single determination;

x = the corresponding concentration on the regression line;

n = number of determinations carried out ($n \geqslant 14$);

—the curve must be linear, i.e. the standard deviation (s) of the differences between the measured responses (y_i) and the corresponding value of the responses calculated from the regression line (z_i) divided by the mean value ($\bar{y}$) of all the measured responses shall not exceed 0·07:

This shall be calculated from:

$$\frac{s}{\bar{y}} = \leqslant 0{\cdot}07$$

where

$$s = \sqrt{\frac{\sum_{i=1}^{n} (y_i - z_i)^2}{n - 1}}$$

$$\bar{y} = \frac{1}{n} \sum_{i=1}^{n} y_i$$

y_i = each individual measured response

z_i = the corresponding value of the response (y_i) on the calculated regression line

$n = \geqslant 14$

Prepare two series of at least 7 phials (4.4). Add to each phial volumes of dilute standard VC solution (5.2) and DMA (3.2) or internal standard solution in DMA (3.3) such that the final VC concentration of the duplicate solutions will be approximately equal to 0; 0·050; 0·075; 0·100; 0·125; 0·150; 0·200 etc mg/l or mg/kg of DMA and that all the phials contain the same quantity of DMA that is to be used under point 5.5. Seal the phials and proceed as described under point 5.6. Construct a graph in which the ordinate values show the areas (or heights) of the VC peaks of the duplicate solutions or the ratio of these areas (or heights) to those of the relevant internal standard peaks and the abscissa values show the VC concentrations of the duplicate solutions.

5.4 *Validation of preparation of standard solutions obtained in points 5.1 and 5.2*

Repeat the procedure described under points 5.1 and 5.2 to obtain a second diluted standard solution with a concentration equal to 0·1 mg VC/l or 0·1 mg/kg of DMA or internal standard solution. The average of two gas-chromatographic determinations of this solution must not differ by more than 5% from the corresponding point of the calibration curve. If the

difference is greater than 5%, reject all the solutions obtained in points 5.1, 5.2, 5.3 and 5.4 and repeat the procedure from the beginning.

5.5 *Preparation of the samples of materials or articles*
Prepare two phials (4.4). Weigh into each phial not less than 200 mg, to the nearest 0·1 mg, of the sample obtained from single material or article under investigation which has been reduced to small pieces, Try to ensure that an equal quantity is weighed into each phial. Close the phial immediately. Add to each phial for each gram of the sample 10 ml or 10 g of DMA (3.2) or 10 ml or 10 g of internal standard solution (3.3). Seal the phials and proceed as described under point 5.6.

5.6 *Gas-chromatographic determinations*

5.6.1 Agitate the phials avoiding contact between the contained liquid and the septum (4.4) to obtain a solution or suspension of the samples of material or article (5.5) as homogenous as possible.

5.6.2 Put all the sealed phials (5.3, 5.4 and 5.5) in a waterbath for 2 hours at 60°C ± 1°C to allow equilibrium to be attained. Agitate again, if necessary.

5.6.3. Take a sample from the headspace in the phial. When utilising manual sampling techniques care must be exercised in obtaining a reproducible sample (see point 4.4), in particular the syringe must be pre-warmed to the temperature of the sample. Measure the area (or height) of the peaks relating to the VC and to the internal standard if used.

5.6.4 Remove from the column (4.3) excess DMA using an appropriate method as soon as peaks of DMA appear on the chromatogram.

6. *Calculation of the results*

6.1 Finding by interpolation on the curve, the unknown concentration of each of the two solutions of the sample taking account of the internal standard solution if used. Calculate the amount of VC in each of the two samples of material or article under investigation by applying the following formula:

$$X = \frac{C \times V}{M} 1000$$

where:

X = concentration of VC in the sample of the material or article expressed in mg/kg.

C = concentration of VC in the phial containing the sample of material or article (see under point 5.5) expressed in mg/l or mg/kg.

V = volume or mass of DMA in the phial containing the sample of material or article (see under point 5.5) expressed in litres or kg.

M = amount of the sample of the material or article, expressed in grams.

6.2 The concentration of VC in the material and article under investigation expressed in mg/kg shall be the average of the two concentrations of VC (mg/kg) determined in point 6.1 provided that the repeatability criterion in point 8 is satisfied.

7. *Confirmation of the VC level*
In cases where the content of VC in materials and articles as calculated under point 6.2 exceeds the maximum permissible amount, the results obtained by the analysis of each of the two samples (5.6 and 6.1) must be confirmed in one of three ways:

—by using at least one other column (4.3) having a stationary phase with a different polarity. This procedure should continue until a chromatogram is obtained with no evidence of overlap of the VC and/or internal standard peaks with constituents of the sample of the material or article;

—by using other detectors, e.g. the micro-electrolytic conductivity detector (see Journal of Chromatographic Science, Vol. 12, March 1974, p. 152).

—by using mass-spectrometry. In this case, if molecular ions with parent masses (*m/e*) of 62 and 64 are found in the ratio of 3:1, it may be regarded with high probability as confirming the presence of VC. In case of doubt the total mass spectrum must be checked.

8. *Repeatability*
The difference between the results of two determinations (6.1) carried out simultaneously or in rapid succession on the same sample, by the same analyst, under the same conditions, must not exceed 0·2 mg VC/kg of material or article.

Fd Cosmet. Toxicol. Vol. 18, pp. 537 to 540
Pergamon Press Ltd 1980. Printed in Great Britain

REVIEWS OF RECENT PUBLICATIONS

Developments in Sweeteners—1. Edited by C. A. M. Hough, K. J. Parker and A. J. Vlitos. Applied Science Publishers Ltd., London, 1979. pp. xii + 192. £15.00.

This is part of a *Developments Series*, each volume of which is intended to bring together papers dealing with the latest trends and developments in a specific area. However, the first chapter in this volume on sweeteners begins with a look at the past, dealing comprehensively with the history of cane sugar which is traced from a record of its cultivation in Asia as early as 1000 BC, through the days when it was a valuable rarity and Venice was the centre for sugar refining, and on to the well-documented period of the slave trade and the establishment of the Caribbean sugar industry. The history of sugar beet, a comparatively modern phenomenon, is described more briefly. Methods of production and refining, sugar and politics, and sugar and health are also dealt with in this chapter. On the latter subject the author concludes that the association between sucrose and degenerative diseases appears to be dubious, and that only in the case of dental caries can sugar be implicated, and then merely as one of a number of contributory factors.

In later chapters, however, other authors extol the virtues of alternative sweeteners for their roles in reducing calorie intake or, for instance in the case of xylitol, in reducing the incidence of dental caries. The studies on the effects of xylitol on dental health carried out at the University of Turku are described and the Huntingdon study on xylitol (*Food Chemical News* **20** (25), 33) is mentioned briefly.

In general, this book does not deal with the toxicology of sweeteners, except to point out that the costs of the stringent toxicity studies that must be undertaken for a new sweetener to be accepted, at least in the western world, are prohibitive for many of the sweeteners that have been discovered. Miraculin, one of the protein sweeteners, was one that did not gain FDA acceptance (*Federal Register* 1977, **42**, 26467), while it proved difficult to propagate the plant from which monellin was produced, and the sweetener itself was found unstable to heat and low pH. However, studies of the structure of monellin and of the relationship between structure and sweetness are reviewed. Thaumatin, obtained from *Thaumatococcus daniellii* is considered the brightest hope among the protein sweeteners.

Other chapters deal with the synthesis and properties of aspartame and with the glucose and fructose syrups. Less common sweeteners such as stevioside, glycyrrhizin, and the urea-based and oxime sweeteners are also described, and the final chapter deals with the theory of sweetness. Cyclamate and saccharin, the two compounds that probably spring to mind most readily at the mention of sweeteners, are not covered in this volume. Each chapter is contributed by different authors, almost all of whom are from the sugar or sweetener industry. Many references are given and a summary is provided at the start of each chapter. The volume provides an interesting background for those who would like to know more about the production and properties of sugar and of past, present and future sweeteners.

Patty's Industrial Hygiene and Toxicology. Volume III: **Theory and Rationale of Industrial Hygiene Practice.** Edited by L. V. Cralley and L. J. Cralley. John Wiley & Sons Ltd., Chichester, 1979. pp. x + 752. £27.50.

The great strides made in industrial hygiene practice over recent decades have for the first time made it worthwhile to add a third volume to this classic textbook. Volume I (General Principles) was revised in 1978 (*Cited in F.C.T.* 1979, **17**, 291) and a new Volume II (Toxicology) is being prepared.

Some of the more general chapters in this volume review subjects of overall relevance, including analytical measurements, measurement of worker exposure, and biological indicators of chemical dosage and burden. Data automation, statistical design and data analysis requirements, and the problem of keeping an inventory of emissions into workroom air are also considered in some depth, as are more specific topics, such as the evaluation of exposure to chemical agents, biological agents, radiation, noise, temperature, vibration and pressure.

One of the more controversial chapters, by Drs P. J. Gehring and K. S. Rao of the Dow Chemical Company, deals with the extrapolation of toxicological data. The fact that 1,4-dioxane is toxic and carcinogenic to rats only at levels involving a saturation of its normal metabolic pathway is adduced to justify the present TLV of 50 ppm, at which concentration the substance is detoxified readily by man. The great variation in dose-response curves for several hydrocarbons is elegantly used to illustrate the need for different exposure standards for these materials, and the value of linear pharmacokinetics is emphasized by the ability of such studies to elucidate interspecies differences in susceptibility to cataracts from 2,4-dinitrophenol. The authors also give the somewhat alarming news that unpublished chronic studies on certain chemicals at Dow Chemical USA have revealed toxic manifestations not observed in prior 6-month studies, a finding that could obviously have widespread implications for the future of toxicity testing.

Health surveillance programmes in industry are reviewed by Dr. W. C. Cooper, who discusses the value of medical examinations before, during and after employment, as well as hazard-oriented screening tests such as sputum and urine cytology. Increasing emphasis has been placed in recent years on engineering controls, and a 36-page chapter is devoted to their philosophy and management. Personal protection, which is now generally regarded as a 'last

ditch' or emergency method rather than a routine defence, also receives its due share of attention. The concluding chapters, dealing with job safety and health laws (up to mid-1978) and with compliance and projection, will be of interest mainly to American readers.

There is considerable overlap between some of the subjects covered in this volume and those in Volume I, and it is surprising in some cases that another chapter on the same subject was considered necessary. Some overlap also occurs between chapters within Volume III. However, the book covers enough new ground to deserve a place on the shelves of all industrial companies, and increases the eagerness with which a revised Volume II is anticipated.

Potential Industrial Carcinogens and Mutagens. Studies in Environmental Science 4. By L. Fishbein. Elsevier Scientific Publishing Company, Amsterdam, 1979. pp. x + 534. Dfl. 150.00.

The literature on the carcinogenicity and mutagenicity of industrial chemicals has been expanding rapidly in recent years, making it increasingly difficult for anyone to keep abreast of the current status of such materials. A determined effort to overcome this problem has been made in the publication cited above, which systematically reviews the relevant data on 176 organic chemicals of industrial utility that have been indicted as carcinogens and/or mutagens. The chemicals are grouped according to chemical structure, and information is included on synthesis, trace impurities, production volumes, use patterns, environmental occurrence, chemical and biological reactivity, populations estimated to be at risk, and TLVs or MACs. Introductory chapters discuss combination effects in chemical carcinogenesis, such as initiation and promotion, and aspects of epidemiology and risk assessment, including the thorny question of whether a threshold may be estimated for carcinogens.

Data on carcinogenicity have been derived to a large extent from IARC monographs, but subsequent NCI and other studies have been added where necessary. The information included is commendably up-to-date by the standards of most books, writing evidently having been completed only in the year before publication (which may provide some excuse for the number of obvious printing errors). From the point of view of completeness, it is disappointing that some important classes of organic carcinogens, such as polycyclic aromatic hydrocarbons and mycotoxins, have been omitted, and inorganic substances such as the heavy metals have also been ignored, but this doubtless lays the way open for another book in the same vein! One could also take issue with the implication in the preface that most environmentally-induced cancers are caused by industrial chemicals, rather than by factors such as smoking, drinking and overnutrition, although the role played by the last three factors does receive some mention in a later chapter. However, the book will be of chief value for its orderly summaries of mutagenicity and carcinogenicity data on the various classes of industrial organic chemicals, and it well deserves a place on the shelves of any company that manufactures or uses such substances.

Contemporary Topics in Immunobiology. Vol. 8. Edited by N. L. Warner and M. D. Cooper. Plenum Press, New York, 1978. pp. xiv + 272. $33.00

Volume 8 of Contemporary Topics in Immunobiology maintains the high standard of the previous issues in the series and brings to the reader's attention new trends and knowledge in the field of immunobiology. It opens with an extremely interesting contribution by J. F. A. P. Miller on the role of genes of the major histocompatibility complex (MHC) in controlling the function of T lymphocytes. T lymphocytes act in different ways in immune responses and may be divided into various subsets. The exact manner by which the MHC controls T-cell activities and T-cell-dependent functions is not known, but evidence is accumulating that MHC gene products are intimately involved in delivering an activating signal to these lymphocytes. Different alleles may be responsible for the varying efficiency with which cells can recognize a determinant or a self-determinant.

Several chapters in the book are devoted to the further characterization of lymphocyte subpopulations in the light of new knowledge of immunoglobulin membrane receptors. The discovery that T lymphocytes have receptors for IgM and IgG has enabled investigators to discriminate between functionally distinct subpopulations of T cells and has indicated the fundamental importance of the balance between helper and suppressor T cells in the regulation of immune responses.

In another important chapter, R. C. Burton, S. E. Chism and N. L. Warner discuss the development of *in vitro* assays that demonstrate not only the existence of a specific subpopulation of T lymphocytes responsible for mediating cytotoxicity, but also their response to tumour-associated antigens. These authors report some success in experiments in which well-defined tumour-associated antigens rather than whole tumour cells were used to induce cytotoxic T cells *in vitro*. A second technique, that of presenting soluble tumour antigens *in vitro* on macrophages also indicates that it may be possible in the future to induce cytotoxicity *in vitro* with specificity for particular tumour antigens. Such techniques may lead both to a better definition of human tumour antigens and to improved therapeutic treatment.

The following chapter considers the potential role of various cell types in allogeneic and anti-tumour immunity and then concentrates on the *in situ* tumour response as contrasted with systemic immunity. It discusses the finding that non-T lymphocytes, monocytes and macrophages may also infiltrate the tumour site and participate in graft destruction. A further chapter brings exciting new evidence for naturally occurring cytotoxic cells, known as natural killer cells. These cells exist in healthy, normal mammals and display specific cytolytic activity against various target cells. The authors (R. Kiessling and O. Haller) present experimental evidence for a protective role of natural killer cells against transplantation of certain lymphoma cells. They postulate that these cells may act

as regulators of haemopoiesis, thus regulating various cell compartments which are subjected to continuous physiological proliferation.

There is also a contribution on the subject of B-lymphocyte heterogeneity and another on the role of the immune response in protecting the host against parasitic infections. The range of topics thus provides a cross-section of contemporary immunobiology and should be of interest to all interested in its range and applications.

Biology of the Lymphokines. Edited by S. Cohen, E. Pick and J. J. Oppenheim. Academic Press, New York, 1979. pp. xvi + 626. £27.60.

This book can be highly recommended to everyone interested in the role played by soluble cell products in many of the cell interactions involved in regulating the immune response. The volume brings together a number of distinguished contributors, who elegantly and concisely present the various functions of the mediators in cellular immunity—that is the soluble cell products of all categories except the classical antibodies. Lymphokines are, by definition, materials possessing a biological activity that is assessed by an *in vitro* procedure. Their relevance to events *in vivo* can be demonstrated in two ways, as is discussed in the introductory chapter. First, a lymphokine produced in tissue culture is active when introduced into the intact animal, and, secondly, lymphokines can be isolated from animal tissues or body fluids, particularly from those derived from sites where an immunologically mediated inflammatory reaction is taking place.

The second chapter in the book discusses the ability of lymphokines to mobilize, attract and activate a variety of circulatory cells that can participate in local inflammation, and demonstrates the important link between the immune system and the inflammatory system. Several subsequent chapters deal with particular lymphokine activities. Lymphokines can inhibit the spontaneous *in vitro* motility of phagocytic cells and in the light of recent evidence it is postulated that this action is composed of three main phases: the interaction of the factor with membrane receptors of the phagocytic cell, the generation of second messengers in the cytoplasm, and an effect on the cytoskeleton.

A consideration of the importance of lymphokines in reactions such as allograft rejection, tumour immunity and certain autoimmune diseases follows a chapter on the relationship between lymphokines and cell-mediated cytotoxicity. The situation with regard to techniques for measuring lymphokine production in man is brought up to date. It is pointed out that measuring lymphokines in human diseases has proved of value in determining antigens to which the host is sensitized, providing possible explanations for the mechanism of tissue destruction in certain diseases, and elucidating the mechanisms of several immunodeficiency states.

The process of activation of lymphokine production occupies several chapters. Recent findings indicate that both T and B lymphocytes are capable of producing most lymphokines, but in certain instances macrophages are also required.

Amplification factors, which cause lymphocytes to divide and/or synergistically augment cell division induced by other lymphocyte stimulants, are other important soluble factors in the cell. They can be produced by mononuclear phagocytes as well as by lymphocytes, and their biological effects, their biochemistry and their effect on the antibody response receive considerable attention in this volume. Emphasizing the need for these mediators to be purified, possibly by production of specific antibodies, so that their effect on *in vivo* as well as *in vitro* immune responses can be evaluated, the authors envisage that they may prove to be of therapeutic utility in some immunodeficiency states or in adjuvant therapy.

One particularly fascinating chapter characterizes the interferons and discusses their ability to regulate the immune response as well as to protect cells against viruses. It demonstrates that interferons can have immunosuppressive or immunoenhancing effects, depending on the experimental conditions, and can be products of T or B lymphocytes or macrophages. Macrophages themselves produce many soluble immunoregulatory products and the possible relationship between the capacity of activated macrophages to release soluble mediators and to kill target cells is discussed in a separate contribution.

Lymphokine research can be expected to contribute greatly to the expansion of a biochemical and pharmacological approach to immunological problems. This book, so lucidly presented, offers the basis for a greater understanding of the multiple involvement of lymphokines, as well as providing up-to-date reviews.

The Mammalian Testis. By B. P. Setchell. Elek Books Ltd., London, 1978. pp. ix + 450. £18.00.

It has long been recognized that exogenous chemicals may interfere with reproduction, although in studying such effects physiologists and toxicologists have focused their attention primarily on the female. In recent years, however, it has become clear that many chemicals may damage the male gonads, either directly or indirectly *via* interference with the complex hormonal control of spermatogenesis. The detection and analysis of impaired testicular function calls for a thorough knowledge of the normal structure and function of the testis. For this purpose it would be difficult to find a better starting point than Dr. Setchell's excellent book.

Following a description of the overall organization of the mammalian testis, three chapters deal with the development of the testis and its vascular and nervous supplies. The greater part of the book is devoted to the two major functions of the testis, spermatogenesis and the production of androgens. These processes are clearly described and the essential relationships between them are emphasized. A further chapter on the hormonal control of the testis cogently reviews the considerable advances that have been made in this area over the past decade. Of obvious importance to the toxicologist is the extent to which chemicals may gain access to the germinal epithelium. The existence of a blood–testis barrier is now well established and in an extensive chapter Dr. Setchell discussed the implications of this both for the entry of substances and

for the production and composition of fluid within the tubules. A final chapter deals with naturally occurring and induced abnormalities of the testis. Included is a section on chemically-induced damage although this is little more than a list of compounds and is not comprehensive. However, since the book is primarily concerned with the normal structure and function of the testis, this is small criticism.

This readable and up-to-date account of the mammalian testis should be invaluable to those involved in reproductive toxicology. The book is well produced, copiously illustrated and referenced and altogether thoroughly informative.

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Information Section

ARTICLES OF GENERAL INTEREST

DIOXIN DANGERS TO MAN

Objective comment in an area of fast-moving bandwagons is difficult. Pronouncements on the dangers and value of the 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) herbicides (*Review of the Safety for Use in the U.K. of the Herbicide 2,4,5-T*; Reference Document; UK Advisory Committee on Pesticides, 1979) have aroused some public interest. The publication of the Oregon study and the apparent discrepancy between subsequent government actions in the USA and UK have ensured that future developments will be followed by an even wider audience. The present earnest discussion on the acceptability of the 2,4,5-T herbicides is in effect concerned with the evaluation of a safe level of dioxin. The manufacture of 2,4,5-T and related compounds from trichlorophenol can result in the final product being contaminated with small amounts of 2,3,7,8-tetrachlorodibenzodioxin (dioxin), a singularly unfortunate choice of impurity since this is one of the most toxic compounds known to man. A review of the status of dioxin in order to add perspective to the various government actions (or inactions) seems opportune. The present article will concentrate on the more recent epidemiological aspects of the debate; we hope to review the extensive animal data in future articles.

The findings of the so-called Oregon study have catalysed current events on 2,4,5-T in the USA. In response to some earlier threatened EPA action on the herbicide, a group of eight women from Alsea, Oregon who had suffered a total of 13 miscarriages over a 5-yr period, had informed the Agency of their suspicions that forest spraying with 2,4,5-T may have been implicated. The results of an epidemiological study initiated to investigate the allegations were published in March 1979 (*Federal Register* 1979, **44**, 15874). Comparisons were made of the spontaneous abortion rates over the period 1972–1977 for the study region, a 1600-square-mile area of rural Alsea in which there had been extensive use of 2,4,5-T as a forest spray, a control rural area where there had been little or no use of 2,4,5-T and a control urban area (close to Alsea). The spontaneous abortion index of 80·8 for rural Alsea was significantly higher than the indices for either of the two control areas (43·8 and 65·4). Calculations of the monthly spontaneous abortion index indicated that there was a striking increase in abortions in the study area for the months June and July; 2,4,5-T forest spraying was known to have occurred mainly during the months of March and April but also in May, July and August. It was also calculated that there was a statistically significant correlation between the amount of 2,4,5-T used and the abortion index 2–3 months later. The EPA noted that on the basis of the relative toxicities of 2,4,5-T and dioxin in animal experiments, dioxin rather than 2,4,5-T should be considered the likely causative factor. Beyond this supposition, however, they provided no quantitative data on the exposure to dioxin (or indeed 2,4,5-T) of those residing in the study or control area.

Some critics have argued that the areas of land chosen by the EPA investigators were not carefully matched and could not be regarded as truly representative of an area sprayed with 2,4,5-T and a control area. Differences in hospital admissions for miscarriage could also have varied among the regions chosen for study, thus invalidating the study's conclusions (*Nature, Lond.* 1980, **284**, 111). Whilst no detailed critique of these findings has yet been forthcoming from the UK authorities, the view of the Advisory Committee on Pesticides has been made known in a reply to a parliamentary question on 26 July 1979. They did not accept that the stated abortion rates were valid "in either scientific or statistical terms" and concluded that the study did not afford any grounds for further restrictions on 2,4,5-T use.

The results of a recent Swedish study, (Hardell & Sandström, *Br. J. Cancer* 1979, **39**, 711), suggesting that 2,4,5-T and related herbicides may pose a carcinogenic hazard has played only a peripheral role in the dioxin debate so far. In this carefully designed experiment, patients with soft-tissue sarcomas were compared with a control group with respect to their exposure to 2,4,5-T, 2,4-dichlorophenoxyacetic acid and chlorophenols. Since soft-tissue sarcoma is recognized as a rare tumour type, the investigators considered a matched case-control study was the most appropriate method for identifying possible causative factors. Four controls, matched for sex, age and place of residence, were selected for each of 51 cancer patients; an estimation of exposure to the phenoxyacetic acids and chlorophenols was achieved by means of a questionnaire completed by the individuals themselves or, in the case of the 31 cancer patients who had died and their controls, by the next-of-kin. Exposure to these chemicals mainly resulting from employment in some aspect of forestry was registered in 36·5% of the cancer patients but only in 9·2% of the 206 controls. It was calculated that exposure was associated with an almost six-fold increase in the risk of developing soft-tissue sarcoma. When the phenoxyacetic acids were considered separately the calculated increase in risk was slightly less. It was not possible from the study to separate the effects of the herbicides themselves from their dioxin or dibenzofuran impurities and no data on the levels of dioxin found in herbicides typically used in Sweden were reported.

Media awareness of dioxin's toxicity is not new. An unhappy history includes a number of industrial accidents where reactors manufacturing trichlorophenol have gone out of control with subsequent formation and escape of dioxin. In all but one of these incidents the dioxin remained within the factory confines, and since only a limited number of workmen were subsequently troubled by symptoms of dioxin poisoning, popular interest was minimal. The same cannot be said of the latest dioxin accident, that occurring at the ICMESA plant at Seveso in July 1976 where uniquely the dioxin contaminated a large area surrounding the factory. The population of 733 living in the area of maximum contamination (Zone A) were not evacuated until 14 days after the accident (Pocchiari *et al. Ann. N.Y. Acad. Sci.* 1979, **320**, 311). Average soil dioxin levels in Zone A of between 15 and 580 $\mu g/m^2$ were recorded, a level with potentially serious toxicological consequences as indicated by the 25% animal mortality in this area. In the area of secondary dioxin contamination, designated Zone B, there were 0·3–0·5% animal deaths. Nevertheless, some 4000 people were living in Zone B, and no evacuation was attempted. Monitoring of the health of the Seveso population is now into its fourth year. Two of the most recent reports, one from Reggiani (*Arch. Tox.* 1979, Suppl. 2, 291) of Hoffman La Roche which owns ICMESA and the other from members of an Italian research institute (Pocchiari *et al. loc. cit.*) provide clinical data up to the end of 1978.

Chloracne, a characteristic feature of dioxin poisoning, developed in 7% of the population of Zone A, children making up some two-thirds of this total, and in 1·2% of those in Zone B. However, the number of chloracne cases was greatly reduced over the 18–24 month surveillance period. A similar improvement has not been seen in the neurological studies. In the first screening in 1977, 30 out of 446 people (6·7%) seen from Zone A were found to have clinical signs of neurological damage, mainly involving the peripheral nervous system. By 1978, the percentage of people affected had increased to 11·7 (24 out of a smaller sample of 205). There was no correlation between skin lesions and the neurological symptoms (Pocchari *et al. loc. cit.*).

Although almost a third of the 1650 Seveso residents examined had enlarged livers, all but 8% of this total could be ascribed to either alcohol or viral hepatitis (Pocchiari *et al. loc. cit.*). Biochemical evidence of liver damage—increase in serum enzyme levels—was seen in Zone A individuals at a higher incidence than in controls, and the percentage of people involved had increased between the first screening (July–October 1976) and the second screening (October 1976–January 1977). Nevertheless, by the third and fourth screenings (covering a period up to March 1978), the percentage of abnormal values had decreased and was below that of the first examination, suggesting that the initial toxic injury may have been repaired. Cytogenetic screening identified only a slight increase in the incidence of chromosomal aberrations in lymphocytes taken from the peripheral blood of people from Zone A (125) or a group of 59 ICMESA workers when compared with controls.

The immunological status of 45 children aged from 3 to 7 of Zone A, 21 of whom had chloracne, was comparable with that of a group of 45 controls. Serum immunoglobulin and circulating complement levels, lymphocyte response *in vitro* and percentages of the lymphocyte subpopulations as well as a full haematological examination were carried out (Pocchiari *et al. loc. cit.*).

Because of dioxin's potent teratogenicity and general embryotoxicity in experimental animals, the gynaecological and obstetric aspects of the Seveso monitoring have been afforded the most publicity (Reggiani *loc. cit.*). The relative proportion of spontaneous abortions seen in the Seveso population in the two 6-month periods following the ICMESA accident were in the range 9–11%. This was no different from the totals for previous years and was still lower than the "15–20% of all pregnancies generally accepted for the western countries". More detailed examination of the data showed for example an increase in abortions in the fourth quarter of 1976 to 21% for Zone A alone, but Reggiani thought that this and other fluctuations were related to factors other than TCDD contamination. Although there was an increase in the number of congenital malformations from 4/3902 live births in 1976 to 38/2774 in 1977, this was, in the opinion of Reggiani *et al.*, a consequence of previous under-reporting. Even the higher 1977 figure, 1·36% of live births, was significantly lower than the average incidence of 2·5–3·0% seen in "western countries" or the 2·32–2·97% found in other parts of Italy. Both the polymorphism of the malformations and their geographical distribution also weigh against a dioxin-related effect. Embryomorphological examination of the 34 aborted foetuses of Seveso women revealed no signs of abnormalities; all of the foetuses had the normal number of chromosomes and the abnormal chromosome patterns observed reflected those seen in the amniotic fluid. The postnatal development of a large proportion of the 2000 babies born to mothers living in Zone A and B or in other parts of Seveso since the ICMESA explosion is currently being followed by regular paediatric examination (Pocchiari *et al. loc. cit.*).

Commenting on the Seveso prospective survey Hay (*Ann. N.Y. Acad. Sci.* 1979, **320**, 321) drew attention to the logistic problems of monitoring a large population exposed to small dioxin concentrations. To overcome them he thought it might be better to study a smaller group of the Seveso population who had been exposed to the highest dioxin levels and compare them with a non-exposed control group. He also suggested a more productive research effort might be aimed at a detailed examination of those groups of workers subjected to high levels of dioxin as a result of accidents at trichlorophenol plants. Exploring this avenue, however, may also have its difficulties if experiences from the UK company, Coalite and Chemical Products, are typical (Hay, *Nature, Lond.* 1980, **284**, 2). An investigation of the 126 workers of this company, exposed to dioxin between 1968 and 1971 during the manufacture of 2,4,5-T, was originally conducted as a result of pressure from the Health and Safety Executive. The results, which the company has not yet published in full, evidently indicated that the workforce had a greater incidence of impaired liver function than did a control group of 31. Furthermore, the higher levels of serum cholesterol and triglyceride

and the lower high-density lipoprotein levels which were also seen in the dioxin-exposed cohort, are factors commonly associated with an increased risk of cardiovascular disease. At present only a second, and smaller, study involving eight Coalite workers suffering from chloracne has appeared in the medical press (Walker & Martin, *Lancet* 1979, **I,** 446). Again, increases in serum cholesterol and triglyceride (and δ-glutamyl transpeptidase) and reduced serum high-density lipoprotein was observed. Calls for the full data from the larger study have so far been unsuccessful; the Health and Safety Executive has no legal power to demand the medical records of the Coalite workers as the company no longer manufactures 2,4,5-T.

According to Hay (*Nature, Lond.* 1980, **283,** 613) a world-wide total of at least 16 trichlorophenol-dioxin plant accidents have occurred and so there is plenty of scope for evaluating dioxin's toxicity profile in man. Unfortunately, as the Coalite episode indicates, the companies involved may be loath to come forward with data. Hay also notes that Monsanto are about to publish some of their epidemiological findings and it is to be hoped that this will encourage further reports; at present only limited studies following a few of these episodes have been published. Preliminary indications are that Monsanto's study of 228 workers exposed to dioxin, 117 as a result of a plant accident in 1949, produced reassuring results.

All of the epidemiology recently published suffers the same handicap—ignorance of the exposure levels responsible for the observed effects. Even in the case of the Seveso incident, where scientific curiosity has been substantial, estimates on exposure vary widely. Pocchiarri *et al.* (*loc. cit.*) considered that about 2 kg of dioxin was distributed unevenly over about 110 hectares of the most heavily contaminated land. Other guesses of the total amount of dioxin released have ranged up to 5 kg (*Federal Register* 1978, **43,** 17123) and even higher. Uncertainties over dose are even more marked in the Oregon study. The only quantitative data indicates that 2,4,5-T typically used in the USA had a dioxin content ranging from less than 0·01 ppm up to 0·025 ppm (*ibid* 1979, **44,** 15874), but how this relates to the intake of any specific individuals or groups is impossible to estimate.

It does seem reasonable to assume that the Seveso population will have been subjected to a greater body burden of dioxin than those non-occupationally exposed to 'impure' 2,4,5-T. The two circumstances are not entirely comparable in that Seveso is effectively an acute toxicity problem (at least with respect to residents of Zone A) whereas 2,4,5-T herbicide use results in chronic exposure to dioxin.

Prospective surveys facilitate investigations of toxic effects that would not be possible in retrospective studies, thus the potential value of the Seveso disaster. Although the survey now covers a period of over 2 yr since the accident (from the point of view of published data), it is still too early to draw conclusions on dioxin's toxic effect in man. For example, one of dioxin's target organs is the thymus, with consequent damage to the immune system. The relatively long half-life of lymphocytes in man means it could still be too early to see clinical immunological effects in the Seveso population. All the effects being observed at present are the early manifestations of dioxin's toxicity. It will be several decades before the Seveso population provide valid data on carcinogenicity.

One aspect of the Seveso study has been disappointing, and this concerns embr otoxicity, an unfortunate weakness since effects on reproduction were highlighted by the Oregon study. Many of the Seveso women, pregnant at the time of the ICMESA explosion, evidently travelled to other countries for abortions because of difficulties in obtaining the operation in Italy and in this way valuable information was lost (Hay, *Ann. N.Y. Acad. Sci.* 1979, **320,** 311).

Multiple misfortune surrounding trichlorophenol manufacture may enable us eventually to obtain a direct but qualitative understanding of dioxin's toxicity in man. It will take some strong lobbying and a number of years more before the available epidemiological data are fully exploited, and even then the nature of the various accidents will probably preclude a quantitative appreciation. The epidemiology must be supplemented, therefore, by experimental toxicology. Future articles will attempt to review the more important animal studies and identify the areas requiring further investigation.

[J. Hopkins—BIBRA]

MORE EXPERIMENTAL DATA ON HEXACHLOROBENZENE

Hexachlorobenzene (HCB) is a fungicide which has become a persistent and widespread global pollutant. Its toxicity to man was well documented after an episode in Turkey when seed treated with HCB was consumed in bread, resulting in a syndrome of porphyria and liver damage, with a mortality rate in children under 5 yr of 95% (*Cited in F.C.T.* 1967, **5,** 429). Many reports have since been published of the toxic effects, metabolism and tissue distribution of HCB in animals (*ibid* 1976, **14,** 351; *ibid* 1978, **16,** 287).

The carcinogenicity of HCB in golden hamsters has been reported by Cabral *et al.* (*Nature Lond.* 1977, **269,** 510) who fed the animals a diet containing 50, 100 or 200 ppm HCB (more than 99·5% pure) for life. At wk 50, survival among the treated animals was 71%, similar to that in the controls. After 70 wk the survival of males and females fed the highest level had declined compared with that of the other groups, and the males in this group showed marked reduction in weight. Controls showed a tumour incidence of 10%, compared with 95% in those fed 200 ppm HCB. There was a dose-response relationship in the mean number of tumours per hamster and in the percentage of animals with more than one tumour. Alveolar adenomas of the thyroid appeared in treated animals but not in the controls, and the highest incidence was in males fed 200 ppm HCB. Hepatomas, which were also absent from controls, appeared in 47% of the group

fed 50 ppm HCB and in 85% of those fed 200 ppm HCB. Liver haemangioendotheliomas affected 11·6% of females and 35% of males fed 200 ppm HCB, and were absent from the controls. Adrenal neoplasms showed some increase in the treated animals but no clear dose-response relationship was apparent.

The same group (Cabral *et al. Int. J. Cancer* 1979, **23,** 47) also demonstrated the carcinogenic effect of HCB in mice. Swiss mice were fed 50, 100 or 200 ppm HCB for 101–120 wk. Survival rates were reduced in both males and females fed the highest dose and growth rate was depressed in the treated animals, particularly in the males fed 100 or 200 ppm HCB. No liver-cell tumours were found in mice fed 50 ppm HCB or in the controls, but they occurred in 10% of both sexes fed 100 ppm and in 34% of the females and 16% of the males fed 200 ppm. The finding of lymphomas in only 11% of those fed 200 ppm HCB, but in 35% of the controls, was probably due to the shorter lifespan of the treated animals. Similarly, there was a greater number of animals with lung tumours among the controls than among treated animals, but the numbers of nodules per mouse were similar in all groups. Of 60 mice fed 300 ppm HCB for 15 wk and then returned to the control diet, most males died early but this effect was less apparent in females. Two mice in this group developed liver-cell tumours, and the lymphoma incidence was relatively low in the males, but otherwise the occurrence of tumours in this group was similar to that in the controls. No evidence of metastasis of any liver-cell tumours was found in this study. In the hamster study (Cabral *et al.* 1977, *loc. cit.*) some metastasis of the hepatic haemangioendotheliomas was detected.

The effect of HCB on rhesus monkeys has been investigated by Knauf & Hobson (*Bull. envir. Contam. Toxicol.* 1979, **21,** 243). Adult females were given 8, 32, 64 or 128 mg HCB/kg/day, as a suspension in 1% aqueous methylcellulose, by gastric intubation for 60 days. The highest and lowest dose levels and the vehicle alone were each given to two monkeys, and the intermediate doses to one monkey only. Blood was taken at 2-wk intervals for a wide range of serum analyses and for blood cell counts and haemoglobin and haematocrit determinations. At the end of the treatment all the animals except one control and one given 8 mg/kg/day were killed. Tissue samples were taken for HCB determinations and a histopathological study (Iatropoulos *et al. Toxic. appl. Pharmac.* 1976, **37,** 433) was carried out. Of the six treated monkeys, five (not specifically identified) began to lose weight by wk 4, but there were no significant changes in the haematological values. Appetites were apparently depressed by the treatments but no details are given. In all but one (8 mg/kg/day) animal, cholesterol values were significantly lower than the pretreatment value after treatment for 3, 5 and 8 wk, but the reason for this is not clear. On day 60, one monkey given 128 mg HCB/kg/day showed depression of serum potassium and raised serum glutamic–oxalacetic transaminase and urea nitrogen levels. This animal showed marked weakness and lethargy on day 52, while the monkey given 64 mg HCB/kg/day (no. 627) showed severe tremors and muscular weakness during days 58–60. Serum levels of HCB were not directly related to the dose ingested, but they showed a general rise as the cumulative dose increased. Tissue levels of HCB were higher in fat and bone marrow than in other tissues, and in the adrenals the cortex showed a consistently higher level than the medulla. The HCB levels in serum and non-fatty tissues seemed to be directly related to the individual amounts of body fat, which appeared to serve as a protective reservoir. The animal that showed severe signs of neurological damage (no. 627) showed a much higher level of HCB in the brain (and other tissues) than the others and was virtually devoid of any adipose tissue.

Iatropoulos *et al.* (*loc. cit.*) reported that the principal morphological findings in these monkeys were cortical atrophy of the thymus, and degenerative changes in the ovary, involving the primary follicles, germinal epithelium and stroma, in the liver, resembling those seen in porphyria, and in the kidneys. There were no changes in the skin or nervous tissue. The mechanism by which HCB or its metabolites induce such organic lesions is not understood but data continue to accumulate on the distribution and metabolism of HCB in various species. Yang *et al.* (*J. agric. Fd Chem.* 1978, **26,** 1076) used tracer doses of [^{14}C]HCB to study these aspects in rhesus monkeys and rats. In monkeys given a single iv dose of [^{14}C]HCB (between 0·22 and 0·38 mg/kg), tissue concentrations were determined after 100 days, 6 months and 1 yr, respectively. During the intervening period, the animals were kept in metabolism cages for regular collection of faeces and urine. Fat and bone marrow showed the highest accumulation of ^{14}C, followed by adrenal tissue, and the activity in the liver and central nervous tissue was also relatively high. The general pattern was an inverse relationship between the radioactivity level in each tissue and the length of the experimental period. The main route of ^{14}C excretion in these monkeys was the faeces, which accounted for 17 and 28% of the dose in the 100-day and 1-yr periods, respectively. Urinary excretion was of little importance, amounting to less than 2% of the dose in 3–6 months. Biliary excretion is thus an important mechanism for HCB disposal in the rhesus monkey. About 90% of the faecal radioactivity, but none of the urinary ^{14}C, was in the form of unchanged [^{14}C]HCB. Pentachlorophenol was a major faecal metabolite and was accompanied by traces of pentachlorobenzene. As in the rat, the urinary activity was in the form of polar metabolites. The general picture in rats given an iv dose of [^{14}]-HCB was similar to that in the monkey, but the rate of metabolism was somewhat less slow. In rats HCB binds to the erythrocytes and the level of radioactivity in whole blood is consequently much higher than that in the plasma, but this effect was not demonstrated in the monkeys.

In a study of the transplacental transfer of HCB (Courtney *et al. Envir. Res.* 1979, **19,** 1), 10, 50 or 100 mg HCB/kg was given to mice in a single intragastric dose on day 11 or 16 of gestation, or daily between days 6 and 11 or 6 and 16. Rats were given 10 or 50 mg HCB/kg on days 6–12 or 12–16 of gestation. The animals were killed 24 hr after the last dose and the HCB content of maternal and foetal tissues was determined. The concentration of HCB in foetal and placental tissue of both rats and mice was dose-

dependent, with levels in the foetuses being generally lower than those in the corresponding placentae. Multiple small doses produced greater concentrations of HCB in mouse maternal and foetal tissue than did an equivalent amount given in one dose. In rats, foetal and placental accumulation of HCB was greater when the dose was given late in gestation. Irrespective of the dose, foetal resorptions (defined as dead and resorbed foetuses) showed higher HCB concentrations than live foetuses. Courtney & Andrews (*Toxicology Lett.* 1979, **3,** 357) have shown that in mice HCB is readily mobilized from maternal depots during gestation and transferred across the placenta to the foetuses. Following intragastric doses of 10, 50 or 100 mg HCB/kg, all maternal tissues, except the spleen, showed a dose-related accumulation. Whether treatment was given before implantation (on days 0–5 of gestation) or after implantation (on days 6–11) had no significant effect on the HCB levels in maternal blood or liver, but there was a greater concentration in the body fat in mice treated before implantation. By day 12 of gestation foetal and placental HCB concentrations were significantly higher in the animals treated before implantation than in those similarly treated on days 6–11. The foetal and placental levels resulting from treatment on days 6–16 were in turn higher than those following pre-implantation treatment, but it is clear that the maternal body burden of HCB may be an effective source for the foetus even when no maternal exposure occurs during gestation. Just as very thin animals may be more susceptible to the toxic effects of HCB (Knauf & Hobson, *loc. cit.*) because of a lack of depot fat to absorb the offending material, so a mobilization of fat stores for one reason or another (in starvation, for example, or during the later stages of gestation) could lead to delayed exposure of the animals to effective doses of HCB.

[P. Cooper—BIBRA]

MORE ON ISOCYANATE SENSITIZATION

The question of what constitutes a safe working level of toluene diisocyanate (TDI) is still the subject of debate. The view that a TLV of 0·02 ppm (0·14 mg/m^3) protects most workers derives from indications that exposure to TDI below this level is not associated with a measurable deterioration in pulmonary function (*Cited in F.C.T.* 1980, **18,** 199), but other authors have stressed the difficulty of establishing the atmospheric level of TDI below which sensitization will not be induced in susceptible individuals. The continuing importance of this field of study was underlined by the NIOSH recommendations in 1978 (*ibid* 1980, **18,** 199), and a few more recently published papers are reviewed here.

Reference has already been made (*ibid* 1979, **17,** 90) to a prospective study, which began about 7 yr ago among workers at a new TDI-producing plant and is still producing data. During this study, it was found that although exposure for several years to concentrations at or sometimes slightly above the TLV had no demonstrable effect on pulmonary function, about 5% of these workers developed a clinical sensitivity to TDI. Butcher *et al.* (*J. Allergy clin. Immunol.* 1979, **64,** 146) have now reported further work in which they attempted to establish the mechanisms underlying this bronchial hyperreactivity. Selected workers with a history of sensitivity to TDI, as evidenced by wheezing, tightness of chest and shortness of breath, were used for this study. They had developed these symptoms within a year of first exposure, and usually within 3 months. Provocative inhalation challenge was used to test 28 workers for reaction to TDI, and 14 of these were also given a challenge with methacholine. TDI challenge was initially with a 15-min exposure to 0·005 ppm, but this was increased to 0·01 and 0·02 ppm on successive days if the airway response was insufficient to produce a 20% reduction in the 1-sec forced expiratory volume (FEV_1). The same criterion was applied to methacholine challenge tests involving 1–5 breaths of concentrations of 5, 10 and 25 mg/ml. All individuals who reacted to TDI with a reduced FEV_1 and immediate and/or delayed (by at least 1 hr) bronchospasms also reacted positively to methacholine, although the reverse was not the case. Changes in plasma histamine, total complement levels or split products of complement were not observed, but TDI reactors showed decreased lymphocyte cAMP dose-response slopes when stimulated with isoprenaline, prostaglandin E_1 or TDI. These findings confirm that reactivity to TDI is not a non-specific irritative reaction, but they fail to provide evidence in support of an immunological mechanism. Rather they suggest a pharmacological mechanism involving impairment of the adrenergic response.

Another extension of a previous study has been published by Pham *et al.* (*Ann. occup. Hyg.* 1978, **21,** 271). In their earlier paper, this group (Pham *et al. ibid* 1978, **21,** 121) reported that although they found no abnormal incidence of bronchitic or asthmatic symptoms or allergic reactions among workers in a factory where the average atmospheric isocyanate levels were below 0·02 ppm, their observations led them to suspect that such atmospheric conditions could give rise to diffuse interstitial pulmonary fibrosis, with involvement of an allergic component. The investigation was therefore extended to employees of another factory where large amounts of TDI were handled and where some workers encountered higher levels of exposure. Of the 125 employees studied, 11 (group I) were not exposed to isocyanates or other industrial chemicals, 29 (group II) were exposed only indirectly and slightly, and 85 (group III) had direct and substantial exposure to TDI. The mean concentration in the general factory atmosphere was close to the TLV, but in some areas where injection or moulding took place the level occasionally reached 1·9 ppm (14 mg/m^3). Contact eczema was seen in seven workers in group III. Chronic rhinorrhoea, tightness of the chest, wheezing during work, chronic asthma, chronic bronchitis and dyspnoea showed an increased incidence in groups II and III, and abnormalities of ventilatory function were also

more frequent in these groups than in group I. There was no significant inter-group difference in gas-exchange capacity, as measured by a carbon monoxide transfer factor test. It was noted that at exposure levels above the TLV, respiratory signs and symptoms became more common and acute, whereas at lower exposure levels the disturbances were more chronic and symptoms were milder, but lung-function tests again indicated the possibility of some degree of fibrosis.

Immunological changes in 25 workers with a history of long-term exposure to TDI have been reported by Karol *et al.* (*J. envir. Sci. Hlth* 1979, **C13,** 221), who took serum samples for examination by immunoenzymatic and radioimmunoassay techniques. Tolyl-specific IgE antibodies were detected only in workers who showed clinical hypersensitivity to TDI and who had been in contact with the compound during the 6 months prior to testing; in one who had not been exposed to TDI for at least 2 yr no IgE antibodies could be found. No tolyl-specific IgG antibodies could be detected in workers in whom the IgE antibodies were found, nor in other workers who had been exposed to TDI for many years. It is unlikely that IgG antibodies were present at an undetectable level, since the radioimmunoassay used was extremely sensitive. The authors suggest that the unusual finding of IgE antibodies without IgG antibodies may be attributable to the high reactivity of TDI and its consequent ability, when inhaled in very low concentrations, to stimulate production of IgE, which is produced predominantly in the respiratory and gastro-intestinal tracts. Another suggestion involves a possible immunological action through a suppressor mechanism, by analogy with a recent report (Thomas *et al. Immunology* 1978, **35,** 41) that sensitization of mice with picryl chloride, another highly reactive chemical, induced suppression of IgG antibodies without suppressing IgE antibodies.

TDI does not have a total monopoly of isocyanate-induced health problems. Cockcroft & Mink (*Can. med. Ass. J.* 1979, **121,** 602) described the case of a 44-yr-old man who, having been a spray-painter for 28 yr, had developed a productive cough and increasing shortness of breath, particularly at night, some 12–18 months after the introduction of a new spray paint containing a hexamethylene diisocyanate (HMDI) hardener. He wore a protective mask while spraying, had no history of chest or atopic disease, and had not smoked for 5 yr. Although in a series of tests he was able to tolerate 325 ml of a 50% solution of isocyanate-free paint sprayed over a 5-min period, he could tolerate no more than a 2-min spraying of 130 ml, followed by a 5-min exposure to the atmosphere so created, when tested with an otherwise similar paint dilution containing 0·6% added HMDI. With the control (HMDI-free) inhalation, the FEV_1 showed a reduction of less than 10% in 6 hr; with the HMDI exposure the FEV_1 fell by 18% at 10 min, and this fall was followed by a spontaneous improvement and then by a late asthmatic response in which the maximal FEV_1 fall was 41% at 3 hr. When a histamine inhalation test was performed on this patient 16 hr after the end of a 16-hr occupational exposure to the paint spray, the concentration of histamine required to produce a 20% reduction in the FEV_1 (PC_{20}) was reduced 15-fold, and 6 hr after the 7-min test exposure to HMDI there was a further eightfold fall in the PC_{20}, which only returned to normal in a further 6 days.

[P. Cooper—BIBRA]

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ABSTRACTS AND COMMENTS

NATURAL PRODUCTS

Citral and fertility

Toaff, M. E., Abramovici, A., Sporn, J. & Liban, E. (1979). Selective oocyte degeneration and impaired fertility in rats treated with the aliphatic monoterpene, Citral. *J. Reprod. Fert.* **55**, 347.

The aliphatic monoterpene citral (3,7-dimethyl-2,6-octadienal) occurs naturally in the essential oils of lemon and lime, in lemongrass oil and in the essential oils of other species, and it is widely used as a food flavouring and as a fragrance raw material in the cosmetics industry. No adverse macroscopic effects were observed in rats fed high levels of citral (up to 10,000 ppm) in the diet for 13 wk, but citral had teratogenic effects on chick embryos when injected into the eggs (Opdyke, *Fd Cosmet. Toxicol.* 1979, **17**, 259). The authors cited above have now studied the effects of citral on reproduction in female rats.

Each of a group of 28 virgin Wistar rats was given an ip injection of 300 mg citral/kg body weight (as a solution in neutralized olive oil) on the day of pro-oestrus for six consecutive cycles. Citral dissolved in ethanol was administered daily at a level of 460 mg/kg/day to the shaved dorsal skin of groups of 19 or 17 Wistar rats for 60 or 100 days, respectively. Control animals were treated with neutralized olive oil ip (22 rats) or topically with ethanol (19 rats) and a further control group of 28 rats was left untreated. Some of the rats were killed on the first day of pro-oestrus following the final treatment. The remainder were mated. The resultant offspring were counted, and weighed on days 1, 7 and 21 after birth. Both mothers and offspring were killed 21 days after delivery and the mothers were autopsied. The numbers of implantation sites were recorded, the reproductive organs and viscera were examined macroscopically and the ovaries and uteri were examined histologically.

No toxic effects were observed in any of the treated rats. Citral had no effect on the duration of the oestrus cycle or on the histology of the uteri of either the non-pregnant rats or those killed 21 days after delivery. However, citral administered by either route significantly reduced the number of implantations and the size of the litters and significantly increased the number of post-implantation losses compared with the vehicle-treated and the untreated controls. Neonatal mortality was significantly higher among the offspring of rats treated with citral for 100 days; all these offspring died soon after birth.

The ovaries of treated rats were smaller than those of control rats. The histological appearance of the ovaries of citral-treated rats killed 21 days after delivery was similar to that of the citral-treated non-pregnant rats. The numbers of normal primordial-primary and intermediate follicles were significantly lower in treated rats than in the corresponding controls and the number of primordial-primary follicles showing oocyte degeneration was significantly increased. Significant increases in oocytic degeneration of intermediate as well as primordial-primary follicles occurred in the non-pregnant rats that were treated ip with citral and in those to which citral was administered topically for 60 days. The incidence of degenerative changes (atresia) involving both oocytes and follicles was similar in both the control and the treated groups; citral only increased the incidence of the type of atresia that affected the oocytes alone. In all of the rats treated topically with citral for 100 days the number of corpora lutea was significantly reduced compared with the group treated for 60 days.

The results indicated that citral did not interfere with the endocrine status of the rats since it did not affect the numbers of follicles showing characteristics of physiological atresia and since it affected neither the oestrus cycle nor the histology of the uterus. The apparently specific effect of citral on the oocytes but not on the follicles merits further investigation. In view of the reduced number of implantation sites, the increased post-implantation loss and the smaller litters among the treated rats and, in one group, the increase in neonatal mortality, it is possible that treatment with citral resulted in the release of defective ova. The doses given in this study were very high (the authors note that the LD_{50} for citral administered ip is 60 mg/kg/day). However, citral is used extensively in foods and cosmetics and it is possible that similar effects on ovarian histology could occur in humans exposed for long periods to smaller doses; further studies would appear to be warranted.

Methylxanthines and dye absorption

Nakamura, J., Takamura, R., Kimura, T., Muranishi, S. & Sezaki, H. (1979). Enhancement effect of methylxanthines on the intestinal absorption of poorly absorbable dyes from the rat small intestine. *Biochem. Pharmac.* **28**, 2957.

The authors cited above have previously suggested that the poor intestinal absorption of phenol red (PR) is due to its low affinity for the intestinal mucosa and its poor lipid solubility, while bromophenol blue (BPB) is rapidly bound to the brush borders and transport from the intestinal epithelial cells to the blood vessel is the absorption barrier for this dye (Nakamura *et al. Chem. pharm. Bull., Tokyo* 1976, **24**, 683). The study described below was undertaken to determine whether the methylxanthines, theophylline and caffeine, have any effect upon the intestinal absorption of PR and BPB in rats.

Male Wistar albino rats were anaesthetized, the

small intestines were cannulated and the bile ducts were ligated. A solution of the dye was perfused through the cannulated intestine at a rate of 5 ml/min. At the end of the perfusion period the amount of dye that had disappeared from the perfusion fluid was determined spectrophotometrically. The level of dye in the intestinal tissues was measured and the absorption of the dye was calculated. The presence of 5 mM-theophylline or 5 mM-caffeine did not alter the pH (6·5) of the buffered perfusate or the apparent partition coefficients of the dyes. However, the disappearance of PR and BPB from the intestinal lumen was significantly increased by the presence of either of the methylxanthines. The tissue accumulation and net absorption of BPB were also increased, indicating that the presence of caffeine or theophylline increased the transport of BPB from the intestinal lumen into the portal vein.

When the intestine was pretreated by perfusion with 1, 3 or 5 mM-theophylline for 10 min and then perfused with the dye solution the disappearance of PR from the luminal solution was increased. Similarly, pretreatment with 3 or 5 mM-theophylline increased the removal of BPB from the perfusion fluid and pretreatment with caffeine also enhanced the intestinal transfer of both dyes. These results indicate that theophylline and caffeine alter the permeability characteristics of the intestinal mucosa and thus enhance the uptake of these dyes, which are usually poorly absorbed. However, a single iv dose of 15 μmol theophylline or 25 μmol caffeine had no effect on the intestinal transfer of PR or BPB, although each enhanced the absorption of salicylate. Therefore it seems that the enhanced absorption of the dyes was not due to a methylxanthine-induced increase in blood flow. It is possible that the increased absorption may be related to a methylxanthine-induced accumulation of cyclic AMP. The disappearance of PR and BPB from the luminal solution was significantly increased when the lumen was pretreated with 0·5 mM-isoproterenol, which is known to produce intracellular accumulation of cyclic AMP. Further investigation is required to clarify this possible relationship to the cyclic-AMP system.

AGRICULTURAL CHEMICALS

DBCP—a possible mechanism?

Sandifer, S. H., Wilkins, R. T., Loadholt, C. B., Lane, L. G. & Eldridge, J. C. (1979). Spermatogenesis in agricultural workers exposed to dibromochloropropane (DBCP). *Bull. envir. Contam. Toxicol.* **23,** 703.

Lee, I. P. & Suzuki, K. (1979). Induction of unscheduled DNA synthesis in mouse germ cells following 1,2-dibromo-3-chloropropane (DBCP) exposure. *Mutation Res.* **68,** 169.

Published evidence for the interference of 1,2-dibromo-3-chloropropane (DBCP) in human spermatogenesis has been derived largely from studies of workers exposed industrially during the manufacture of this nematocide (*Cited in F.C.T.* 1978, **16,** 498; *ibid* 1979, **17,** 555; *ibid* 1980, **18,** 99). The first study cited above covered a wider range of occupational groups involved in some way with DBCP, including farmers, agricultural workers, research workers, commercial applicators and sales personnel (together designated as 'users') as well as formulators. The sperm counts of these 73 subjects were compared with those recorded by MacLeod & Wang (*Fert. Steril.* 1979, **31,** 103) in a study of 9000 males with no known occupational exposure to toxic chemicals. The latter group showed higher sperm counts than either DBCP users or formulators, the frequency of sperm counts below 20 million/ml being 15, 32·5 and 75% respectively, in these three groups. Within the user group, however, the median counts in sales personnel and researchers did not show statistically significant differences from the 'MacLeod group'. In both formulators and users, a significant negative correlation was demonstrated between sperm count and the "DBCP use-index" (calculated by dividing the total weight used [handled] in the lifetime by the total number of days on which DBCP was used). A low sperm count was also frequently associated with high serum levels of follicle-stimulating hormone (FSH) and luteinizing hormone, but as in other DBCP studies in which FSH was found to be high, testosterone levels were unaffected.

DBCP is thus likely to affect spermatogenesis when encountered under conditions of use involving relatively severe exposure as well as under manufacturing conditions, but little still seems to be known about the mechanism underlying this effect (*Cited in F.C.T.* 1979, **17,** 555). To determine whether DBCP damages germ-cell DNA, Lee & Suzuki (cited above) treated prepubertal and adult male mice ip with the maximum tolerated dose (MTD), the LD_{05} or the LD_{10} (100–130 and 80–120 mg/kg, respectively, for the two groups) and subsequently assayed unscheduled DNA synthesis in premeiotic germ cells isolated 2, 4, 8 and 16 hr after treatment and in mature sperm. The single MTD (100 mg DBCP/kg) given to prepubertal mice induced statistically significant unscheduled DNA synthesis in premeiotic germ cells. This increased linearly for the first 8 hr after treatment, and with a higher dose the unscheduled DNA synthesis was still statistically significant after 16 hr. No effect on the DNA in mature sperms was seen with any dose, however, Failure to induce unscheduled DNA synthesis in mature spermiogenic cells has previously been reported after treatment of mice and rabbits with various chemical mutagens and after *in vitro* UV irradiation. The authors speculate that a reactive aliphatic epoxide produced by dehydrobromination of DBCP may react with DNA (and perhaps other macromolecules) in the premeiotic germ cells causing saturation of the germ-cell DNA-repair capacity and consequent germinal aplasia.

The dioxin saga—monkey reproduction

Barsotti, D. A., Abrahamson, L. J. & Allen, J. R. (1979). Hormonal alterations in female rhesus monkeys fed a diet containing 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Bull. envir. Contam. Toxicol.* **21,** 463.

Considerable publicity has been given recently to the possibility that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) has long-term effects on reproduction in man (*Cited in F.C.T.* 1980, **18,** 541). Experiments with rodents have shown that TCDD has teratogenic potential and that when it is administered to pregnant rats, it is capable of inducing the microsomal enzymes regulating steroid metabolism in the perinatal offspring (*ibid* 1977, **15,** 483). Reduced spermatogenesis has been observed in monkeys exposed to TCDD in their diet (Allen & Carstens, *Am. J. vet. Res.* 1967, **28,** 1513). In the study cited above, Barsotti *et al.* investigated the effect of low levels of TCDD on progesterone and oestradiol levels, menstruation and fertility in non-human primates.

Seven adult female rhesus monkeys with normal menstrual cycles and normal reproductive histories were fed approximately 3 μg TCDD/kg body weight (500 ppt in the diet) over 9 months. Exposure resulted in anaemia within 6 months, in severe pancytopenia within 9 months and in the deaths of five animals between months 7 and 12 after the start of the experiment. The length of their menstrual cycles and the intensity and duration of menstruation were not altered appreciably during the first 6 months of treatment. Nevertheless, after 6 months, measurement of progesterone and oestradiol levels revealed anovulatory patterns for both steroids in two of the animals, decreases in progesterone to 51·9 and 47·3% and in oestradiol to 43·2 and 50·4% of pretreatment values in two other animals, and a 27·6% decrease in progesterone levels, but normal oestradiol levels, in a fifth.

The monkeys were mated after 6 months and all eight of the untreated control animals conceived and delivered healthy infants. Of the seven treated animals the two that had normal progesterone and oestradiol levels after 6 months of treatment also conceived, but one later aborted a partially resorbed foetus. The animal that had maintained normal oestradiol but not progesterone levels also aborted during her pregnancy. The two monkeys that had reduced levels of both hormones and one that had an anovulatory pattern of steroid hormones failed to conceive, whilst the remaining anovulatory animal was not bred due to her debilitated state. Prolonged implantation bleeding in the monkeys that later aborted and intense sporadic menstruation in two that had not become pregnant, exacerbated their anaemic condition. There was a marked variation in the individual responses to this dose of TCDD. The only treated animal able to carry her infant to term had shown minimal effects of TCDD exposure throughout the experiment; her infant was healthy and when it had been weaned the mother's hormones returned to normal levels. The other animal that recovered fully had previously shown an anovulatory hormone pattern and had been unable to conceive. Her progesterone and oestradiol levels, and platelet and white blood cell counts gradually returned to normal and she eventually became pregnant and gave birth to a healthy infant.

It appears that if monkeys exposed to TCDD survive and are allowed to recover, they may re-establish a normal menstrual cycle and recover their ability to reproduce. The study showed that TCDD could interfere with the balance of certain steroid hormones in the monkeys before the more debilitating toxic effects were manifest. This indicates that reproductive dysfunction was not caused by severe pancytopenia. The authors further suggest that the transplacental induction of foetal microsomal enzymes by TCDD might have led to steroid imbalance and the observed foetal mortalities.

Diphenylamine in mice

Kronevi, T. & Holmberg, B. (1979). Acute and subchronic kidney injuries in mice induced by diphenylamine (DPA). *Expl Path.* **17,** 77.

The development of structural and functional changes in the rat kidney as a result of long-term ingestion of diphenylamine (DPA) has been followed in detail (*Cited in F.C.T.* 1979, **17,** 552). In the present study the effects of acute and subchronic administration of DPA were investigated and the results again demonstrated the nephrotoxicity of this material.

A single oral dose of 600 mg DPA/kg to 150 female mice was followed by 55 deaths in 10 days, 46 of which occurred within 5 days of the treatment. Autopsy of these mice revealed dehydration; the small intestine was flaccid and its contents were watery. Acute nephrosis, characterized by the swelling and vacuolization of the proximal tubular epithelia, the presence of cellular debris in the tubular lumen and the dilation of some tubules, was seen on microscopic examination of the kidneys. The 95 survivors, which were observed for a further month, showed no signs of toxicity.

In the subchronic investigation, 20 male mice received ten weekly doses of 1400 mg DPA/kg by gavage. Five animals died within 2 days of the initial dose and only half survived for longer than 4 months. The kidneys of the animals that died 3–4 months after the initial exposure were pale and irregular and the microscopic changes, which were present throughout, were most pronounced in the cortex where all normal structure had been lost. Dilation or atrophy of most of the proximal, distal and collecting tubules was observed, and many of the tubules contained desquamated epithelial cells or other deposits. In some cases the glomeruli were reduced in size and the tufts had become hyalinized.

Examination of the liver in both sexes revealed some activation of the reticulo-endothelial cells and the presence of a yellowish-brown deposit which, in the females, was shown to contain iron and therefore was assumed to be haemosiderin. The possibility that the deposit was of haematogenous origin in the males was not ruled out although no iron was detected.

OCCUPATIONAL HEALTH

Arsenic on the nerves

Feldman, R. G., Niles, C. A., Kelly-Hayes, M., Sax, D. S., Dixon, W. J., Thompson, D. J. & Landau, E. (1979). Peripheral neuropathy in arsenic smelter workers. *Neurology, Minneap.* **29,** 939.

Inorganic arsenic exposure has been associated with internal malignant neoplasms (*Cited in F.C.T.* 1979, **17,** 309), angiosarcoma of the liver (*ibid* 1976, **14,** 507) and lung, skin and lymphatic cancers (*Federal Register* 1975, **40,** 3392). Neurological effects may be an early sign of chronic arsenic intoxication, and epidemiological studies of the incidence of peripheral neuropathy in industrially exposed workers may therefore have considerable value.

A population of 70 workers in a copper-smelting foundry which produced arsenic trioxide as a by-product was compared with a control group of 41 workers comprising 28 workers from an aluminium plant and 13 employees of the city where the smelting plant was located. Forty of the 111 subjects showed clinical neuropathies, but there were alternative explanations other than arsenic exposure in 10 of them. Sensory neuropathies were more frequent among the arsenic-exposed subjects, affecting 30%, with motor components in 13%. The incidence of sensory neuropathy in the controls was 12%. The lowest values for nerve-conduction velocities were found in the arsenic-exposed workers. It was found that there was a correlation between neurological state and arsenic concentrations in the urine, hair and nails, but this did not include blood arsenic levels. Mean arsenic levels in the hair and nails of the high-exposure workers were 182·6 and 72·8 ppm, respectively, and of the low-exposure workers 8·9 and 21·1 ppm, compared with control values of 0·6 and 1·2 ppm, respectively. Arsenic workers at sites of high exposure had the highest urinary arsenic levels, 378 μg/litre urine compared with 74·1 μg/litre in the low-arsenic group. Arsenic was found in the blood of only seven of the 111 men, four in the exposed group and three not exposed. Chronic industrial exposure to arsenic thus affects peripheral nerve function to an extent detectable by routine clinical and electrophysiological tests. Neurological abnormalities may be evident when the possible carcinogenic effects of arsenic are not yet observable and may thus provide a screening method.

Chromosomes lead astray?

Verschaeve, L., Driesen, M., Kirsch-Volders, M., Hens, L. & Susanne, C. (1979). Chromosome distribution studies after inorganic lead exposure. *Hum. Genet.* **49,** 147.

In a number of previous investigations increased frequencies of chromosomal aberrations have been found in cultured lymphocytes of persons exposed to lead. In a study of 26 workers exposed to lead at a smelter, Nordenson *et al.* (*Hereditas* 1978, **88,** 263) reported a significant increase in the occurrence of gaps and chromatid and chromosome aberrations; the frequency of damage increased with the blood level of lead.

In the present study, nine workers occupationally exposed to inorganic lead (mean age 38·7 yr) with no history of genetic disorders and in normal health were compared with twelve controls (mean age 40·5 yr). The degree of exposure was reflected by the blood levels of lead which ranged from 50 to 84 μg/100 ml in the exposed group (mean 65·6 μg/100 ml) and from 10 to 25 μg/100 ml in the controls (mean 16·2 μg/100 ml). Haemoglobin levels were normal in the lead-exposed group but urinary levels of δ-aminolaevulinic acid indicated a high exposure to lead, being elevated to 14,040–57,050 μg/litre (mean 26,341 μg/litre), far above the normal limit of 1400 μg/litre. Chromosome analysis performed on lymphocytes (100 cells/subject) from 48-hr cultures showed that in the exposed group there was a significant increase in aneuploidy, due to an increase in hypoploidy, as well as a statistically significant increase in type-B cell aberrations (single chromatid breaks, gaps and isogaps). There was no significant difference between the control and exposed groups in the centromere to metaphase-centre distances. When the distances between the centromeres of homologous and non-homologous chromosome pairs were measured, 23 combinations were significantly ($P < 0{\cdot}05\%$) different between the two groups. No clear pattern emerged, however, apart from the consistent dissociation of one specific chromosome pair. This absence of a clear alteration pattern suggests a non-specific action of lead on the spindle apparatus and the lack of any striking alterations implies that the primary target of lead is not the spindle but the chromosomes. A general dissociation of acrocentric chromosomes in the lead-exposed group suggests a secondary effect of lead on the factors regulating the nucleolus organization.

Chronic effects of magenta, paramagenta and phenyl-β-naphthylamine

Green, U., Holste, J. & Spikermann, A. R. (1979). A comparative study of the chronic effects of magenta, paramagenta, and phenyl-β-naphthylamine in Syrian golden hamsters. *J. Cancer Res. clin. Oncol.* **95,** 51.

Magenta (a mixture of 4-toluidine derivatives) and one of its components, paramagenta, are widely used as dyes in the textile and paper industries. Their manufacture is, however, associated with a significantly high risk of bladder cancer amongst the production workers (Case & Pearson, *Br. J. ind. Med.* 1954, **11,** 213). Furthermore, sc administration of paramagenta has been shown to induce local sarcomas in rats (Druckrey *et al. Naturwissenschaften* 1956, **43,** 543). It has also been suggested that phenyl-β-naphthylamine (PNA), an antioxidant used in the rubber industry, might be linked with occupational bladder cancer because of the carcinogenicity of one of its metabolites, β-naphthylamine (*Cited in F.C.T.* 1978, **16,** 301). The present investigation was carried out to compare the biological effects of chronic exposure to magenta, paramagenta and PNA in the hamster.

Six groups of 40 male and 40 female Syrian golden hamsters were treated intragastrically (ig) twice weekly for life, with 400 or 600 mg magenta, 300 or 600 mg paramagenta, or 37·5 or 75 mg PNA/kg body weight. The higher dose level of each compound was not tolerated by the hamsters, and most of these animals died within the first 10 wk of treatment. During treatment, they showed rapid weight loss, haemorrhagic enteritis, stomach ulcers and diarrhoea. The three lower dose levels were, however, well-tolerated, and the body-weight developments of the hamsters thus treated were similar to those of the controls, as were their average lifespans. Moreover, macroscopic alterations and microscopic lesions were similar in both treated and control hamsters, and no significant increase in tumour incidence (including bladder cancers) was observed in the former.

Since magenta and paramagenta are only slightly water soluble, and may not be absorbed by the intestinal tract, the authors suggest that the local sarcomas observed in rat sc tissue following paramagenta administration could have been due to its physical properties. The authors also suggest that since no neoplastic growths were caused by ig administration of the lower PNA dose, biologically ineffective amounts of β-naphthylamine were produced by the metabolism of this quantity of antioxidant.

Mixed hazards of printing

Greene, M. H., Hoover, R. N., Eck, R. L. & Fraumeni, J. F. (1979). Cancer mortality among printing plant workers. *Envir. Res.* **20,** 66

The suggestion that printing workers have increased risks of certain cancers due to their exposure to a wide range of potentially hazardous chemicals is not new (Carter *et al. Fd Cosmet. Toxicol.* 1969, **7,** 53). The potential hazards associated with this industry change rapidly with the advent of new printing processes.

Among male employees of the Government Printing Office in Washington there were 347 cancer deaths during the period 1948–1977 (262 in whites and 85 in non-whites). The observed numbers of cancer deaths among different categories of print workers were compared with the numbers expected on the basis of figures for the Washington area. The proportion of lymphatic and haemopoietic cancers was significantly elevated among the print workers, with multiple myeloma accounting for half the excess. The frequencies of hepatobiliary and colon cancer were also increased but there was no increase in the proportion of rectal cancer deaths. An excess of prostate cancer was seen only in non-whites, of bladder cancer only in whites and of skin melanoma in both racial groups. There was a decreased frequency of deaths from some other cancers but none of these deficits were statistically significant. The excess of multiple myelomas was confined to composing-room employees whose main exposure is to lead. Until the early 1960s, bindery workers were exposed to benzene, which may therefore have been implicated in their leukaemia. The excess of colon cancers was thought to be related to socio-economic and lifestyle factors rather than to any occupational exposure. Solvents to which these workers were exposed included toluene, xylol, ethyl acetate and 1,1,1-trichloroethane, none of which are known or suspected carcinogens. The authors point out that this study was limited by the small numbers involved and by other methodological problems.

Tissue distribution of styrene

Plotnick, H. B. & Weigel, W. W. (1979). Tissue distribution and excretion of ^{14}C-styrene in male and female rats. *Res. Commun. chem. Path. Pharmac.* **24,** 515.

In man, exposure to styrene vapour has been shown to cause irritation of the eyes and nose, and depression of the central nervous system (*Cited in F.C.T.* 1968, **6,** 811; *ibid* 1979, **17,** 299). Similar effects have also been reported in experimental animals, and mild kidney and liver damage have also been observed (Spencer *et al. J. Ind. Hyg. Toxicol.* 1942, **42,** 295). In addition, human workers exposed to styrene have been reported to suffer an increase in glucose tolerance (Chmielewski, *Bull. Inst. mar. trop. Med. Gdansk* 1976, **27,** 177). A previous study (Savolainen & Vainio, *Toxicology* 1977, **8,** 135) indicated that following ip injection of styrene into rats, the liver, brain, kidney and duodenal contents of styrene were higher than those of the blood, lungs and spinal cord. In the present investigation, an attempt has been made to correlate tissue levels with target organ toxicity by studying the tissue distribution and excretion of styrene and its metabolites after oral administration to rats.

Uniformly ring-labelled [^{14}C]styrene was administered to male and female rats by oral intubation in a single dose of 20 mg/kg body weight. Rats were killed 2, 4, 8, 12, 24, 48 or 72 hr after administration, and the ^{14}C content of the major tissues, organs and body fluids was determined. Styrene was found to be well absorbed from the gastro-intestinal tract, less than 10% of the administered dose being found in the stomach and intestines 8 hr after treatment. Furthermore, excretion of styrene in the urine was rapid, with more than 60% of the dose appearing in the urine within 8 hr of administration. Whilst urinary excretion accounted for 90% of the dose within 24 hr of administration, faecal excretion accounted for less than 2%. After 48 hr, tissue levels of the radioactive label were below the limit of detection. Peak tissue levels were attained within 4 hr of administration. The organ with the highest concentration of radioactivity at all time intervals studied was the kidney, followed by the liver and the pancreas. No significant differences were observed between the male and female rats. The authors point out that since the kidney and liver are the two organs in which pathological changes have been observed following styrene exposure, there may well be a relationship between tissue distribution and styrene toxicity in these organs. They also suggest that there could be a relationship between the high levels of styrene and/or its metabolites in the pancreas and the reported increase in glucose tolerance in workers exposed to styrene.

ENVIRONMENTAL CONTAMINANTS

Cadmium in the body of smokers

Ellis, K. J., Vartsky, D., Zanzi, I., Cohn, S. H. & Yasumura, S. (1979). Cadmium: in vivo measurement in smokers and nonsmokers. *Science, N.Y.* **205,** 323.

Cadmium levels approximately twice as high as those of nonsmokers have been found in the renal tissue of smokers at autopsy (*Cited in F.C.T.* 1978, **16,** 499). The paper cited above further investigates the effect of smoking on the cadmium body burden *in vivo.*

A statistically significant relationship was demonstrated between kidney and liver cadmium values measured using the partial body neutron activation technique in 20 healthy, non-occupationally exposed, male volunteers (12 smokers and 8 nonsmokers). The urinary level, assessed by atomic absorption spectrophotometry, was found to be elevated when the kidney content was high but the plasma cadmium value, estimated by the same method, did not appear to be related to liver and kidney levels.

The mean cadmium levels for the organs and urine and the urinary β_2-microglobulin level were higher for smokers than for nonsmokers but only the values for the liver and kidney were significantly elevated. Cadmium levels in the livers and kidneys of smokers were 4·1 μg/g and 5·8 mg respectively compared with 2·3 μg/g and 3·1 mg respectively for nonsmokers. The smokers had an average smoking history of 38·7 pack-years (no. of packs/day × no. of years smoking) and it was calculated that smokers increased their body burdens of cadmium by 680 μg/pack-year above the amount accumulated from the diet. The data indicated that a male American nonsmoker (aged 52) had a total body burden of roughly 19 mg cadmium compared with 35 mg for a smoker (aged 50). Smoking may thus double the body burden of cadmium in man. This estimate is in agreement with that of an earlier author (*loc. cit.*).

The lack of a relationship between blood or urine data and kidney or liver burden of cadmium indicates that *in vivo* measurement techniques may be essential to determine cadmium levels in the liver and kidney.

Differing fates of oral and inhaled mercury

Fang, S. C. (1980). Comparative study of uptake and tissue distribution of methylmercury in female rats by inhalation and oral routes of administration. *Bull. envir. Contam. Toxicol.* **24,** 65.

Studies on the toxicity of mercury and on its environmental distribution have featured prominently in toxicological research in recent years. However, there have been relatively few studies on inhaled mercury. The results of one study on inhaled inorganic mercury in humans (*Cited in F.C.T.* 1979, **17,** 311) showed maximal accumulation of mercury in the red blood cells immediately after exposure and sequential accumulation of mercury in the plasma during the 20 hr following exposure and indicated that blood mercury levels can be used to monitor recent exposure to mercury. In the study cited above the uptake and distribution of inhaled methylmercury is compared with that of methylmercury given orally.

In the inhalation studies, groups of adult female Wistar rats were exposed continuously either to air containing ^{203}Hg-methylmercury chloride (^{203}Hg-MMC) at an initial concentration of 140 nmol/litre (air flow rate, 500 ml/min) for 6, 12, 18 or 24 hr, or to air containing initial concentrations of 50, 100 or 140 nmol ^{203}Hg-MMC/litre for 24 hr. The rats were killed at the end of the exposure period. For the oral studies, groups of rats were given either 3 or 9 μmol ^{203}Hg-MMC in corn oil and were killed 1, 2, 3 or 4 days after dosing. After inhalation of ^{203}Hg-MMC, the accumulation of ^{203}Hg in the organs and tissues increased linearly with the time of exposure and in proportion to the initial vapour concentration. (Only the initial ^{203}Hg-MMC concentration at the inlet of the metabolism chamber was determined; the true vapour concentration in relation to pulmonary uptake was unknown.) Excluding the hair, the accumulation of ^{203}Hg was greatest in the blood, followed in decreasing order of magnitude by that in the stomach, kidney, liver, spleen, lung, pancreas, heart, intestine, muscle and brain. The accumulation of ^{203}Hg in the lung was not high compared with that in other tissues, and therefore it seemed that inhaled mercury is rapidly transported throughout the body. After oral doses the highest level of ^{203}Hg accumulation occurred in most organs on day 1 after dosing and the levels decreased slowly from day 1 to day 4. However the level of ^{203}Hg in the brain steadily increased throughout the 4-day period. Significant differences were observed between the blood:kidney, blood:brain and blood:muscle ^{203}Hg ratios between the groups dosed orally and those that inhaled ^{203}Hg. This suggested a greater retention of ^{203}Hg in the blood after pulmonary uptake, but this apparent effect may have been due to the method of calculation used; the ratios for rats dosed orally were means for rats killed 1–4 days after dosing while the values for rats that inhaled ^{203}Hg were means for animals killed after 24 hr of continuous dosing. In the rats dosed orally, the blood:tissue ^{203}Hg ratios remained fairly constant throughout the 4 days after treatment, with the exception of the blood:brain ^{203}Hg ratio which gradually declined during this time. Determinations of ^{203}Hg in subcellular fractions of liver, kidney and brain did not reveal any significant differences between the groups in subcellular ^{203}Hg distribution.

Morphology of mercurial nephritis

Hinglais, N., Druet, P., Grossetete, J., Sapin, C. & Bariety, J. (1979). Ultrastructural study of nephritis induced in Brown Norway rats by mercuric chloride. *Lab. Invest.* **41,** 150.

It is known that chronic mercuric chloride exposure induces a two-phased nephropathy in Brown Norway rats (Druet *et al. Annls Immun., Paris* 1978, **129C,** 777), with fixation of antiglomerular basement membrane (anti-GBM) antibody as the first phase followed by an immune complex-type nephritis with disseminated extrarenal deposits in the second. The

study cited above was designed to demonstrate the morphological evolution of this condition.

Brown Norway rats were given sc injections of mercuric chloride in a 2-mg/ml aqueous solution at a dose of 1 ml/kg body weight three times/wk. One group was studied for 15 days while another received the same dosage regime for 2 wk followed by 2 ml/kg weekly for 6 wk. Kidney samples were examined at the end of both studies and biopsies were taken twice between days 6 and 14 from the kidneys of some of the rats in the first group.

On days 8 and 9 of exposure, electron-microscopy studies demonstrated an influx of monocytes into the glomerular and interstitial capillaries and a focal detachment of the glomerular endothelial cells. Later (on days 14 and 15) subendothelial heterogeneous material and scattered subepithelial deposits appeared in the glomeruli. These initial lesions are thought to be a result of anti-GBM antibodies, which are produced in this strain of rat, since no glomerular alteration was observed at a similar stage of mercury intoxication in PVG/c or Lewis rats used as controls. The presence of monocytes rather than polymorphonuclear cells in the glomerular capillary walls on days 8 and 9 suggests a pathogenic role for these cells.

However, the anti-GMB antibodies exhibit only a transient pathological effect since the sequential ultrastructural study showed that no cellular infiltration or glomerular endothelial alterations persisted at day 60. Instead, an immune-complex process occurs, with granular deposition of IgG in the arteriolar walls of all the animals and in the glomeruli of half of them. The transition between the two phases occurs at about day 15.

PCB-induced gastric hyperplasia in the monkey

Silverman, S., Rosenquist, C. J. & McNulty, W. P. (1979). Radiographic study of gastric hyperplasia induced by polychlorinated biphenyls in the rhesus monkey. *Investve Radiol.* **14,** 65.

Becker, G. M., McNulty, W. P. & Bell, M. (1979). Polychlorinated biphenyl-induced morphologic changes in the gastric mucosa of the rhesus monkey. *Lab. Invest.* **40,** 373.

These complementary studies examine the macroscopic and microscopic progression of the unusual type of gastric hyperplasia that has been reported to occur in polychlorinated biphenyl (PCB) intoxication in the rhesus monkey (Allen & Norback, *Science, N.Y.* 1979, **179,** 498) and the pig (Hansen *et al. Am. J. vet. Res.* 1976, **37,** 1021).

In the study described in the first paper cited above three immature monkeys (1–2 yr of age) received 3,4,3′,4′-tetrachlorobiphenyl (TCB) in the diet, initially at a level of 3 ppm. The severity of the toxic reaction after 30 days necessitated the reduction of the dose level; for 3 wk the treated diet was fed on Mondays and Thursdays only and a final dietary level of 0·3 ppm was selected as suitable for the remainder of the experiment. On day 29 the initial radiographic examination of the stomach revealed thickening of the gastric wall along the greater curvature of the distal body and antrum and a rigid narrowing in the region of the junction of the antrum with the body of the stomach in two of the treated monkeys, one of which died shortly after this examination. The next inspection of the surviving monkey from this pair was made on day 62 of the experiment. It showed marked progression of the lesion by continued spreading and thickening, and both the serosal and mucosal surfaces had become irregular and nodular. This animal was quite sick and died several hours after this examination. No gastric distress had been observed in the remaining test animal and although a possible lesion was seen at the examination on day 62, it had not progressed by the final radiographic examination on day 99 of the investigation. The gastric mucosa of the three control monkeys remained normal throughout. In the two treated animals that died, autopsy revealed a broad thick lesion occupying portions of the body and antrum of the stomach. Microscopically, the rolled margins of the mucosal lesions showed hyperplasia of the mucous glands and disappearance of the zymogenic parietal cells. The flat, coarsely granular centre was composed of alternating regions of ulceration and disorderly growth of mucous glands deep into the submucosa. The fundic mucosa remained structurally normal but the parietal cells had disappeared and mucous cells were found scattered among the zymogenic cells.

In the second study cited, similar cellular changes were examined in detail in five young monkeys (7–8 months of age) fed diets containing 3–100 ppm PCB (Aroclor 1242). One control received an untreated diet. Baseline data were obtained from tissue samples taken at laparotomy from the body of the stomach near the greater curvature at the start of the experiment. Further samples were taken on day 12 and thereafter at monthly intervals for 6 months. The initial set of tissue samples permitted assessment of the normal types and distribution of cells in the gastric mucosa of this species. A dramatic decrease in the numbers of parietal cells with a concomitant increase in the number of mucous neck cells was noted soon after the start of the experiment. As treatment continued the neck region of the gastric gland increased in extent and the lumina of the neck and base became dilated. The mucous neck cells from the treated animals showed markedly irregular apical surfaces and an almost total absence of microvillae. These cells were virtually devoid of endoplasmic reticulum and Golgi bodies and a disorderly configuration of the whole cell body was noted. Zymogen cells were reduced in number but the remaining cells showed marked dilation of the rough endoplasmic reticulum. In later specimens (after 189 days on 10 ppm), the zymogen granules were degenerating and enormous numbers of autophagic vacuoles were seen. Subsequently large autophagic vesicles appeared in the lumen of the gastric gland. In all the treated animals the early changes were followed by total mucous conversion of the gastric epithelium, growth of the gastric glands down through the muscularis mucosae into the submucosa and eventually the formation of cysts lined by mucus-secreting columnar epithelial cells.

It appeared that even at low doses there was a possible failure of germinative cells to differentiate into parietal and zymogen cells and the ultrastructural changes were strongly suggestive of cell injury and

eventual cell death in the remaining population of recognizable zymogenic and parietal cells. The gradual loss of these cells, it was suggested, was due to normal attrition plus failure to be replaced by differentiation of immature cells. However, as reports indicate that the normal half-life of a parietal cell is greater than 23 days and at the highest dose they disappeared in only 12 days, conversion to mucus-secreting cells may also have been involved.

According to Silverman *et al.* (*loc. cit.*) it is uncertain whether these lesions would progress or regress if PCB administration were discontinued and it is also unclear whether they represent benign or premalignant changes. The cellular pattern of the mucosal changes was thought to be benign but the infiltration of the mucosa into the submucosa and muscularis suggests potential malignancy. It is as yet unknown whether similar lesions occur in humans.

Another constituent of pulp-mill effluent

Chu, I., Villeneuve, D.C., Secours, V. & Viau, A. (1979). Absorption, distribution and metabolism of epoxystearic acid in the rat. *Bull. envir. Contam. Toxicol.* **22,** 462.

In the last issue we referred to toxicity studies on chlorinated guaiacols formed as waste products of pulp and paper manufacture (*Cited in F.C.T.* 1980, **18,** 447). Another compound that is present in pulp-mill effluents and is therefore likely to be found as a contaminant of rivers, and perhaps also of drinking-water, is 9,10-epoxystearic acid. There is evidence that, like the chlorinated guaiacols, this acid is toxic to fish (Leach & Thakore, *J. Fish. Res. Bd Can.* 1975, **22,** 1249), and to throw some light on its possible toxicity to mammals, Chu *et al.* (cited above) have studied its fate in the rat.

Male Sprague–Dawley rats were given ^{14}C-labelled epoxystearic acid in a single orally intubated dose of 15·6 mg/kg (20 μCi/kg) in corn oil or in a single iv injection of 7·8 mg/kg (10 μCi/kg). Groups of the intubated rats were used either for blood sampling at intervals from 0·25 to 48 hr after dosing or for the collection of urine and faeces (and sometimes also of expired air) for 6 or 24 hr or 7 days, and were then killed for tissue analysis. Bile samples were collected from the injected rats and the urine and faeces of these rats were analysed for possible metabolites.

Absorption from the gut was rapid, ^{14}C being detected in the blood 15 min after treatment and reaching a peak at 1 hr. Radioactivity was distributed throughout the tissues but concentrations were lowest in the muscle, testes and brain. The highest concentrations found 6 hr after dosing were in the liver (equivalent on average to 19·5 μg epoxystearic acid/g wet tissue), but by 24 hr the liver levels had declined and the highest concentrations (30 μg/g) were in the body fat. Thereafter the order of accumulation in the different tissues remained unchanged, but the levels gradually declined (to 1·5 and 3·8 μg/g in liver and fat, respectively, by day 7).

Radioactivity was excreted mainly as $^{14}CO_2$, 43% of the dose being accounted for in the expired air within 6 hr and 75% within 5 days. The radioactivity in the urine, faeces and bile accounted for 6·4, 10·6 and 3·8% of the dose, respectively, and was mainly present as unchanged epoxystearic acid, but the urine also contained a small amount of a hydroxylated derivative. These findings suggest that metabolism of epoxystearic acid involves β-oxidation and that little bioaccumulation of this compound is likely to occur.

COSMETICS, PHARMACEUTICALS AND HOUSEHOLD PRODUCTS

The hazards of hair sprays

Palmer, A., Renzetti, A. D., Jr. & Gillam, D. (1979). Respiratory disease prevalence in cosmetologists and its relationship to aerosol sprays. *Envir. Res.* **19,** 136.

Studies have indicated an increased cancer incidence among female beauticians (*Cited in F.C.T.* 1977, **15,** 654; *ibid* 1978, **16,** 631), and there has been considerable debate about the safety of one group of substances, the hair dyes, to which such workers are occupationally exposed since it has been found that certain hair-dye components are carcinogenic in animals (*Food Chemical News* 1978, **19** (43), 38; *ibid* 1978 **19,** (46), 60). The hair sprays, another group of products to which beauticians and cosmetologists are heavily exposed, have also come under attack as a cause of pulmonary thesaurosis, which has been suggested to be allergic in origin (*Cited in F.C.T.* 1974, **12,** 434). The results of an epidemiological study of the incidence of respiratory disease among female cosmetologists have now been reported.

The study was carried out in the state of Utah in the USA and the study population consisted of 213 practising cosmetologists from 55 salons, 262 student cosmetologists from nine colleges and 569 controls, loosely matched for geographical area, age and smoking habits. Salon air samples were taken using both area and personal sampling techniques and the atmospheric levels of particulates were determined. Each subject was questioned about respiratory symptoms and all non-pregnant subjects were given a chest X-ray. Determinations of forced vital capacity (FVC), forced expiratory volume at 1 sec, and forced expiratory flow rates at 50 and 75% of the FVC and between 25 and 75% of the FVC were also made for each participant. Closing volume and lung diffusion capacity were measured in every tenth participant and sputum samples were obtained from all subjects aged more than 30 yr and from 10% of those younger than 30 yr.

There was a slight but insignificant increase among qualified cosmetologists in the incidence of abnormalities indicated by X-rays and by measurements of the diffusing capacity of the lung that suggested the presence of thesaurosis or sarcoidosis. No relation-

ship was found between these findings suggestive of thesaurosis and a history of allergy, although there was a slightly higher prevalence of results indicating thesaurosis among allergic cosmetologists than among their non-allergic peers. The findings relating to obstructive lung disease were less ambiguous. The cosmetologists showed a highly significant increase in the prevalence of abnormal and borderline respiratory symptoms compared with the controls. These differences were not eliminated after adjustments for age and smoking habits. Furthermore the increased prevalence of respiratory symptoms was greater (but not significantly so) among employees from small salons, who were generally exposed to higher atmospheric levels of hair-spray particulates and who also had the lowest smoking rates. The normal FVC and forced expiratory volume values showed no evidence of restrictive or large-airway obstructive diseases. The results of pulmonary function tests indicated an increase in the incidence of small-airway obstruction among small-salon employees. There was also evidence from the whole group of cosmetologists that the degree of obstruction increased with increasing time of exposure. The percentage of cosmetologists with atypical sputum cytology was significantly higher among both student and qualified cosmetologists than among the controls. Again the greatest difference from the controls occurred among employees of small salons. These findings indicate the need for investigation of a relationship between exposure to hair sprays and the risk of lung cancer.

[Several previous epidemiological studies of the incidence of cancer among cosmetologists have suffered from the lack of reliable data concerning the smoking habits of the women (*ibid* 1978, **16,** 631). This criticism cannot be levelled at the present study and its results would seem to merit further investigation.]

More on the hair-spray hazard

Gebbers, J.-O., Tetzner, C. & Burkhardt, A. (1979). Alveolitis due to hair-spray. Ultrastructural observations in two patients and the results of experimental investigations. *Virchows Arch. Abt. A path. Anat. Histol.* **382,** 323.

In the epidemiological study described above higher incidences of abnormal respiratory symptoms, small airway obstruction and atypical sputum samples were found among cosmetologists exposed to hair sprays than among non-occupationally exposed controls. Polyvinylpyrrolidone (PVP) and polyvinyl acetate (PVA) are high-molecular-weight polymers used in cosmetic hair sprays, which have produced a macrophage reaction in the lungs of experimental animals (*ibid* 1967, **5,** 591; *ibid* 1975, **13,** 407). Numerous macrophages filled with granules staining with periodic acid-Schiff reagent (PAS; usually taken as indicative of the presence of PVP) were found in the lungs of a woman who was suffering respiratory symptoms and who had been a heavy user of hair sprays for many years (*ibid* 1974, **12,** 434).

The authors cited above report that two patients, a 48-yr-old housewife and a 60-yr-old hairdresser, developed lung disease attributable to extensive exposure to hair sprays. They were non-smokers and lacked any history of exposure to other inhaled dusts or gases; both improved within 6 months of withdrawal from further exposure to hair sprays. Histological examination of lung tissue showed alterations of the parenchyma, with active chronic inflammation, macrophagocytic granulomas containing multinucleated giant cells of the foreign-body type, and a few lymphocytes and granulocytes and intra-alveolar inclusions. The granulomas were situated in the interstices of the alveolar septa and in the peribronchiolar tissue. The intra-alveolar and interstitial macrophages and the giant cells contained PAS-positive cytoplasmic inclusions. On ultrastructural examination, lamellar inclusions appeared in the secondary lysosomes of macrophages and giant cells.

Similar alterations occurred in the lungs of mice, rats and guinea-pigs that were injected sc with extracts of hair spray or with saline suspensions of PVP and PVA. In cell cultures of human monocytes hair-spray extract containing PVP and PVA induced maturation and aggregation, with the appearance of PAS-positive cytoplasmic inclusions. Multinuclear giant cells developed in these cultures.

These observations indicate that lung disease induced by hairsprays results from a prolonged and extensive response of the local mononuclear-phagocyte system (MPS). The MPS is initially overstimulated by the large quantities of hair spray inhaled at a time and by the large size of the particles to be ingested. This heavy demand on and possible exhaustion of the local MPS could lead to a partial blockade of the system. The resulting functional reduction may cause an increased susceptibility to infectious diseases of the lung.

Musk ambrette sensitization

Kochever, I. E., Zalar, G. L., Einbinder, J. & Harber, L. C. (1979). Assay of contact photosensitivity to musk ambrette in guinea pigs. *J. invest. Derm.* **73,** 144.

Musk ambrette (2-methoxy-3,5-dinitro-4-methyl-*tert*-butylbenzene; MA) is used as a musk substitute in the food and cosmetics industries. It produced no sensitizations at a concentration of 20% in a maximization test (Opdyke, *Fd Cosmet. Toxicol.* 1975, **13,** 681) but it has recently been implicated as the photosensitizing agent present in an aftershave lotion (Raugi & Storrs, *Archs Derm.* 1979, **115,** 106). Experiments were carried out using guinea-pigs to determine whether MA would induce photosensitization in this species, and whether it is a phototoxic or a photoallergic agent. When sensitization was attempted on shaved, unstripped nuchal skin (2·5 cm × 2·5 cm; induction dose: 0·1 ml 5 or 10% MA) none of the 20 animals responded to challenge doses (0·1 ml 2·5, 5 or 7% solution) of MA and UV light. A significant incidence of photosensitization was achieved, however, when the skin was stripped with cellophane tape as part of the induction process (induction dose: 0·1 ml 10% MA; challenge dose: 0·1 ml 5 or 7% MA). None of the animals responded to MA in the absence of irradiation. In tests to determine whether MA was phototoxic, doses of 0·1 ml 1–50% MA were applied to a 2·5 × 3·5 cm area of shaved skin and the area

was irradiated with UV light. None of the animals showed erythema after 24 hr. The mechanism of photosensitivity of MA thus appears to be photoallergic rather than phototoxic.

Cresol on the brain

Savolainen, H. (1979). Toxic effects of peroral o-cresol intake on rat brain. *Res. Commun. chem. Path. Pharmac.* **25,** 357.

Cresols are used in disinfectants and occur as contaminants in many technical chemicals. They are also metabolites of toluene in rats (Bakke & Scheline, *Toxic. appl. Pharmac.* 1970, **16,** 691) and humans (Angerer, *Int. Archs occup. envir. Hlth* 1979, **43,** 63). The study cited above was designed to investigate the toxic effects of *o*-cresol on the nervous system, with special reference to the glial cells. Male Wistar rats were given drinking-water containing 0·3 g *o*-cresol. Their drinking rate and weight gain were measured weekly, and groups of ten rats were killed for biochemical evaluation after 4, 10, 15 or 20 wk of treatment. The drinking rate of the treated rats was significantly greater than that of the controls after 4 wk of treatment but it then decreased and after 20 wk it was significantly less than that of the controls. No significant effect on weight gain was observed. Cerebral RNA was significantly increased at wk 4 but not at the other time intervals. At wk 20 the levels of glutathione and azo reductase activity were significantly lower in the brain homogenates from the treated animals. In the glial cells the concentration of glutathione did not alter during exposure, but after 20 wk the acid proteinase activity exceeded that in the controls, and after 10 and 20 wk 2′,3′-cyclic-nucleotide 3′-phosphohydrolase activity was considerably higher than in the controls. It is concluded that despite lack of evidence that *o*-cresol or other cresols accumulate in the body tissues, several weeks' exposure to water contaminated with *o*-cresol is potentially toxic for the central nervous system.

CARCINOGENICITY AND MUTAGENICITY

Senescent metabolism

Baird, M. B. & Birnbaum, L. S. (1979). Increased production of mutagenic metabolites of carcinogens by tisses from senescent rodents. *Cancer Res.* **39,** 4752.

Cancer incidence rises with increasing age. This may result from some factors intrinsic in the ageing process or from an increased duration of exposure to carcinogens in the environment. Alternatively, the length of the latent period may simply produce an increase in the likelihood of showing cancer symptoms with increasing age. Hepatic metabolism of xenobiotics becomes altered in ageing organisms (Baird *et al. Nature, Lond.* 1971, **233,** 565). Microsomal mixed-function oxidases, together with other enzymes, both activate and detoxify a variety of carcinogens and age-related alterations in the metabolism of carcinogens may contribute to the increase in cancer incidence with age. The authors cited above set out to substantiate this hypothesis by comparing the activating capacities of microsomes and microsomal homogenates from young rodents with those from old rodents in the *Salmonella typhimurium* mutagenicity assay.

Both rats and mice were pretreated with a single dose of Aroclor 1254 to induce hepatic microsomal mixed-function oxidase activity. Liver homogenates (S-9) and sterile hepatic microsomes from the treated animals were prepared. The mutagenic metabolites of benzo[*a*]pyrene (B[*a*]P) and 2-fluorenamine (2-FA) produced were estimated using the *S. typhimurium* plate assay (Ames test). When the results of the Ames test using S-9 mix from 700-day-old rats was compared with that using S-9 mix from 300-day-old rats, it was found that the reversion frequency produced by the B[*a*]P metabolites was roughly doubled in strain TA98 and was slightly increased in strain TA100. With 2-FA the increase in revertants was more than three-fold in strain TA98 and almost double in strain TA100. Similar results were obtained when S-9 mix from 100-day-old mice was compared with that from 800-day-old mice using strain TA98.

B[*a*]P is metabolized in rodents by microsomal aryl hydrocarbon hydroxylase but non-microsomal mechanisms further metabolize the intermediate epoxide. However, it was found that even when using sterile hepatic microsomes, those from old rats produced more mutagenic metabolites of B[*a*]P than did those from young rats. The age-related effects were eliminated by heating or by incubating the plates in the absence of NADPH and this confirmed that the age-related differences were enzymic. There were no significant age-related differences in microsomal NADPH-cytochrome-c-reductase or *P*-450 content after induction with Aroclor. Results similar to those for Aroclor-treated rats were obtained in a comparison of age-groups using 2-FA in strain TA98 when the S-9 mix or microsomes were prepared from rats that had not been pretreated. The age-related response is thus not simply an altered response to induction by the Aroclor. The results of this study support the suggestion that age-related alterations in carcinogen metabolism contribute to the increasing incidence of cancer in old age.

Fd Cosmet. Toxicol. Vol. 18, p. 557
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MEETING ANNOUNCEMENTS

OCCUPATIONAL CANCER

An International Symposium on the Prevention of Occupational Cancer, organized by the Helsinki Institute of Occupational Health in collaboration with WHO and IARC is to be held in Helsinki on 21–24 April 1981. The symposium will cover the epidemiology of occupational cancer, the methods of risk evaluation, and the prevention of occupational cancer. The languages of the conference will be both English and French with simultaneous interpretation in the main conference room. Further details may be obtained from the Secretary, International Symposium on Prevention of Occupational Cancer, Institute of Occupational Health, Haartmaninkatu 1, SF-00290 Helsinki 29, Finland.

INTERACTIONS OF COLLOIDS

An International Conference on all aspects of physical and chemical interactions of hydrocolloids in food systems will be held on 13–17 July 1981. Further details may be obtained from "Gums and Stabilizers for the Food Industry", The North E. Wales Institute, Kelsterton College, Connah's Quay, Deeside, Clwyd CH5 4BR.

ENVIRONMENTAL MUTAGENS CONFERENCE

The Third International Conference on Environmental Mutagens sponsored by the International Association of Environmental Mutagen Societies and the Environmental Mutagen Society of Japan will be held in Japan in September 1981. The main conference will take place in Tokyo on 21–24 September and will be followed by a conference at Mishima on 25–26 September and a satellite meeting in Kyoto on 26–27 September. The first two meetings will cover a very broad range of topics but the satellite meeting will consider individual sources of environmental mutagens such as food, air and water. The address from which further information may be obtained is: Third International Conference on Environmental Mutagens, Kyobashi, P.O. Box 236, Tokyo 104-91, Japan.

THIRTEENTH INTERNATIONAL CANCER CONGRESS

The Thirteenth International Cancer Congress will be held in Seattle, USA, on 8–15 September 1982. The congress is sponsored by the International Union against Cancer and will cover a broad range of topics including "environment and cancer" and "changing life styles and cancer". Further information may be obtained from David Siegel, Operations Manager, 13th International Cancer Congress, c/o Fred Hutchinson Cancer Research Center, 1124 Columbia St., Seattle, WA 98104, USA.

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FORTHCOMING PAPERS

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

Effects of FD & C Red No. 40 on rat intrauterine development. By T. F. X. Collins and T. N. Black.

Comparison of effects of dietary administration of butylated hydroxytoluene or a polymeric antioxidant on the hepatic and intestinal cytochrome *P*-450 mixed-function-oxygenase system of rats. By S. C. Halladay, B. A. Ryerson, C. R. Smith, J. P. Brown and T. M. Parkinson.

Syncarcinogenic action of saccharin or sodium cyclamate in the induction of bladder tumours in MNU-pretreated rats. By U. Green, P. Schneider, R. Deutsch-Wenzel, H. Brune and J. Althoff.

Mutagenic activity of pyrazine derivatives: A competitive study with *Salmonella typhimurium*, *Saccharomyces cerevisiae* and Chinese hamster ovary cells. By H. F. Stich, W. Stich, M. P. Rosin & W. D. Powrie.

N-Nitroso compounds from reactions of nitrite with methylamine. By M. W. Obiedzinski, J. S. Wishnok and S. R. Tannenbaum.

Effect of alcohols on nitrosamine formation. By T. Kurechi, K. Kikugawa and T. Kato.

Changes in the nitrate and nitrite contents of fresh vegetables during ongoing cultivation and postharvest storage. By J. K. Lin and J.-Y. Yen.

Mutagenicity testing of coffee: A study of problems encountered with the Ames salmonella test system. By H. U. Aeschbacher, C. Chappuis and H. P. Würzner.

The occurrence of ochratoxin A in mouldy flour and bread. By B. G. Osborne.

Pathological changes in rats fed the crambe meal glucosinolate hydrolytic products, 2*S*-1-cyano-2-hydroxy-3,4-epithiobutanes (*erythro* and *threo*) for 90 days. By D. H. Gould, M. R. Gumbmann and M. E. Daxenbichler.

Three-generation reproduction study of rats ingesting 2,4,5-trichlorophenoxyacetic acid in the diet. By F. A. Smith, F. J. Murray, J. A. John, K. D. Nitschke, R. J. Kociba and B. A. Schewtz.

Comparative induction of aryl hydrocarbon hydroxylase activity *in vitro* by analogues of dibenzo-*p*-dioxin. By J. A. Bradlaw, L. H. Garthoff, N. E. Hurley and D. Firestone.

Comparative distribution, excretion and metabolism of di-(2-ethylhexyl) phthalate in rats, dogs and miniature pigs. By G. J. Ikeda, P. P. Sapienza, J. L. Couvillion, T. M. Farber and E. J. van Loon.

The variability of dietary fibre in laboratory animal diets and its relevance to the control of experimental conditions. By A. Wise and D. J. Gilburt.

[*Contents continued*]

Aims and Scope

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

Some other Pergamon Journals which may interest readers of *Food and Cosmetics Toxicology*:

Annals of Occupational Hygiene

Archives of Oral Biology

Atmospheric Environment

Biochemical Pharmacology

Chronic Diseases

European Journal of Cancer

Health Physics

Journal of Aerosol Science

Life Sciences

Toxicon

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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.

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

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

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

e.g. Hickman, J. R., McLean, D. L. A. & Ley, F. J. (1964). Rat feeding studies on wheat treated with gamma-radiation 1. Reproduction. *Fd Cosmet. Toxicol.* **2,** 15.

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

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

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

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

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

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