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* Except where indicated otherwise, these items have been contributed by the staff of the British Industrial Biological Research Association. Comments on them will be welcomed and should be sent to the Assistant Editor at BIBRA.
†By R. J. L. Allen, Brentford, Middlesex.

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Research Section

THE RELIABILITY OF A PROCEDURE FOR THE DETERMINATION OF NITROSAMINES IN FOOD

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(*Received 16 October 1974*)

Abstract—A procedure is described for the clean-up process used in this Laboratory prior to the analysis of foodstuffs for the presence of traces of nitrosamines. Recoveries from this procedure were estimated by the gas chromatographic separation of the individual nitrosamines and subsequent detection by a Coulson electrolytic conductivity detector. The ability of the detector to give a reproducible response has been assessed, as was the retention stability of the GLC system. A similar study on a combined gas chromatographic–mass spectrometric system used for the confirmation of the presence of nitrosamines is also described. The value of the Coulson detector for screening purposes prior to mass spectrometric confirmation was assessed using samples of cheese, fish, meat and some other commodities.

INTRODUCTION

It is generally recognized that trace amounts of nitrosamines can occur in some foodstuffs, several papers having been published in recent years indicating that these compounds can be present in cured meat products, fish and cheese (Crosby, Foreman, Palframan & Sawyer, 1972; Fazio, Damico, Howard, White & Watts, 1971; Fazio, White & Howard, 1971; Fiddler, Piotrowski, Pensabene, Doerr & Wasserman, 1972; Fong & Chan, 1973; Panalaks, Iyengar & Sen, 1973; Sen, 1972; Wasserman, Fiddler, Doerr, Osman & Dooley, 1972). *N*-Nitrosodimethylamine (NDMA) is the most commonly occurring nitrosamine in these commodities as purchased, but *N*-nitrosopyrrolidine (NPYR) has been detected in cooked bacon (Fazio, White, Dusold & Howard, 1973; Sen, Donaldson, Iyengar & Panalaks, 1973).

The level at which these compounds occur is so low that it is necessary to carry out clean-up and concentration stages in order to detect them, especially if a quantitative estimation is required. This clean-up is usually achieved by means of distillation and column chromatography (Wasserman, 1972). The nitrosamines are separated using gas-liquid chromatography (GLC) (Crosby *et al.* 1972; Rhoades & Johnson, 1970) and preferably are detected using a nitrogen-specific detector. Alternatively derivatives such as nitramines (Sen, 1970) or fluorinated amides (Alliston, Cox & Kirk, 1972), which can be detected by electron capture, are prepared. It has been recommended (International Agency for Research on Cancer, 1975) that combined gas chromatography–high-resolution mass spectrometry (GLC–MS) should be used to confirm the presence of nitrosamines. A gas-chromatographic method using one of the specific detectors mentioned above may be used as a screening process.

A method for the routine determination of traces of nitrosamines in a large number of food samples should preferably be rapid and must be reliable over an extended period of time. One of the most effective clean-up procedures developed at this Laboratory and based on these criteria involves steam distillation of the sample followed by solvent extraction of the distillate and concentration by evaporation. Separation of the nitrosamines from each other and the remaining extraneous material is achieved by GLC.

This study has been undertaken to assess the overall reliability of the clean-up and concentration procedures and the GLC–MS confirmation. The value of a nitrogen-specific detector for screening is also discussed.

EXPERIMENTAL

Clean-up procedure. The procedure adopted for the determination of steam-volatile nitrosamines requires 250 g of foodstuff, which is comminuted and slurried in a total of 250 ml water, including that originally present in the sample. To the slurry are added 100 g sodium chloride and two antifoam tablets together with 250 μl of a solution (10 μl/litre) of *N*-nitrosodi-*n*-propylamine (NDPA), a nitrosamine that has not been detected in foodstuffs. (The recovery of the NDPA is compared with that anticipated to assess the clean-up procedure for each sample.) The mixture is steam-distilled and the first 400 ml of distillate is collected. After addition of 80 g sodium chloride and 4 ml 10 N-sulphuric acid to the distillate, the nitrosamines are extracted into 4 × 40 ml redistilled dichloromethane. The combined extracts are washed with 70 ml 1·5 N-sodium hydroxide and the organic layer is dried over sodium sulphate. The dried extract is placed in a Kuderna-Danish flask and the volume is reduced to 2·5 ml by evaporation at 45°C; 800 μl hexane are added and the evaporation is continued to about 250 μl. The final volume is measured with a 500 μl syringe and the concentration factor is calculated. The concentrate is stored at 10°C in a glass vial fitted with a septum cap.

Gas-chromatographic screening procedure. The screening procedure is carried out using a Varian 2700 chromatograph fitted with a Coulson electrolytic conductivity detector operating in the reduction mode (Palframan, Macnab & Crosby, 1973). A stainless-steel column, 6·3 m × 1·8 mm i.d. and filled with 15% FFAP on diatomite CLQ 80–100 BS mesh, is used. The column is operated at 140°C with helium carrier-gas flowing at 25 ml/min. The sample size normally used is 4 μl.

Gas chromatographic–mass spectrometric method. Details of this method have previously been published (Gough & Webb, 1973). In brief, a Pye model R chromatograph is connected to an AEI MS-902 mass spectrometer via a silicone membrane separator (Gough & Webb, 1972). The chromatograph contains a peak-cutting system to prevent unnecessary contamination of the mass spectrometer and a carrier-gas pressure-programming device to minimize sample analysis and turn-round time. Two stainless-steel columns are used, one before and one after the vent. The first column, 2·4 m × 2 mm i.d. contains 15% Carbowax 20M on A.W. Chromosorb W, while the second column, 5·4 m × 2 mm i.d. contains 5% Carbowax 20M also on A.W. Chromosorb W. The column temperature is 145°C and the helium carrier-gas flow rate varies from 6 to 25 ml/min as the pressure increases. The sample size used is again normally 4 μl. The mass spectrometer is operated at a resolution of 7000 and the gas chromatograph conditions are such that species originating in the antifoam tablets, which can give rise to trimethylsilyl ions in the ion source of the mass spectrometer (Dooley, Wasserman & Osman, 1973; Gough & Webb, 1973), are not co-eluted with the NDMA.

RESULTS AND DISCUSSION

Gas-chromatographic method

It was decided to use the gas chromatograph with Coulson detection to monitor the recovery of the nitrosamines from the various stages of the procedure, so a preliminary study was carried out to assess the reliability of this approach. A standard solution was prepared containing concentrations of 10 μl/litre of each of the following: NDMA, *N*-nitrosodiethylamine (NDEA), NDPA, *N*-nitrosodi-*n*-butylamine (NDBA), *N*-nitrosopiperidine (NPIP) and NPYR. Replicate 4 μl injections were made over a 7-hr period on two different occasions and the detector response and retention times were measured. Retention times and Kovats retention indices at 140°C, together with the change in value per 10°C over the range 140–170°C are given in Table 1.

Table 1. *Gas-chromatographic retention data for six nitrosamines using the Coulson detector*

	Retention times* (min)					
	Series 1		Series 2		Retention indices	
Compound†	$\bar{x}$	σ	$\bar{x}$	σ	I (140°C)	δI/10°C
NDMA	9·9	0·19	9·8	0·15	1312	3·7
NDEA	12·9	0·22	12·8	0·15	1390	4·3
NDPA	21·4	0·22	21·4	0·35	1523	4·3
NDBA	43·2	0·36	43·2	0·25	1687	4·7
NPIP	51·0	0·29	51·0	0·18	1715	10·0
NPYR	58·0	0·28	58·6	0·26	1738	9·0

$\bar{x}$ = Mean retention time σ = Standard deviation
I = Kovats retention index
*Seven determinations in each series.
†For identification of abbreviations, see text.

Retention times based on chart-paper measurements could not be measured to better than 15 sec, which proved to be of the same order as the standard deviation of these measurements. It was thus concluded that the reproducibility of retention time was sufficiently accurate to enable peak-height measurements to be used for response data. The response values given in Table 2 are normalized with respect to the mean NDPA response. The noise level is 2 units on this scale. Agreement between the two sets of experiments is good, even though the coefficient of variations is rather high for the NPYR response. Under similar conditions the variation of response of a flame ionization detector is about 3% for each nitrosamine.

Table 2. *Gas-chromatographic response data for six nitrosamines using the Coulson detector*

	Response values*					
	Series 1			Series 2		
Compound†	$\bar{x}$	σ	v	$\bar{x}$	σ	v
NDMA	175	18·8	10·7	176	19·3	10·9
NDEA	171	6·7	3·9	170	8·8	5·2
NDPA	100	5·5	5·5	100	2·8	2·8
NDBA	44	2·6	5·9	45	2·1	4·7
NPIP	53	4·9	8·9	53	3·6	6·8
NPYR	33	7·6	23·0	32	7·3	22·8

$\bar{x}$ = Mean response σ = Standard deviation
v = Coefficient of variation
*Seven determinations in each series: values normalized with respect to $\bar{x}$ for NDPA.
†For identification of abbreviations, see text.

Clean-up procedure

Using the Coulson detector, the reliability of the distillation, extraction and clean-up stages was assessed. Recoveries of NDMA, NDEA and NDPA from the distillation were quantitative at the 10 μl/litre level. The recovery of NDBA varied between 75 and 100% and that of the two heterocyclic nitrosamines from 60 to 107%. The efficiency of various solvents in extracting nitrosamines from aqueous solution has been studied previously and it was concluded that extraction was virtually complete using dichloromethane (Neurath, Pirmann & Dünger, 1964). Losses during evaporation were expected to be more pronounced for the low dialkylnitrosamines, although it has been found that in practice there is little difference in loss between the various nitrosamines, and the recoveries are generally over 75%.

In order to study losses for the complete process, samples of cheese, fish and meat were selected which were known to give clean chromatograms when analysed by the procedure. Nitrosamines were added at the 0·01 μl/kg level (i.e. 10 μl/litre in the final extract, assuming no losses). An aqueous solution of the same concentration was analysed for comparison. Recoveries are given in Table 3, from which it can be seen that the recovery of any given nitrosamine is

Table 3. *Overall recoveries of added nitrosamines from food samples analysed using gas chromatography and the Coulson detector following the described clean-up procedure*

Compound†	No. of determinations...	Mean recovery (%)* from Aqueous solution 9	Cheese 17	Fish 5	Meat 7
NDMA		64 (10·7)	79 (7·0)	69 (5·1)	67 (9·8)
NDEA		78 (10·9)	91 (9·4)	87 (8·8)	78 (10·1)
NDPA		80 (15·5)	87 (10·9)	82 (7·1)	81 (9·4)
NDBA		80 (21·0)	74 (11·5)	72 (5·6)	77 (6·3)
NPIP		84 (13·0)	84 (11·5)	84 (7·2)	80 (13·7)
NPYR		59 (14·0)	61 (9·6)	60 (9·5)	65 (32·9)

*With standard deviations in parentheses.
†For identification of abbreviations, see text.

Table 4. *Effects of storage conditions on the ageing of aqueous solutions of* N-*nitrosodimethylamine* (*NDMA*)

Container and storage conditions		Concn of solution (μl/litre)...	Percentage of NDMA remaining: After 4 months 5	After 4 months 100	After 8 months 5	After 8 months 100
Clear borosilicate glass:	daylight		0	1	0	1
	darkness		98	101	100	104
Al foil-wrapped:	daylight		99	100	100	101
	darkness		101	100	101	100
Brown borosilicate glass:	daylight		86	92	67	86
	darkness		103	102	104	104

similar for all commodities and in most instances varies by about 10%.

Long-term stability

For adoption of the procedure as a routine technique, long-term stability of the nitrosamine standards and the GLC retention times is essential. Stability of detector response is less critical, provided frequent calibration is carried out. Nitrosamines can decompose photochemically (Ballweg & Schmähl, 1967), so the effects of different methods of storage on the stability of aqueous solutions of NDMA at the 5 and 100 μl/litre levels were studied. The results are summarized in Table 4. Results were obtained by comparing the detector response to the aged solutions and to fresh standards prepared on the day of analysis. Storage in clear glass vessels wrapped in aluminium foil is both satisfactory and convenient. Using a standard stored in this way, variation in retention time and detector response was followed over a period of 6 months. A summary of the 67 determinations for each nitrosamine is given in Table 5.

Comparison with Table 1 shows excellent agreement of mean retention times and although the standard deviations are greater, retention stability is quite satisfactory. The mean detector responses do not show such good agreement with the short-term variations recorded in Table 2 and are rather poor compared with the stability attainable using non-selective GLC detectors over similar periods of time.

Variations in overall recoveries from various commodities to which NDPA had been added were followed over a 6-month period. Thirty different samples of each commodity were analysed. Mean recoveries (88, 87 and 83% for cheese, fish and meat, respectively, with 96% for the aqueous solution) agreed well with those observed in the short-term study (Table 3) and the standard deviations (10, 16, 16 and 19, respectively) were not substantially worse. In both the short- and long-term studies, the variation in recovery was greatest for the aqueous solutions.

Gas chromatographic–mass spectrometric method

For comparative purposes, short- and long-term retention and response variations have been measured using standard solutions of nitrosamines. As with the Coulson detector, short-term variations were measured over 7 hr and the long-term variations over 6 months. In view of the nature of the specific ion-monitoring system on the mass spectrometer, retention times can only be measured to ± 8 sec. Using

Table 5. *Long-term retention time and detector response variations measured over a 6-month period using a Coulson detector*

Compound†	Mean retention time* (min) $\bar{x}$	σ	Mean response* with respect to NDPA $\bar{x}$	σ	v
NDMA	9·4	0·28	226	51·9	22·8
NDEA	12·8	0·33	178	32·4	18·2
NDPA	21·4	0·49	100	20·0	20·0
NDBA	43·4	0·92	46	10·4	22·4
NPIP	51·4	1·06	60	15·8	26·2
NPYR	58·3	1·24	52	18·5	35·3

$\bar{x}$ = Mean retention or response σ = Standard deviation
v = Coefficient of variation
*For each nitrosamine 67 determinations were carried out.
†For identification of abbreviations, see text.

this system, the nitrosamine is determined by comparison with a reference compound at 4-sec intervals. Over the 7-hr period, no variations in retention time were observed on seven replicate analyses for each nitrosamine. The coefficient of variation of response varied from a minimum of 4% for NDMA to a maximum of 13% for NDEA. The long-term variations are summarized in Table 6. There was no correlation between response changes and the ageing of the mass-spectrometer ion source.

The variations in retention times are negligible even over an extended period of time and hence the resetting of the mass spectrometer to monitor the molecular ion of each nitrosamine in turn on the basis of the appropriate retention time is reliable. The procedure adopted is to monitor the molecular ion

Table 6. *Long-term retention time and response variations with the gas-chromatographic-mass spectrometric system*

	Mean retention time* (min)		Mean response with respect to NDPA		
Compound†	$\bar{x}$	σ	$\bar{x}$	σ	v
NDMA	11·4	0·40	717	262	36
NDEA	14·7	0·50	475	242	51
NDPA	20·0	0·71	100	57	57
NDBA	29·8	0·82	28	14	50
NPIP	32·7	0·94	213	84	39
NPYR	36·2	1·09	194	102	53

$\bar{x}$ = Mean reading σ = Standard deviation
v = Coefficient of variation
*A total of 60 readings per sample.
†For identification of abbreviations, see text.

Table 7. *Comparison of results from gas-chromatography–Coulson detector screening of foodstuffs for nitrosamines and from confirmatory studies using gas chromatography–mass spectrometry*

	No. of samples			Percentage of samples	
Compound*	With interfering peaks	Positive by Coulson	Positive by GC–MS	Confirmed by GC–MS	Rejected by screening
		Cheese (60 samples)			
NDMA	18	24	21	50	30
NDEA	4	20	16	67	60
NDPA	0	60	60	100	—
NDBA	0	27	18	67	55
NPIP	0	22	17	77	63
NPYR	0	18	17	95	70
		Fish (33 samples)			
NDMA	6	12	9	50	45
NDEA	8	8	7	44	51
NDPA	3	30	33	100	—
NDBA	2	12	7	50	58
NPIP	3	10	7	54	60
NPYR	2	8	7	70	70
		Meat (61 samples)			
NDMA	17	12	18	62	52
NDEA	10	18	8	29	54
NDPA	2	59	61	100	—
NDBA	2	14	9	56	74
NPIP	0	12	5	42	80
NPYR	0	13	7	54	79
		Brine (28 samples)			
NDMA	3	19	21	96	22
NDEA	10	2	5	42	57
NDPA	0	28	28	100	—
NDBA	0	1	1	100	97
NPIP	0	8	6	75	71
NPYR	0	0	0	—	100
		Seasoning (18 samples)			
NDMA	17	1	2	11	0
NDEA	14	2	2	12	11
NDPA	7	11	11	61	0
NDBA	2	0	0	0	89
NPIP	0	18	17	94	0
NPYR	4	6	2	20	44
		Flour, bread, pastry (10 samples)			
NDMA	2	2	1	25	60
NDEA	0	4	1	25	60
NDPA	0	10	10	100	—
NDBA	0	3	1	33	70
NPIP	0	1	1	100	90
NPYR	0	1	1	100	90

*For identification of abbreviations, see text.

region for each nitrosamine over a period of time which is at least equal to the standard deviations given in Table 6. Standards are run at the beginning and end of each day to check that the retention times and response values are within these limits and agree sensibly with each other.

Comparison of the two methods

Because of the high cost of a mass spectrometer it is imperative that it be used at maximum efficiency. A screening method that can minimize the number of samples requiring mass-spectrometric confirmation and so release the instrument for other work is one way of achieving this. Consideration of Table 7 indicates that a substantial proportion of samples is eliminated by screening. The total number of samples analysed in each commodity group is given. Interference of the GLC nitrosamine peaks is likely to be more common at short retention times in view of the clean-up procedure used, so many samples giving apparently positive results by the screening process require mass spectrometric confirmation for NDMA and NDEA only. The number of samples in which gross interference made it impossible to detect the apparent presence of a particular nitrosamine using the Coulson detector is given in Table 7, together with the number of apparently positive results obtained from the screening process, the number confirmed by mass spectrometry and the percentage eliminated by the screening process. The proportion of results confirmed by mass spectrometry is derived from the number of runs in which interfering peaks precluded any GLC identification plus those samples that showed an apparent positive using the Coulson detector. The proportion eliminated by screening is calculated from the difference between the total number of samples analysed and the sum of the number of samples giving interference or a positive reaction on the Coulson detector.

The GLC time lost for each sample requiring MS confirmation is 35 min, compared to an equal saving of MS time for each sample eliminated by screening. Considering the relative capital value of the two instruments and the high proportion of samples eliminated by the screening process, we consider this approach to be cost-effective.

The cheeses were derived mainly from cows milk and 29 different varieties were included in the sample. Both fresh fish and processed products such as smoked salmon and kippers were examined, while the meat samples included cooked luncheon meat, ham, bacon, sausages and some fresh meat. The brines were curing solutions used for the preparation of some of the bacon samples. The analyses of a selection of spice/curing-salt premixes used in the preparation of meat pies are included under the heading 'seasoning'.

The figures given in Table 7 do not necessarily represent the occurrence of nitrosamines in normally available foodstuffs, some of which are the subject of a separate study. The occurrence of gross interference using the GLC method was highest in the region of NDMA for all the foods examined. Nevertheless about half of the apparently positive results for NDMA obtained from cheese, fish and meat, a total of 154 samples, were confirmed by mass spectrometry. For the curing brines, more interference was encountered in the region of NDEA. In general, there were few gross interfering peaks in the region of NDBA, NPIP and NPYR. Mass spectrometry confirmed about half of the Coulson positives for these compounds in the case of fish and meat and somewhat more in the case of cheese.

Alternative clean-up procedures and GLC conditions have not resulted in any worthwhile improvement in screening reliability within the limitation of the need for a routine technique to be rapid and reliable over an extended period of time.

CONCLUSIONS

It is concluded from the foregoing that quantitative determination of nitrosamines should be based on mass spectrometric measurements and that these have a short-term reproducibility of about $\pm 10\%$. In general, recoveries of nitrosamines from the clean-up procedure also vary by about 10%, so provided a recovery check is made by the addition of a known amount of NDPA to each sample and the mass spectrometer is calibrated daily, the quantitative results can be quoted to $\pm 20\%$ with confidence.

The response of the Coulson detector varies by a similar extent for the dialkylnitrosamines but by about 23% for NPYR. In the analysis of foodstuffs, about half of the positive results obtained with the Coulson detector are shown to be false by GLC-MS, so this technique is essential for the confirmation of nitrosamines. Nevertheless the use of a Coulson detector substantially reduces the work load on the mass spectrometer and gives an indication of the levels of nitrosamine to be expected.

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SHORT-TERM TOXICITY OF ETHYLENE CHLOROHYDRIN (ECH) IN RATS, DOGS AND MONKEYS

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Abstract—Oral dosage studies on ethylene chlorohydrin (ECH) were carried out in rats, dogs and monkeys of both sexes for periods of at least 90 days. The responses of the animals to three graded dosage levels of ECH were compared with those of controls fed unsupplemented rations appropriate for the respective species. Doses were administered to rats by intubation, to dogs in their diet, and to monkeys by syringe. All dose mixtures were freshly prepared each day. Behaviour, growth, survival, blood parameters and urine analyses were recorded. Terminally, gross and microscopic examinations were made of organs and tissues and the major organs were weighed.

In the rats, no adverse findings were seen with dose levels of 30 and 45 mg ECH/kg body weight/day, but with 67·5 mg/kg, growth was depressed in both sexes and mortality was high. In dogs, ingestion of the ECH diet was followed by severe emesis, which limited the highest intake that could be retained to approximately 18–20 mg/kg/day regardless of dietary level. The dogs showed some variations in haemoglobin and haematocrit readings, but these were not correlated with dosage. The treated dogs did not grow, but all survived. The monkeys also failed to increase in weight at treatment levels up to 62·5 mg/kg, but showed no noteworthy differences from the controls. Gross and histopathological examinations disclosed no consistent dose-related abnormalities in any of the species.

INTRODUCTION

Ethylene oxide is a highly effective agent for the sterilization of grains, spices and other food products originating from plants, as well as of medical and surgical supplies. Ethylene chlorohydrin (ECH) has been shown to result from the reaction of ethylene oxide with sodium chloride added to or naturally present in food. The potential hazard of ECH as an indirect food additive was therefore investigated in 90-day studies in rats, dogs and monkeys. Before the 90-day studies were initiated, however, exploratory tests were conducted to establish procedures for ensuring the accuracy of the dosage of this volatile compound.

EXPERIMENTAL

Test material. The sample of ECH (2-chloroethanol) used was obtained from Distillation Products Industries, a division of Eastman Kodak Co., Rochester, N.Y.

Animals. The studies were carried out on 200 weanling albino rats (FDRL strain), 32 pure-bred beagle dogs (7–9 months old) and 16 imported adult monkeys (*Macaca mulatta*). The animals of each species were assigned to four groups consisting of equal numbers of each sex. Each animal was housed individually in a raised-bottom cage supplied with fresh water at all times. The rats were fed Purina Laboratory Chow, the dogs Purina Dog Meal and the monkeys Rockland Farms Monkey Chow. All were examined daily for survival, appearance and behaviour. Body weights and food intake were recorded weekly. The neurological reflexes of the dogs were examined periodically.

Dosing procedures. The rats were fasted overnight and then allowed a 1-hr feeding period. Doses of 0, 30, 45 or 67·5 mg ECH/kg body weight were given daily by gavage in the form of freshly prepared aqueous solutions in such concentration that the specified dose could be administered in a volume of 10 ml/kg body weight. Food remained available for *ad lib.* consumption until the end of each workday.

Doses were administered initially to the dogs in the form of a freshly prepared wet mash, providing 0, 600, 900 or 1350 ppm ECH (dry basis). Because of the severe emetic response, it was necessary to reduce the concentrations in several stages to ensure retention. The levels of ECH were then gradually increased as long as it was found that the doses could be retained. The actual total intakes were calculated at the end of the test period.

In contrast to the dogs, the monkeys retained their daily doses of 0, 30, 45 or 62·5 mg ECH/kg body weight, given as freshly prepared mixtures in apple sauce administered orally by syringe. In addition to the basal diet, the animals were given fresh fruit daily.

Initially and at wk 6 and 12, ten rats of each sex from each group and all the dogs and monkeys were subjected to the following determinations: haemoglobin, haematocrit, total and differential leucocyte counts, prothrombin time, blood urea nitrogen, blood glucose, serum glutamic–oxalacetic transaminase (SGOT), serum alkaline phosphatase (SAP) and urinary analyses. The rats and monkeys were killed at wk 12 and the dogs at wk 15. Gross autopsies were carried out and the liver, kidneys, heart, gonads, adrenals, thyroids and pituitaries were weighed. Haematoxylin/eosin-stained sections of 26 organs from ten rats of each sex from the control and highest dose-level groups and liver and kidneys from representatives of the lower-dose groups were examined microscopically. The same organs from all dogs and monkeys were weighed and similarly examined.

RESULTS

Rat study

Preliminary tests in which the rats received ECH in the diet at levels intended to provide 0, 30, 45 and 67·5 mg/kg/day were terminated at wk 6 when gas-chromatographic analyses indicated a lack of stability of the compound in the diet. At this point, the males on the above diets had gained 171, 184, 184 and 171 g in body weight and the females 100, 102, 103 and 99 g, respectively. Clinical examinations disclosed no abnormalities or differences among the groups. Thereafter aqueous solutions of ECH were given daily by intubation. The observations recorded here cover the subsequent 12-wk period.

During the first 3 wk after conversion to intragastric dosage, many of the rats on the highest dose level ate poorly and showed signs of laboured breathing. Subsequently they became moribund and were killed but the remainder (eight males and six females) survived for the rest of the 12-wk period. In the control and two lower dosage groups, all but two rats (one female in each of the control and 45-mg/kg groups) survived. Over the full test period, growth of the surviving males in the highest dosage group was retarded, but at the two lower dosages and in all the females, growth was comparable to that of the relevant controls (Table 1). Food utilization (EFU) in all groups was comparable over the 12-wk period, even though at the highest level the food intake was reduced.

No behavioural abnormalities were noted in the surviving rats. The clinical blood tests disclosed no deviations from normal nor significant differences among the groups, at any of the test periods. SGOT values were somewhat higher and SAP values lower at wk 6 and 12 than initially but within each period were about the same in the test and control groups.

In the rats killed at the end of the study, the organ weights of the ECH-treated rats were comparable, with few exceptions, to those of the controls, both in terms of absolute and relative weights. Relative liver weight was slightly lower in the males on the two lower dosage levels than in the controls (3·41 and 3·27 v. 3·84% of body weight, respectively) but was higher than the control value in females given the highest dose level (4·48 v. 3·55% in the controls). Relative thyroid weights were lower than in the controls in the males given 30 or 67·5 mg/kg (0·0082 and 0·0080 v. 0·0099% in the controls) and in females given 30 or 45 mg/kg (0·0121 and 0·011 v. 0·0156%). In the rats of both sexes that died, the relative weights of all organs except the ovaries (which were of course immature at this age) were significantly increased. These elevations were related, at least in part, to the terminal weight losses due to the malnourished state of the rats.

Table 1. *Growth response and survival of rats given ECH by intubation in doses of 0–67·5 mg/kg/day for 12 wk*

ECH dose level (mg/kg/day)	Mean body weight (g) Initial	Net gain by wk 12	Efficiency of food utilization (weight gain (g)/100 g food)	Survival* (%)
		Males		
0	264	111	5·8	100
30	276	91	5·9	100
45	276	94	6·6	100
67·5	263	73	5·4	32
		Females		
0	184	51	3·7	96
30	187	49	4·3	100
45	187	49	4·0	96
67·5	183	46	4·0	24

* Each dosage group consisted initially of 25 males and 25 females.

The gross autopsy findings in the rats in the control and two lower dosage groups and in the survivors at the highest level, were not noteworthy. In those that died (the majority of the 67·5-mg ECH/kg group) the livers were dark, with alternate pale and granular areas, gastro-intestinal tissues were reddened (and/or bloody), some adrenal and pituitary glands were haemorrhagic and most lungs were red or dark red.

The microscopic findings in the rats that survived the study, including those on the highest dosage, were scattered and suggested no relation to dosage. The rats in the 67·5-mg/kg group that died within a short period after the change from dietary to intragastric dosage showed a high incidence of subacute myocarditis (in both sexes), a few cases of colloid depletion in the thyroid (in one male and four females), fatty changes in the liver (in one male and five females), thyroid congestion (in four males) and a high incidence of congestive pulmonary changes (in both sexes). The cardiac tissues of the surviving rats in this group and of five males and five females given 45 mg/kg disclosed no abnormalities.

Dog study

The dietary concentrations originally planned for the dogs (600, 900 and 1350 ppm) would have provided a daily dose of 15, 22·5 and 33·8 mg/kg body weight had they eaten the expected 25 g food/kg each day. Except at the lowest level, however, severe emesis with marked loss in body weight occurred and repeated adjustments of dietary levels were made. Lower concentrations resulted in retention of food and recovery of body weight. Except for one bitch on the highest dose level, which lost 2·1 kg, the net loss in body weight at the end of the study was less than 1 kg. It was calculated that over the entire 15-wk period of treatment, the males consumed daily an average of 25, 26, 21 and 20 g and the females 31, 28, 28 and 24 g food/kg body weight in the control and three test groups. From the concentrations fed and amounts of food eaten, the estimated mean daily doses of ECH for the males were 13·3, 18·4 and 18·3 mg/kg and for the females 16·9, 20·3 and 19·3 mg/kg in the original low, intermediate and high dosage groups, respectively. It appeared therefore that these were the maximum doses that could be tolerated. All dogs survived the observation period. No behavioural abnormalities were noted in the dogs in the control or '600-ppm' groups. At the two higher levels (i.e. when concentrations were higher than 600 ppm ECH in the diet) severe vomiting occurred for several hours after ingestion. A few of the dogs developed a tendency towards a squatting posture but otherwise they appeared normal.

In the dogs given the two higher levels of ECH, the haemoglobin and haematocrit values were depressed at wk 6, haemoglobin ranging from 11·5 to 16·2 g 100 ml compared with 14·0–17·8 g/100 ml in the controls and haematocrit from 33 to 53% compared with a control range of 47–56%. Later, at wk 12, the levels increased in all the dogs except two females, which still showed haemoglobin levels of about 13 g/100 ml and haematocrits of approximately 40%. However, statistical analyses by the Duncan ranking procedure showed that these differences were not significant. The values for total and differential leucocyte counts, prothrombin time, blood glucose and blood urea nitrogen, SGOT and SAP and the urinary findings were all within the normal ranges.

The organ weights of the dogs disclosed no dose-related effects. Expressed as a percentage of body weight, liver weight was raised in one male on the lowest and one on the highest dose level (4·38 and 4·35%, respectively, compared with a mean of 2·5% in the controls), and in one female in each of the intermediate and high dosage groups (4·48 and 5·59%, respectively, compared with a mean of 2·88% in the controls). Various incidental findings were observed both grossly and microscopically in the dogs, but there was no indication of dose-related effects. It is particularly noteworthy that there were no histopathological changes in the gastric mucosa despite the severe emetic effect of ECH in these animals.

Monkey study

All monkeys survived and showed no behavioural abnormalities. They gained or lost only minimal amounts of weight (0·5 kg or less). Though considerable variation was noted in the haematological and clinical chemistry determinations, no significant deviations from normal levels or trends with time were noted. Organ weights were comparable on an absolute basis but when expressed in relation to body weight the weights of the brain, adrenals and thyroid glands of the ECH-dosed monkeys were slightly increased. However, no gradation was observed among low, middle and high dosage groups. The testes of all ECH-dosed monkeys were smaller than those of the controls but no dose-related effect was apparent.

The gross and microscopic examinations of the monkey tissues disclosed only scattered findings such as are commonly seen in this species, namely evidence of residues of pulmonary and parasitic infections. It should be noted that the monkeys used in this study had been imported.

DISCUSSION

From the observations reported here, it is apparent that at high dosages ECH is unpalatable to the species of animals employed in this study. The rats and monkeys retained all doses up to the highest level administered (67·5 and 62·5 mg ECH/kg body weight/day, respectively). No adverse effects were noted at dosage levels up to 45 mg/kg/day in the rats or at any level in the monkeys. Food intake and body weights were diminished and deaths occurred in rats dosed at 67·5 mg/kg/day. Clinical findings and post-mortem examinations in both species were negative except in the case of the rats that died (or were killed in a moribund state) after a short period of intubation with doses of 67·5 mg/kg; these showed a high incidence of myocarditis and fatty liver. On the basis of doses retained by rats and monkeys, therefore, 45 mg/kg body weight/day may be regarded as a no-adverse-effect level.

Despite the severe emetic effect, which limited the mean daily intake of the dogs to approximately 20 mg ECH/kg body weight, all survived. Only the lowest concentration fed to the dogs (600 ppm) was consistently retained Clinical and post-mortem findings, including histopathology, failed to demonstrate any dose-related effects in the dogs even in the gastric mucosa of those animals that vomited with great frequency.

Wesley, Rourke & Darbishire (1965) demonstrated the presence of chlorohydrins in foods fumigated with ethylene oxide or propylene oxide. Earlier, a 220-day feeding study had been reported (Ambrose, 1950) in which male rats were able to tolerate up to 800 ppm ECH in the diet without adverse effect but showed growth retardation when fed dietary concentrations of 1200 ppm or more. On the basis of customary assumptions of food intake by adult rats (50 g/kg/day), these findings indicates a no-effect level of about 40 mg/kg/day, very close to that derived from the present 90-day study.

The toxicological findings reported here, together with analytical data on ECH residues in ethylene oxide-fumigated ground spices (reported by R. L. Hall at an American Chemical Society meeting in September 1968), constituted the evidence upon which the FDA granted a regulation permitting the use of ethylene oxide as a fumigant for ground spices (Code of Federal Regulations 121.1232; Federal Register 1970, *35*, 14545) specifying the same tolerance limits for residues as had previously been established for whole spices similarly treated (CFR 120.151).

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THE POTENTIAL MUTAGENICITY OF DIELDRIN (HEOD) IN MAMMALS

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Abstract—Mutagenicity studies have been carried out in CF1 mice and Chinese hamsters after oral dosing with HEOD, the major constituent of dieldrin. A dominant lethal assay was carried out in which male CF1 mice were dosed orally with 12·5, 25 or 50 mg HEOD/kg. Chinese hamsters of both sexes were given single oral doses of 30 or 60 mg HEOD/kg and 8 or 24 hr after dosing, femoral bone marrow was obtained and analysed for chromosome changes. Host-mediated assays were carried out on male CF1 mice either given a single oral dose of 25 or 50 mg HEOD/kg or dosed orally on five successive days with 5 or 10 mg HEOD/kg. *Saccharomyces cerevisiae* was the test organism and the genetic change measured was mitotic gene conversion. The three test systems showed no evidence of the induction of dominant lethality, chromosome breakage or gene conversion in animals dosed with HEOD. Additional chromosome studies were carried out on short-term lymphocyte cultures from workers currently or previously employed in a dieldrin manufacturing plant. The degree of chromosome damage did not differ significantly from that found in a control group of workers. These findings suggest that HEOD does not present a mutagenic hazard in mammals.

INTRODUCTION

Dieldrin is a persistent organochlorine insecticide, active against a wide variety of insect pests. The major constituent of dieldrin is HEOD (1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-*exo*-1,4-*endo*-5,8-dimethanonaphthalene). Its metabolism in mice and rats has been studied extensively (Baldwin, Robinson & Parke, 1972) and these authors have indicated that the most probable point of metabolic attack is the epoxide ring. Such chemical configurations are present in a number of compounds with mutagenic activity (Fishbein, Flamm & Falk, 1970).

A series of experiments is described here in which the mutagenic potential of HEOD was investigated in a number of widely used test systems. These include the dominant lethal assay in male mice after single oral doses, cytogenetic studies of the bone marrow cells of Chinese hamsters after single oral doses, and a host-mediated assay. In addition, as part of a health-screening programme among workers in a dieldrin manufacturing plant, chromosome studies were carried out on short-term lymphocyte cultures obtained from such workers. The subjects included workers currently employed in the plant, workers previously employed there and, as controls, workers in other areas who had no known contact with the dieldrin manufacturing process.

EXPERIMENTAL

Materials. Recrystallized dieldrin (purity >99% HEOD) was supplied by Woodstock Laboratory, Sittingbourne Research Centre. Ethyl methanesulphonate (EMS) and Endoxan (cyclophosphamide) were obtained from Koch-Light Laboratories, Colnbrook, Bucks., and Colcemid from Ciba Laboratories Ltd., Horsham, Sussex.

Animals. Carworth Farm No. 1 strain (CF1) mice were reared under specific pathogen-free conditions in this laboratory (Walker & Poppleton, 1967). Chinese hamsters were obtained from Fisons Ltd., Pharmaceuticals Division, Loughborough, Leicestershire, or derived from a colony started in this Laboratory.

Dominant lethal assay

In Experiment 1, 40 male CF1 mice of proven fertility were randomly distributed into three treatment groups of eight mice and a control group of 16. Two groups of eight males were dosed orally with 12·5 or 25 mg HEOD/kg in dimethylsulphoxide (DMSO) solution. A positive control group of eight males was dosed orally with 100 mg Endoxan/kg and the 16 control males with the solvent, DMSO. Following dosing, each male mouse was caged with three randomly selected female mice for 7 days. This procedure was repeated weekly with new females for a total of 8 wk.

In Experiment 2, 40 male CF1 mice of proven fertility were again randomly distributed into three treatment groups of eight mice and a control group of 16. The three treatment groups were dosed orally with 12·5, 25 or 50 mg HEOD/kg in DMSO, and the 16 control males with DMSO. Each male was then caged with three randomly selected female mice for 7 days and the procedure was repeated weekly for a total of 5 wk.

In all groups, mating was presumed to have occurred by the mid-week of caging with the females, and 13 days after the presumed mating the females

were killed and the uterus was removed for examination. Non-pregnant animals were noted, and the numbers of early foetal deaths, live foetuses and late foetal deaths were recorded for each pregnant female. The weekly mean values for total foetal implantations and early foetal deaths were analysed by a two-way analysis of variance. One of the assumptions of the analysis is that the error variance is normally distributed. This was so with the values for total implantations, which were analysed untransformed. The variate for early foetal deaths (EFD) basically followed a Poisson distribution and to achieve normality it was necessary to transform the data by $\sqrt{EFD + 0{\cdot}375}$. Differences in the percentage of females pregnant, which has a binomial distribution, were tested in a two-way contingency table using a Chi-square test.

Cytogenetic studies in Chinese hamsters

Chinese hamsters, 3–6 months of age, were randomly distributed into three groups of eight (four males and four females) and dosed orally with 30 or 60 mg HEOD/kg in DMSO or with DMSO alone. Each animal was injected ip with 0·04% Colcemid solution in a dose of 10 ml/kg body weight, 90 min before termination of the experiment. Four animals from each group were killed with an overdose of sodium pentobarbitone 8 or 24 hr after being dosed with HEOD and the femurs were removed. The method for obtaining chromosome preparations from the femoral marrow of small mammals has been described previously (Dean, 1969). All preparations were coded and examined in random order. Cells showing deviation from normal chromosome morphology were noted and photographed for later re-analysis. One hundred cells were analysed from the bone marrow of each animal. Statistical analysis was carried out after square-root transformation of the data.

Host-mediated assays

The indicator-organism used in these assays was the yeast, *Saccharomyces cerevisiae*, strain D4, heteroallelic at the ade_2 (adenine) and trp_5 (tryptophan) loci, and the genetic change observed was mitotic gene conversion. The culture media used, the selection of suitable cultures of *S. cerevisiae* and the standard assay procedure have been described in detail elsewhere (Dean, Doak & Funnel, 1972). In the single-dose experiment, adult male CF1 mice were dosed orally with 25 or 50 mg HEOD/kg in DMSO, 400 mg EMS/kg or DMSO immediately before inoculation of a buffered suspension of yeast cells into the peritoneal cavity. For the second part of the study, adult male mice were dosed orally on each of five successive days with 5 or 10 mg HEOD/kg in DMSO or with DMSO alone. After the fifth dose, a suspension of the yeast was injected into the peritoneal cavity.

The mice were killed by lethal exposure to ether 5 hr after introduction of the yeast cells, and the cell suspension was harvested. After repeated washing of the cells in sterile distilled water, the cell concentration in each sample was adjusted to 5×10^7 cells/ml and 0·1 ml volumes were seeded on eight synthetic-agar plates, four without tryptophan and four without adenine. To measure yeast-cell survival during the experiment, aliquots containing 500 cells were applied to the surface of complete (YEP medium) agar. After incubation for 5 days at 25°C, the colonies were counted and the number of prototrophs/10^6 cells was calculated. Results were analysed by standard analysis of variance after square-root transformation of the data.

Chromosome studies in dieldrin plant workers

Heparinized blood samples were received in the laboratory in the evening of the day of collection after being transported by air from the manufacturing plant. Cultures were prepared immediately on receipt of the samples. Each culture consisted of 6 ml medium 199 (Wellcome Reagents Ltd., Beckenham, Kent), 3 ml foetal calf serum (Flow Laboratories Ltd., Irvine, Scotland) and 0·2 ml phytohaemagglutinin, to which was added 0·2 ml heparinized whole blood. Duplicate cultures were prepared from each sample and incubated at 37°C for 68 hr, 0·15 ml of a 0·2 mg/ml solution of Colcemid being added to each culture 4 hr before termination. The harvesting of the cultures, including hypotonic treatment, fixation and slide preparation, has been described in detail elsewhere (Dean, 1972).

The slides were coded by a person not taking part in the microscopic analysis and the code was not revealed until all karyotyping was complete. Up to 25 cells were photographed from each culture, i.e. 50 cells/blood sample. Chromosome aberrations were recorded during microscopy and confirmed during analysis of photographs. Karyotyping was carried out on full-plate photographs using symbols to identify pairs or groups of chromosomes. Deviations from the normal human male karyotype were confirmed by constructing conventional karyograms from pairs of full plate prints. Two persons carried out the microscopic analysis and microphotography and four persons were involved in the subsequent karyotyping.

After analysis all cells were classified into three major groups (Buckton, Jacobs, Court-Brown & Doll, 1962; Buckton & Pike, 1964) on the following basis:

'A' cells: cells whose chromosomes have no apparent structural abnormality, but may differ numerically from 46.

'B' cells: cells which are structurally normal apart from chromatid-type aberrations, i.e. chromatid gaps, isochromatid gaps or chromatid breaks.

'C' cells: cells with other types of structural abnormality; these may be divided into Cu cells (those with unstable chromosome abnormalities) and Cs cells (those with abnormalities that appear stable and may persist). Cu cells include dicentrics, multicentrics and ring chromosomes, which interfere mechanically with normal cell division, and acentric fragments which, because they lack a centromere, will not attach to the mitotic spindle. Cs cells contain abnormal monocentric chromosomes, which should not interfere with normal mitosis.

The numbers of B and C cells were converted to percentages of the total cells and analysed using the Student's t test. Ages of workers were included as covariates to examine age-related differences. The numbers of Cu and Cs cells were analysed using a Chi-squared test (Forni, Pacifico & Limonta, 1971).

Table 1. *Percentage of pregnancies in mice mated with males dosed orally with HEOD or cyclophosphamide*

Dosage	Pregnancy rate (% of mated females) From mating at wk* 1	2	3	4	5	6	7	8	Mean (wk 1–8)
Experiment 1									
DMSO (control)	83	69	58	60	56	65	56	75	65·1
HEOD: 12·5 mg/kg	79	74	58	58	63	63	71	61	65·9
25·0 mg/kg	67	67	50	63	54	79	67	67	64·3
Cyclophosphamide†	79	62	76	71	67	57	62	81	69·4
Experiment 2									
DMSO (control)	88	88	79	65	63	—	—	—	76·6
HEOD: 12·5 mg/kg	79	88	92	67	75	—	—	—	80·2
25·0 mg/kg	88	83	88	88	71	—	—	—	83·6
50·0 mg/kg	92	75	88	83	88	—	—	—	85·2

*After dosing of males.
†In a dose of 100 mg/kg.
Total numbers of males mated weekly (with three females in each case) were seven in the cyclophosphamide-treated group, eight in each of the HEOD-treated groups and 16 in the control groups. Differences between values for treated and control groups were tested in a two-way table using a Chi-square test and were not significant.

RESULTS

Dominant lethal assay

Clinical findings. All male mice survived oral dosing with 12·5, 25 or 50 mg HEOD/kg although the animals in the two higher dose groups showed clinical evidence of intoxication after 24 hr. These animals appeared completely recovered 48 hr later. One of the eight mice dosed with cyclophosphamide died 7 days after dosing, but the remaining animals survived until the end of the experiment.

Effect on pregnancy. In Experiment 1, the percentage pregnancies in females mated with control males varied from 56 to 83% with a mean of 65·1% (Table 1). In females mated to males dosed orally with HEOD or cyclophosphamide the percentage pregnancies did not differ from the control value except in the first week after the dosing of males with 25 mg HEOD/kg, when 67% of the females were pregnant compared with 83% in the control group, an apparent though not significant difference. In Experiment 2, the percentage pregnancies in females mated to control males varied from 63 to 88% with a mean of 76·6% (Table 1). There were no significant differences between the HEOD-treated and control groups.

Effect on total number of foetal implantations. The number of uterine implantations was recorded for each pregnant mouse and the group mean values were determined weekly (Table 2).

When the weekly mean values for Experiment 1 were analysed individually, fewer implantations were recorded for females mated to males dosed with 12·5 mg HEOD/kg at wk 3 or with 25 mg HEOD/kg at wk 1 than in the control group. Total implantations in females mated to males in wk 1, 2 or 3 after dosing with 100 mg cyclophosphamide/kg were fewer than in the control groups for these weeks. The overall mean value for the 8-wk period showed a significant reduction in foetal implantations in the 12·5 mg HEOD/kg group ($P < 0·05$) and in the cyclophosphamide group ($P < 0·001$) when compared with the overall control mean.

There were no significant differences when dose groups in Experiment 2 were analysed at weekly intervals and therefore overall implantation means were derived for the 5-wk period for each dose group. The overall mean for total implantations in females mated to males in the 25 mg/kg group was significantly higher than the overall control mean.

Effect on early foetal deaths. The mean number of early foetal deaths per pregnant female did not differ from the control values in either experiment when analysed at each weekly interval or when overall mean values were derived for each experiment (Table 3). Females mated to males during each of the first 3 wk after dosing with Endoxan produced a higher number of dead foetuses than the control mice.

Table 2. *Mean foetal implantations in mice mated with males dosed orally with HEOD or cyclophosphamide*

Dosage	Mean total foetal implantations/pregnancy From mating at wk* 1	2	3	4	5	6	7	8	Mean (wk 1–8)	Difference between treated and control overall means
Experiment 1										
DMSO (control)	13·9	11·9	13·3	12·7	12·1	12·4	11·7	12·2	12·6	—
HEOD: 12·5 mg/kg	13·1	11·5	10·6**	11·5	12·9	12·2	10·8	11·5	11·8	0·75 ± 0·31*
25·0 mg/kg	11·4***	12·1	12·2	12·7	11·8	11·9	11·6	11·6	11·9	0·61 ± 0·32
Cyclophosphamide†	11·9**	8·8***	11·4*	11·7	11·1	10·9	11·4	12·1	11·2	1·34 ± 0·32***
Experiment 2										
DMSO (control)	13·7	12·9	13·2	13·1	11·5	—	—	—	12·9	—
HEOD: 12·5 mg/kg	13·5	12·9	13·2	11·8	13·4	—	—	—	13·0	0·08 ± 0·38
25·0 mg/kg	14·0	14·1	13·8	13·9	12·9	—	—	—	13·7	0·84 ± 0·37*
50·0 mg/kg	13·9	13·4	13·8	12·9	13·2	—	—	—	13·4	0·54 ± 0·37

*After dosing of males.
†In a dose of 100 mg/kg.
Total numbers of males mated weekly (with three females in each case) were seven in the cyclophosphamide-treated group, eight in each of the HEOD-treated groups and 16 in the control groups. Values marked with asterisks differed significantly from those of controls when analysed by two-way analysis of variance using untransformed data: *$P < 0·05$; **$P < 0·01$; ***$P < 0·001$.

Table 3. *Early foetal deaths in mice mated with males dosed orally with HEOD or cyclophosphamide*

Dosage	Means†	Mean early foetal deaths/pregnancy: From mating at wk* 1	2	3	4	5	6	7	8	Overall transformed means
Experiment 1										
DMSO (control)	a	0·98	0·93	0·93	1·06	0·94	0·86	1·09	0·92	0·96
	b	0·76	0·66	0·66	0·92	0·68	0·53	0·98	0·64	
HEOD: 12·5 mg/kg	a	0·78	0·87	0·85	0·95	1·06	0·81	0·93	0·83	0·89
	b	0·40	0·55	0·51	0·69	0·91	0·44	0·65	0·48	
25·0 mg/kg	a	1·01	0·79	0·96	0·96	1·03	0·93	0·98	0·95	0·95
	b	0·81	0·41	0·71	0·71	0·85	0·65	0·75	0·69	
Cyclophosphamide‡	a	1·78	1·64	1·29	0·81	1·07	0·98	1·02	1·09	1·09
	b	2·96***	2·48***	1·45**	0·44	0·93	0·74	0·83	0·98	
Experiment 2										
DMSO (control)	a	1·12	1·06	0·93	0·99	1·04	—	—	—	1·03
	b	1·07	0·94	0·68	0·79	0·89	—	—	—	
HEOD: 12·5 mg/kg	a	1·19	1·06	1·03	0·97	1·07	—	—	—	1·07
	b	1·22	0·93	0·87	0·74	0·95	—	—	—	
25·0 mg/kg	a	1·13	1·11	0·92	1·04	0·93	—	—	—	1·03
	b	1·08	1·04	0·65	0·89	0·67	—	—	—	
50·0 mg/kg	a	0·99	0·92	0·93	1·19	1·08	—	—	—	1·02
	b	0·79	0·65	0·67	1·22	0·97	—	—	—	

*After dosing of males.
†a = transformed means; b = detransformed means.
‡In a dose of 100 mg/kg.
Total number of males mated weekly (with three females in each case) were seven in the cyclophosphamide-treated group, eight in each of the HEOD-treated groups and 16 in the control groups. Values marked with asterisks differed significantly from those of controls when analysed by two-way analysis of variance using data transformed by $\sqrt{EFD + 0{\cdot}375}$: $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$.

Cytogenetic studies in Chinese hamsters

Only three of 4800 cells analysed from 48 animals showed aberrant chromosomes (Table 4). These were single chromatid gaps, evenly distributed between treated and control animals. Polyploid cells were also recorded and arithmetic group means varied from 1·75 to 4·75%. Analysis of transformed group means showed no significant differences between treated and control animals.

Host-mediated assay

Mice dosed with 10 mg HEOD/kg for 5 days remained very lethargic during the dosing period and this concentration proved lethal to a small proportion of the animals. No deaths occurred among mice given five daily oral doses of 5 mg/kg. The rate of mitotic gene conversion in *S. cerevisiae* after repeated oral dosing of male mice with 5 or 10 mg HEOD/kg did not differ significantly from the control value in the

Table 4. *Chromosome analysis of femoral marrow cells from Chinese hamsters given single oral doses of HEOD*

Interval before harvesting (hr)	Dose of HEOD (mg/kg)	Cells* showing: Polyploidy No.	%	Transformed means†	Detransformed means	Chromatid gaps No.	%
Males							
8	0	9	2·25	1·49	2·23	0	0
	30	11	2·75	1·36	1·86	1	0·25
	60	7	1·75	1·29	1·66	0	0
24	0	18	4·50	2·08	4·32	1	0·25
	30	19	4·75	1·89	3·56	0	0
	60	9	2·25	1·06	1·12	0	0
Females							
8	0	7	1·75	1·31	1·72	0	0
	30	7	1·75	1·25	1·56	1	0·25
	60	7	1·75	1·10	1·22	0	0
24	0	11	2·75	1·41	2·00	0	0
	30	8	2·00	1·39	1·93	0	0
	60	9	2·25	1·16	1·35	0	0

*In each case a total of 400 cells from four animals was analysed.
†Lowest significant differences between treatment groups = 1·09.

Table 5. *Mitotic gene conversion in* S. cerevisiae *D4 in the host-mediated assay in male CF1 mice given single or repeated oral doses of HEOD or single oral doses of EMS*

Experiment no.	Compound	Dosage (ml/mouse† or mg/kg)	Convertants/10^6 survivors: Adenine locus	Tryptophan locus
1	DMSO‡	0·2 × 5	26	26
	HEOD	5 × 5	26	24
		10 × 5	27	29
	EMS	400	104***	52***
2	DMSO‡	0·2 × 5	12	20
	HEOD	5 × 5	14	23
		10 × 5	12	23
	EMS	400	31***	104***
3	DMSO‡	0·2 × 5	11	13
	HEOD	5 × 5	10	15
		10 × 5	13	13
	EMS	400	47***	38***
4	DMSO‡	0·2	9	9
	HEOD	25	10	8
		50	10	11
	EMS	400	36***	28***
5	DMSO‡	0·2	8	36
	HEOD	25	8	36
		50	10	43
	EMS	400	20***	59***
6	DMSO‡	0·2	7	13
	HEOD	25	8	13
		50	8	15
	EMS	400	21***	26***

†Doses of DMSO.
‡Control groups.
Each group consisted of two mice except the EMS group in Experiment 4 (one mouse only), and values are means of four replicate cultures per mouse submitted to square-root transformation before statistical analysis. Those marked with asterisks differ significantly from the corresponding control: ***$P < 0.001$.

Table 6. *Cell types in leucocyte cultures from dieldrin plant workers*

Group	Code no.	Age (yr)	No. of cells analysed	No. of cells found: Type A <46	Type A 46	Type A >46	Type B	Type C Cu	Type C Cu
Control workers	3	55	48	10	37	—	6	2	—
	5	56	45	9	35	1	6	1	—
	6	50	48	6	42	—	2	1	—
	11	40	47	8	38	—	3	—	—
	12	40	45	9	35	—	3	1	—
	13	54	31	6	25	—	—	—	—
	15	35	29	6	23	—	—	—	—
	16	40	30	5	25	—	—	—	—
	17	4	29	9	20	—	3	—	—
	25	52	18	5	13	—	1	—	—
	26	60	33	3	29	1	1	—	—
	33	43	38	9	29	—	—	—	—
	34	49	43	7	36	—	—	2	1
	35	54	40	6	34	—	3	—	—
	36	19	36	4	32	—	—	1	—
	37	23	41	16	25	—	—	—	—
	38	30	43	9	33	1	3	1	—
Former plant workers	1	44	44	10	34	—	—	—	1
	2	42	45	10	35	—	2	1	—
	4	42	49	12	37	—	2	1	—
	14	41	39	10	28	—	2	1	—
	18	25	38	4	34	—	2	—	—
	20	43	39	16	23	—	—	—	—
	24	35	41	7	34	—	2	—	—
	31	43	49	4	44	—	4	—	—
	32	39	46	8	37	1	2	—	—
Current plant workers	7	43	50	6	44	—	2	—	2
	8	56	33	8	25	—	—	—	—
	9	50	50	15	34	1	2	—	—
	10	64	49	7	41	1	5	—	—
	19	30	33	7	26	—	2	—	—
	21	41	39	13	25	1	1	—	—
	22	28	51	7	43	1	1	—	—
	23	50	38	8	30	—	2	—	—
	27	37	49	16	33	—	2	—	—
	28	55	50	2	48	—	1	—	1
	29	35	51	6	44	—	3	—	—
	30	45	47	12	35	—	2	—	—

three replicate experiments (Table 5). In experiments in which mice were given a single oral dose of 25 or 50 mg/kg, the rates of gene conversion again showed no significant differences from those of control animals. EMS was used at a dose of 400 mg/kg in all experiments and in each case induced a significantly higher rate of gene conversion at both the ade_2 and trp_5 loci than occurred in yeast recovered from control animals ($P < 0.001$).

Chromosome studies on plant workers

Before statistical analysis, the cells were assigned to one of the three cell types (Table 6) and the numbers of B and C cells found in blood samples from former and current plant workers were compared statistically with the numbers from matching controls (Table 7). Chromosomes were analysed from a total of 1574 cells, of which 4·4% (70 cells) showed chromatid type aberrations (B cells) and 1·1% (17 cells) showed chromosome-type aberrations, five cells having stable aberrations (Cs) and 12 unstable aberrations (Cu). No significant differences ($P \leq 0.05$) were detected between the numbers of B and C cells recorded in plant workers and in workers with no history of industrial exposure to dieldrin. The numbers of Cu and Cs cells were too small to permit a full analysis to be carried out, but differences were examined using a Chi-squared test and no significant differences were detected ($P \leq 0.05$). Of the cells analysed, 20% had fewer than 46 chromosomes and this was constant for each group. Occasional polyploid and endoreduplicating cells were recorded but these

Table 7. *Incidence of cell types in three groups of workers*

Group	No. of workers	Mean age (yr)	Incidence of cell types (% of total cells analysed with 95% confidence limits): B cells*	C cells*	Cu cells†	Cs cells†
Control	17	44·4	4·50 (2·70–6·30)	1·34 (0·53–2·15)	1·40 (0·64–2·62)	0·16 (0–0·86)
Former plant workers	9	39·7	4·03 (1·55–6·51)	1·01 (0–2·12)	0·77 (0·16–2·31)	0·26 (0–1·41)
Current plant workers	12	44·5	4·19 (2·05–6·33)	0·50 (0–1·46)	0 (0–0·68)	0·56 (0·12–1·61)

*Differences were analysed using a Student's t test and were not significant.
†Differences were analysed using a Chi-square test and were not significant.

were not increased by occupational exposure to dieldrin. The ages of the subjects were included as covariates with the data. No significant age-related differences were detected.

DISCUSSION

The three mammalian test systems reported here are those suggested by the report of the United States Advisory Panel on Mutagenicity of Pesticides (1969) and are among those recommended by a WHO Scientific Group (1971) concerned with the evaluation and testing of drugs for mutagenicity.

Dominant lethal mutations in mammalian reproduction result in non-viable zygotes, early foetal deaths and sterility and semi-sterility in F_1 progeny. Such effects are a result of structural and/or numerical changes in the chromosomes of the germinal cells in sexually mature animals (Epstein, Bass, Arnold & Bishop, 1970).

In our first study, male mice were mated to females for a total of 8 wk, a period covering one complete spermatogenic cycle in the male mouse (Oakberg, 1957). In this study, an apparent reduction in the number of females pregnant and in the number of foetal implantations per pregnant female followed mating in wk 1 after dosing of the males with 25 mg HEOD/kg. Females in the 12·5 mg HEOD/kg group showed a reduction in foetal implantations when mated 3 wk after dosing of the males. No increase in early foetal deaths occurred at either HEOD concentration. Considerable weekly variations in pregnancy rate and foetal implantations occurred in both treated and control groups and in order to investigate the intermittent low fertility in the early weeks of the study, an experiment was instituted to include the same doses and a higher dose of HEOD. In addition, the animals were housed under improved environmental conditions, resulting in increases in the percentage of females pregnant and in the number of foetal implantations and a reduction in the weekly variation in these values in all groups. In the second experiment, in which males were dosed with 12·5, 25 or 50 mg HEOD/kg, no reduction in foetal implantations was recorded and the values for early foetal deaths did not differ significantly from the control value.

The somatic cells of the Chinese hamster contain 22 individually characteristic chromosomes, making it an excellent subject for chromosome studies, and most types of chromosome abnormality are readily identified. In the present study only chromatid-type aberrations were seen.

The time of maximum chromosome damage following treatment with chromosome-breaking chemicals varies with the nature of the chemical and the mechanism leading to chromosome breakage. In an evaluation of bone marrow cells as a test system, Schmid, Arakaki, Breslau & Culbertson (1971) treated Chinese hamsters with cyclophosphamide and demonstrated a dose-related increase in chromosome damage with a maximum incidence of aberrations at 6–8 hr. Other alkylating agents are known to exert a delaying effect on cell division (Kihlman, 1966). Studies with Chinese hamster cells *in vitro* have shown that mitotic delay by mutagens ranges from 3–4 hr with EMS to 21 hr with ICR-191, an acridine derivative (Kao & Puck, 1969). To allow for possible mitotic delay in the present study, half the animals were killed after 8 hr and the remainder 24 hr after administration of HEOD.

The oral LD_{50} for HEOD in Chinese hamsters is 120 mg/kg. Single oral doses of 30 or 60 mg HEOD/kg failed to induce any demonstrable chromosome damage in Chinese hamster bone marrow cells.

In the host-mediated assay, the compound being studied is influenced by the metabolic processes of the host before reaching the indicator organism, in this case, a yeast. Thus the activity of the compound may or may not be affected by the host. For example, streptozostatin and methylnitronitrosoguanine are able to induce mutations in *Salmonella typhimurium in vitro* and *in vivo*. Dimethylnitrosamine, a potent carcinogen, requires metabolic alteration before it is able to induce gene mutation, while 2-amino-purine nitrate is positive *in vitro* but is detoxified *in vivo* and is unable to induce mutation in the host-mediated assay (Gabridge & Legator, 1969). EMS is metabolized at a rate that permits it to reach a relatively constant, though short-lived, equilibrium in most tissues of the mouse before being hydrolysed (Cumming & Walton, 1970) and thus it is an effective mutagen *in vitro* and *in vivo*.

After single or repeated administration of HEOD in high oral doses, which proved lethal to a proportion of the animals, there was no evidence of a genetic effect on the test organism in the host-mediated assay.

The short-term culture of peripheral blood offers a convenient method for studying the somatic chromosomes of man and has proved useful for monitoring chromosome changes in small populations. The procedure has been used to investigate the effects of methylmercury pollution in a fish-eating community in Denmark (Malling, Wassom & Epstein, 1970) and, in the industrial health field, for studying the effects on workers of exposure to atmospheric benzene (Forni *et al.* 1971; Tough & Court Brown, 1965; Tough, Smith, Court Brown & Harnden, 1970), heavy metals (Leonard, Deknudt & Gilliavod, 1974) and organophosphate insecticides (Czeizel, Kiraly & Ruzicska, 1974). Tough *et al.* (1970) studied the chromosomes of workers at three different factories in which benzene was present in the atmosphere. They found that while the frequency of chromosome aberrations was generally higher than in a normal population, they were unable to indicate a direct correlation between exposure to benzene and an excess of chromosome aberrations in the blood. They concluded that other factors, such as the age of the individual, influenced the frequency of chromosome changes.

The normal human karyotype consists of 46 chromosomes. Deviation from this number may be due to faulty cell division, but more often hypodiploid cells (with less than 46 chromosomes) result from the loss of chromosomes during the processing of cell cultures for microscopic analysis. It has been suggested (Buckton *et al.* 1962) that X-ray therapy may alter the fragility of cell membranes so increasing the loss of chromosomes during processing, and it is also possible that other agents may have a similar effect. In the present study, although the proportion of hypodiploid cells was high, it did not differ significantly between worker groups (Table 7).

Structural deviations from the normal karyotype are due to breakage of chromosomes and the resulting fragments may or may not rejoin. Breaks or gaps in single chromatids at metaphase (B cells) arise subsequent to the DNA synthesis and replication stage of mitosis and are unlikely to persist through another cell division. Chromosome-type breaks, which are induced before or during DNA synthesis, may rejoin to form new chromosomes. These may be ring-forms, dicentrics or tricentrics and, because of mechanical difficulties, they are unlikely to survive a further cell division. Broken chromosomes may also rejoin to form chromosomes with a stable configuration, which, because they are monocentric, should be able to participate in normal mitosis. Since stable chromosome aberrations will only be recognized if the new monocentric differs morphologically from the normal karyotype, counts of Cs cells present an underestimate of the true number of such translocations. Cu cells, however, are morphologically distinct and provide a more accurate index of the frequency of translocations.

In the present study, neither the frequency of Cs cells nor that of Cu cells differed significantly between worker groups. When the age of the individual workers was introduced as a covariate in the statistical evaluation, again no significant differences were recorded.

The results of the dominant lethal assays reported here confirm the findings of Epstein, Arnold, Andrea, Bass & Bishop (1972) who found no evidence of dominant lethal mutations in male mice after single ip injections or repeated oral doses of dieldrin. These results, together with the negative results obtained in dieldrin plant workers, Chinese hamsters and the host-mediated assay suggest that dieldrin does not present a mutagenic hazard.

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MYCOTOXINE IN NAHRUNGSMITTELN. IV. DER EINFLUSS VERSCHIEDENER VERPACKUNGSFOLIEN AUF DAS WACHSTUM VON *ASPERGILLUS FLAVUS* UND DIE BILDUNG DER AFLATOXINE B_1 UND G_1 AUF EINIGEN SCHNITTBROTARTEN

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Zusammenfassung—Das Wachstum von *Aspergillus flavus* sowie die Aflatoxinproduktion auf Schnittbroten wird im wesentlichen von der Sauerstoffdurchlässigkeit der Verpackungsfolien bestimmt: je geringer diese ist, desto schwächer breitet sich der Pilz aus und desto weniger Aflatoxine werden gebildet. Im Extremfall reicht die Sauerstoffkonzentration im engen Raum zwischen Brot und Verpackungsfolie zwar noch für ein Wachstum des Pilzes, jedoch nicht mehr zur Synthese von Aflatoxinen aus. Sauerstoffmangel hemmt stärker die Bildung von Aflatoxin B_1 als die von G_1. Säuregrad und Zusammensetzung des Brotes beeinflussen ebenfalls das Wachstum von *A. flavus* und die Aflatoxinproduktion.

Abstract—The growth of *Aspergillus flavus* and the production of aflatoxins on sliced bread largely depends upon the oxygen permeability of the packaging foil; the lower this factor, the weaker is the growth of the mould and the production of aflatoxins. In the extreme case, the concentration of oxygen in the narrow space between bread and foil is sufficient for fungal growth but is too low for any aflatoxin synthesis. Oxygen deficiency has a more marked inhibitory effect on the formation of aflatoxin B_1 than on that of G_1. The degree of acidity and the composition of the different kinds of bread also affect fungal growth and aflatoxin production.

EINFÜHRUNG

Aspergillus flavus wächst auf den verschiedensten Lebensmitteln pflanzlicher Herkunft und bildet dort auch die hoch carcinogenen Aflatoxine (Reiss, 1972). Bemerkenswerterweise ist über eine Toxinbildung auf Brot nur verhältnismässig wenig bekannt, obwohl *A. flavus* auf diesem Nahrungsmittel wachsen kann (Reiss, 1973a). So konnten in Leinsamen-, Roggen- und Weizenvollkornbrot, die mit einem toxinogenen *A. flavus*-Stamm beimpft worden waren, die Aflatoxine B_1 und G_1 nachgewiesen werden (Frank, 1966 u. 1968).

Bei Untersuchungen von spontan verschimmelten Broten konnten Frank u. Eyrich (1968) Aflatoxin in einem Vollkornbrot nachweisen, nicht jedoch in Mischbrot, Pumpernickel sowie in Leinsamen- und Weissbrot. Aflatoxine B und G wurden von Bösenberg u. Eberhardt (1969) in einem nicht näher bezeichneten spontan verschimmelten Brot, von Hanssen u. Hagedorn (1969) in Land-, Weiss- und Vollkornbrot gefunden. Von verschiedenen spontan verschimmelten Brotsorten enthielt Mischbrot am häufigsten Aflatoxin (Spicher, 1969 u. 1970). In unverpacktem, mit *A. flavus* beimpftem Brot fand Spicher (1973) nur dort die Aflatoxine B_1 und G_1, wo auch Pilzmycel nachzuweisen war. Vergleiche von Pilzwachstum und Menge der gebildeten Toxine ergaben keine Abhängigkeit beider Faktoren voneinander.

Eigene Untersuchungen zeigten, dass in geschnittenem und verpacktem Weizenvollkornbrot, das mit einem toxinogenen Stamm von *A. flavus* beimpft worden war, erst bei Anwesenheit von Sporen in der Probe Aflatoxin B_1 nachweisbar ist (Reiss, 1971). Diese Erscheinung beruht mit grosser Wahrscheinlichkeit auf dem rasch sinkenden Sauerstoffgehalt im Luftraum zwischen Brot und Verpackung. Jemmali u. Lafont (1972) studierten das Verhalten von Aflatoxin B_1 während der einzelnen Phasen der Brotherstellung: der Toxingehalt sinkt während des Knetens des Teiges deutlich ab, während er durch Teiggärung und Backprozess nur relativ wenig beeinflusst wird.

Die bisher bekannten Untersuchungsergebnisse beziehen sich im wesentlichen auf Laibbrote. Seit längerer Zeit ist jedoch zu beobachten, dass der Konsument geschnittenes und verpacktes Brot bevorzugt. Es erschien daher angezeigt, Wachstum von *A. flavus* und Aflatoxinbildung gerade auf solchen Brotarten zu untersuchen. Dabei wurde zunächst überprüft, ob die Durchlässigkeit der Verpackungsfolien für Wasserdampf und Sauerstoff das Verhalten des Toxinproduzenten beeinflussen. Gerade weil die Aflatoxinbildung vom verfügbaren Sauerstoffgehalt abzuhängen schien (Reiss, 1971), war es denkbar, durch Auswahl eines geeigneten Verpackungsmaterials die Bildung von Aflatoxinen zu hemmen oder sogar ganz zu unterdrücken.

EXPERIMENTELLER TEIL

Pilz. Zur Beimpfung der Brote wurde ein aflatoxinogener Stamm von *A. flavus* (Nr 89717; Commonwealth Mycological Institute, Kew, Surrey, England)

Table 1. *Wasserdampf- und Sauerstoffdurchlässigkeit der verwendeten Verpackungsfolien*

Verpackungsfolie	Abkürzung	Hersteller	Dicke (μm)	Wasserdampfdurchlässigkeit* ($g/m^2/24$ h)	Sauerstoffdurchlässigkeit* ($cm^3/m^2/24$ h)
Cellophan GEB 300	GEB	K/W	20	2,5–3,0	*c.* 15
Trespaphan PC†	PNC	K/W	20	<1	10
Polypropylen	PP	T/T	20	*c.* 2	1455
Polyvinylchlorid	PVC	DN/T	20	*c.* 7	100

K/W = Kalle, Wiesbaden T/T = Tohcello, Tokio
DN/T = Dynamit-Nobel, Troisdorf

*Bei 20°C.
†Polyvinylidenchlorid-lackierte Polypropylenfolie.

verwendet, der auf Malzagar (4% Malzextrakt, 0,5% Pepton und 2,5% Agar) kultiviert wurde.

Brotarten. Als Nährsubstrat dienten folgende Brotarten der eigenen Produktion: Grobes Rheinisches Vollkornbrot (Roggenvollkornbrot), Felkebrot (Roggenvollkornbrot mit Anteilen an Weizenschrot), Grahambrot (Weizenvollkornbrot), Weizenkeimbrot (Weizenvollkornbrot mit Zusatz von Weizenkeimen), Leinsamenbrot (Weizenvollkornbrot mit 15% Leinsamen), Bauernschnitten (Mischbrot aus Roggen- und Weizenmehl), Früchtevollkornbrot und Pumpernickel.

Die Brote wurden maschinell geschnitten und je drei Scheiben in den in Tabelle 1 genannten Folien verpackt. Es wurden von jeder Brotart in jeder Verpackung mindestens vier Packungen hergestellt. Die Packungen wurden vor der Beimpfung mit heisser Luft sterilisiert. Schimmelverhütungsmittel wurden nicht zugesetzt.

Verpackungsfolien. Zur Überprüfung der Abhängigkeit von Pilzwachstum und Aflatoxinbildung von der Sauerstoff- und der Wasserdampfdurchlässigkeit des Verpackungsmaterials wurden solche Folien ausgewählt, die sich in ihren Durchlässigkeitswerten möglichst weit unterscheiden (Tab. 1).

Kontrollen. Zur Ermittlung eines möglichen Einflusses einer sich ändernden Atmosphäre im engen Raum zwischen Brot und Verpackung auf das Verhalten von *A. flavus* wurden sterilisierte Stücke jeder Sorte beimpft und in feuchten Kammern bei 22°C bebrütet.

Bestimmung des Säuregrades. Nach der Heissluftsterilisation wurde von jeder Brotsorte in jeder Verpackungsart der Säuregrad (Spicher, 1971) durch Titration mit 0,1 N-NaOH gegen Phenolphthalein (Methode nach Schulerud, siehe *Arbeitsgemeinschaft Getreideforschung*, 1964).

Beimpfung der Brote. Aus der Malzagar-Vorratskultur wurden einige Sporen steril unter die Verpackungsfolie auf das Zentrum der jeweils vordersten Scheibe der Brotpackungen übertragen. Die so beimpften Brotpackungen wurden bei etwa 25°C im Dunkeln gelagert.

Messung des Pilzwachstums. In Abständen von 24 Stunden wurden über einen Zeitraum von bis zu 20 Tagen hinweg die Radien der Pilzkolonien gemessen. Aus den Werten der einzelnen Packungen wurden die Durchschnittswerte bestimmt.

Halbquantitative Bestimmung der Aflatoxine B_1 und G_1. Nach einer Inkubationszeit von jeweils 10 und 20 Tagen wurden die Brote auf die Anwesenheit der Aflatoxine B_1 und G_1 hin untersucht; 1 g der Proben mit starker Mycelbildung wurde in 5 ml Chloroform zerrieben, und nach einer Extraktionszeit von 10 Minuten wurde das Chloroform dekantiert und zum Verdunsten gebracht und der Rückstand in 1 ml Chloroform gelöst. Mit Hilfe einer Mikrokapillare (Desaga, Heidelberg) wurden 2 μl dieser Lösung auf eine Kieselgel-Fertigplatte (Polygram Sil N-HR; Macherey-Nagel & Co., Düren) aufgetragen und aufsteigend mit 3% Methanol in Chloroform (Reiss, 1970) entwickelt. Als Standard wurden je 2 μl von Lösungen von jeweils 0,01 mg Aflatoxin B_1 und Aflatoxin G_1 (Roth, Karlsruhe) in 2 ml Chloroform mitchromatographiert. Nach Trocknung an der Luft wurden die Chromatogramme unter langwelligem UV-Licht (Blak-Ray, UVL-21; Hormuth-Vetter, Heidelberg) auf fluoreszierende Flecke hin untersucht.

Die halbquantitative Aflatoxin-Bestimmung erfolgte unter Zuhilfenahme eines Kodak-Graukeils (Reiss, 1973b). Absolute Werte können mit diesem Verfahren nicht angegeben werden, jedoch lässt sich ein eng begrenzter Konzentrationsbereich ermitteln. Die untersten Nachweisgrenzen betragen für beide Aflatoxine 0,002 μg, das heisst 2 ppb bei einem Ausgangsgewicht der Proben von 1 g.

ERGEBNISSE

Wachstum von A. flavus *auf verschiedenen Brotarten unter dem Einfluss der einzelnen Verpackungsfolien*

A. flavus wuchs gut auf Felke-, Weizenkeim-, Leinsamen- und Grahambrot sowie auf Bauernschnitten (Abb. 1), nicht jedoch auf Pumpernickel (Säuregrad über 12), grobem Rheinischem Vollkornbrot (Säuregrad 10,5) und Früchtebrot (Säuregrad nicht bestimmbar). Als bestes Substrat erwiesen sich Bauernschnitten, gefolgt von Weizenkeimbrot; auf Felke-, Leinsamen- und Grahambrot gedieh der Pilz ähnlich gut.

Von allen Folien reduzierte GEB das Wachstum von *A. flavus* am stärksten; ähnlich verhielt sich auch PNC. Auf der anderen Seite liess die PP-Folie ein besonders starkes Pilzwachstum zu, während PVC in etwa eine Mittelstellung zwischen GEB und PNC einerseits und PP andererseits einnahm.

Aflatoxinbildung in verschiedenen Brotarten unter dem Einfluss der einzelnen Verpackungsfolien

Die Abbildungen 2a und 2b fassen die ermittelten Konzentrationen von Aflatoxin B_1 und G_1 nach Inkubationszeiten von 10 und 20 Tagen zusammen. Gleichzeitig werden auch die Werte der Kontrollen angegeben. Bei allen Brotarten waren die höchsten

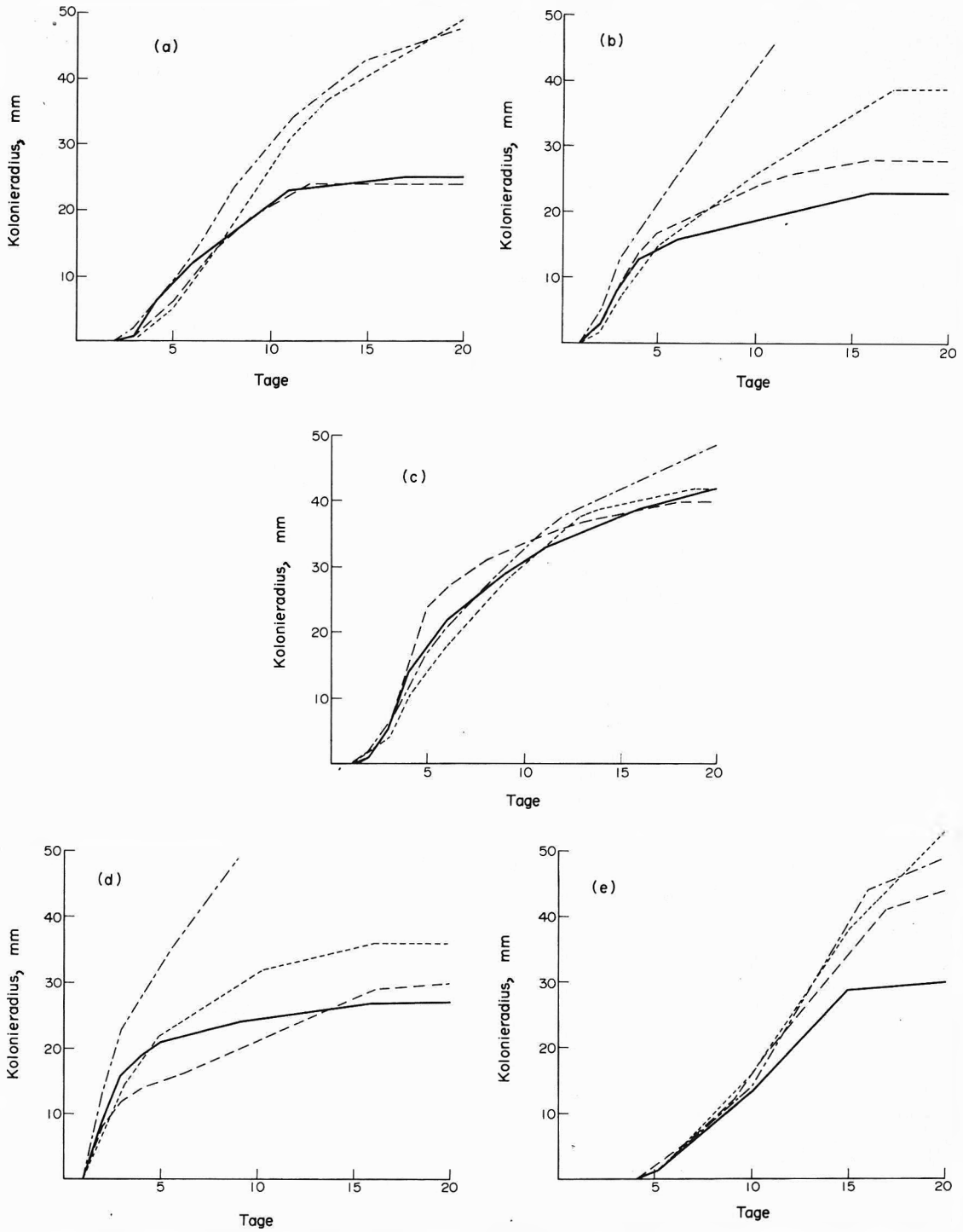

Abb. 1. Wachstum von *A. flavus* auf (a) Felkebroten, (b) Grahambroten, (c) Weizenkeimbroten, (d) Leinsamenbroten und (e) Bauernschnitten in verschiedenen Verpackungsarten: GEB (——), PNC (— — —), PVC (----) und PP (—·—·).

Aflatoxinmengen in den Kontrollbroten ohne Verpackung zu finden. Eine Verpackung reduzierte generell die Toxinbildung, wobei GEB und PNC den stärksten Einfluss hatten, gefolgt von PVC und PP.

DISKUSSION

Die Eigenschaften des verwendeten Verpackungsmaterials beeinflussen in hohem Masse Pilzwachstum und Aflatoxinproduktion. So werden diese beiden Faktoren durch GEB und PNC am stärksten gehemmt, während PVC und PP eine stärkere Ausbreitung von *A. flavus* sowie eine erhöhte Toxinbildung zulassen. Lediglich beim Weizenkeimbrot wird das Pilzwachstum durch die verschiedenen Folienarten nicht wesentlich beeinflusst (Abb. 1c). Wie aus der Tabelle der Eigenschaften der Folien zu erkennen ist, ist die Sauerstoffdurchlässigkeit der

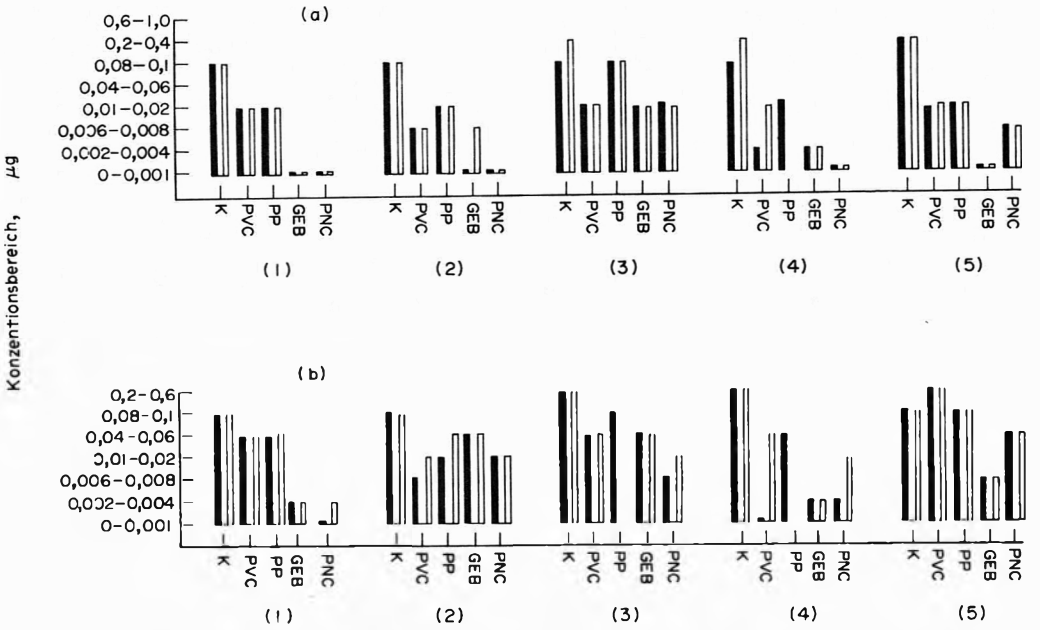

Abb. 2. Konzentrationen der (a) Aflatoxin B_1 und (b) Aflatoxin G_1 nach 10 (▮) und 20 (▯) Tagen auf verschiedenen Schnittbrotarten—(1) Felkebrot, (2) Weizenkeimbrot, (3) Leinsamenbrot, (4) Grahambrot und (5) Bauernschnitten. K = Kontrolle.

massgebende Faktor für den Einfluss einer Folie auf Pilzwachstum und Aflatoxinbildung: GEB und PNC sind praktisch sauerstoffundurchlässig, während PVC und insbesondere PP eine hohe Durchlässigkeit besitzen. Vor allem PP erlaubt dann auch ein rasches Wachstum von *A. flavus* und eine erhöhte Aflatoxin-Produktion. Wahrscheinlich wird bei einem Brot, das in stark sauerstoffundurchlässiger Folie verpackt ist, durch das Mycelwachstum die verfügbare Sauerstoffkonzentration sehr bald so stark erniedrigt, dass eine Synthese der Aflatoxine deutlich gehemmt oder ganz unterdrückt wird. Für die Praxis ergibt sich daraus, dass die sichtbare Anwesenheit eines Pilzmycels nicht notwendigerweise auch das Vorhandensein von Aflatoxinen zur Folge hat. Ist das Brot hingegen in sauerstoffdurchlässigem Material verpackt, dann kann bei einem durch das Mycelwachstum bedingtem Absinken des Sauerstoffgehaltes die den Pilz umgebende Atmosphäre von aussen her immer wieder Sauerstoff aufnehmen. Eine Aflatoxinproduktion ist dann möglich.

Die entscheidende Rolle des Sauerstoffgehaltes in der den wachsenden Pilz umgebenden Atmosphäre wird durch die Ergebnisse von Aflatoxin-Bestimmungen in unverpacktem Brot unterstrichen: alle Brotarten, auf denen *A. flavus* ohne den Einfluss einer Verpackung wachsen konnte, enthielten die höchsten Toxinmengen (Abb. 2a,b). Die bedeutende Rolle der verfügbaren Sauerstoffkonzentration für die Aflatoxinsynthese ist von vielen Autoren aufgezeigt worden Orth, 1973) doch konnte hier zum ersten Mal eine solche Abhängigkeit bei einem verpackten Lebensmittel demonstriert werden.

Ebenso wie in flüssigen und festen synthetischen Medien (Epstein, Steinberg, Nelson u. Wei, 1970) lässt sich auch bei allen Brotarten eine Parallelität zwischen Toxinproduktion und Wachstum feststellen. So ist im allgemeinen überall dort eine gesteigerte Toxinbildung nachzuweisen, wo *A. flavus* am stärksten wächst. Ein Zusammenhang der Konzentrationen von Aflatoxin B_1 mit denen von G_1 existiert nicht (Abb. 2a,b). So wird die Synthese von B_1 durch sauerstoffundurchlässige Folien meist deutlich stärker gehemmt als die von G_1.

Vergleicht man die nach 10 und 20 Tagen gefundenen Toxinmengen (Abb. 2a,b), dann lässt sich feststellen, dass in den meisten Fällen die Höchstmenge schon nach 10 Tagen synthetisiert worden ist und bis zum 20. Tag nur noch selten eine Steigerung eintritt.

Die unterschiedliche Intensität von Wachstum und Aflatoxinbildung auf den verschiedenen Brotarten bei gleicher Verpackung liegt naturgemäss in der Zusammensetzung des Substrats selbst begründet. So ist vermutlich der höhere Säuregrad die Ursache dafür, dass *A. flavus* auf Pumpernickel, grobem Rheinischem Vollkornbrot und Früchtebrot nicht wachsen kann. Auch der Patulin bildende Pilz *Penicillium expansum* konnte sich auf diesen Brotarten nicht entwickeln (Reiss, 1973c). Obwohl sich Leinsamenbrot und Grahambrot nur im Leinsamenanteil unterscheiden, wächst *A. flavus* auf der erstgenannten Brotart besser als auf letzterer. Dies wird wohl auf dem höheren Wasserhaltevermögen der Leinsamen sowie den zusätzlich in diesem Produkt vorhandenen Nährstoffen beruhen. Leinsamenbrot wird dadurch zu einem etwas günstigeren Nährboden, auf dem, wie Abb. 2a und 2b zeigen, auch grössere Mengen Aflatoxin gebildet werden.

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EMBRYOTOXIC AND TERATOGENIC EFFECTS OF CTAB, A CATIONIC SURFACTANT, IN THE MOUSE

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Abstract—Administrations of CTAB, a quaternary ammonium surfactant, to pregnant mice in an ip dose corresponding to 10 or 33% of the LD_{50} (10·5 or 35·0 mg/kg) increased the incidence of malformations, principally cleft palate and minor skeletal defects in the skull and sternum. At the higher dose, CTAB increased foetal mortality. Foetal body weight was increased by administration of CTAB on day 8 of gestation but was reduced by similar treatment on day 12 or 14. Very low levels of radioactivity were found in the foetuses after ip administration of [^{14}C]CTAB to pregnant mice on day 14 of gestation, the amounts probably being too small to cause teratogenic or toxic effects. An appreciable amount (about 3%) of the administered radioactivity was found in the placentae 1–8 hr after the injection. Cationic surfactants are known to affect the permeability of cells and tissues and it is possible that the embryotoxic and teratogenic effects of CTAB are due to a disturbance of the functional integrity of the placenta.

INTRODUCTION

Cationic surfactants exhibit excellent antimicrobial activity and have attained widespread use in bactericidal and fungicidal preparations. Salton (1951), Riemersma (1966), Salt & Wiseman (1968) and others have attributed the antimicrobial activity of cationic surfactants to a membrane-disorganizing effect. Some cationic surfactants have been found to alter the rate of transfer of certain compounds through the intestinal wall (Moore, Zatzman & Overack, 1971; Taylor, 1963), possibly by altering the permeability of the intestinal epithelium. To mammalian tissues, cationic surfactants have been found to be toxic in concentrations ranging from 10 to 500 ppm (Bengmark & Rydberg, 1968; Moore *et al.* 1971; Nissim, 1960; Taylor, 1963). In our laboratory we found certain alkyltrimethylammonium surfactants to be highly toxic to early chicken embryos. The LD_{50} for unincubated eggs was found to be in the region of 50 μg/egg (authors' unpublished findings). No investigation of the embryotoxicity of cationic surfactants in mammals has been reported to our knowledge, and the present study was undertaken to measure the potential embryotoxic and/or teratogenic effect of an alkyltrimethylammonium surfactant in mice.

EXPERIMENTAL

Chemicals. Cetyltrimethylammonium bromide (CTAB), 99% pure, was obtained from E. Merck, Darmstadt, Germany. Trimethyl-[1-^{14}C]cetylammonium bromide ([^{14}C]CTAB), with a radiochemical purity of 99%, was obtained from the Radiochemical Centre, Amersham, Bucks.

Animals. Female NMRI mice, weighing 25–30 g, were used in all experiments. The animals were given commerical pellets and water *ad lib.* and maintained in a 12-hr light-dark sequence under ambient conditions of 20 ± 1°C and 60 ± 5% humidity.

Toxicity. CTAB in aqueous solution was given in single ip injections to four groups each of eight mice. The median lethal dose and its standard error were estimated according to Miller & Tainter (1944).

Embryotoxicity and teratogenicity studies. Female mice were mated overnight with males of the same strain. The presence of vaginal plugs was taken as evidence of pregnancy and the day a vaginal plug was found was designated day 1 of gestation. Aqueous CTAB in a dose of 10·5 or 35·0 mg/kg body weight (calculated on the weight of the animal on conception day) was administered as a single ip dose on day 8, 10, 12 or 14 of gestation. These doses corresponded to 10 and 33% of the LD_{50}. The volume administered was 10 ml/kg body weight and control animals received an equal volume of water. The animals were weighed at regular intervals and killed on day 19 of gestation. The foetuses and placentae were removed and the numbers of living and dead foetuses and the sex and weight of each foetus were recorded. The foetuses were examined for external gross malformations under a stereomicroscope and then cleared in KOH and stained with Alizarin Red S for detection of skeletal anomalies and determination of the state of ossification. The Student's *t* test was used for comparison of body weights and the binomial test for proportions was used to evaluate the frequency of anomalies and resorptions.

Distribution studies. Pregnant mice were given an ip dose of CTAB (10% of the LD_{50}) containing about 1 μCi [^{14}C]CTAB on day 14 of gestation. The animals were decapitated 1, 8 or 24 hr after the injection. Blood samples were collected and the placentae and foetuses were removed. Foetal livers and samples (0·1–0·2 g) from maternal livers were taken for the determination of radioactivity. The placentae and the foetuses were homogenized in water (1:3) and aliquots (0·2–0·3 g) of these homogenates were taken for the determination of radioactivity. Tissues were dissolved in Protosol (NEN Chemicals, Dreieichentiain bei Frankfurt/M., Germany) by incubating overnight at 55°C and a toluene-based scintillation fluid was added. Blood plasma (0·1–0·2 ml) was counted in Aquasol (NEN Chemicals). All samples were counted

Table 1. *Effects of CTAB administered ip to mice on day 8, 10, 12 or 14 of gestation*

	Effects of a dose (mg/kg) of								
	0 on day	10·5 on day				35·0 on day			
Parameter	8–14†	8	10	12	14	8	10	12	14
Maternal weight gain‡ (g§)	22·56 ± 0·76	23·14 ± 1·09	23·74 ± 1·18	21·49 ± 1·26	21·53 ± 1·47	24·23 ± 0·98	19·84 ± 2·86	21·15 ± 1·23	20·08 ± 2·22
Live foetuses:									
total no.	212	84	88	84	93	64	69	80	81
% of implantations	95·1	93·3	89·8	92·3	94·9	65·3	71·9	88·9	79·4
Foetal weight (g§)	1·26 ± 0·009	1·30 ± 0·012**	1·25 ± 0·017	1·27 ± 0·013		1·32 ± 0·015***	1·25 ± 0·017	1·16 ± 0·013***	1·21 ± 0·014**
Dead implantations:									
total no.	11	6	10	7	5	34	27	10	21
% of all implantations	4·9	6·7	10·2	7·7	5·1	34·7***	28·1***	11·1*	20·6***
Malformed foetuses:									
total no.	11	21	20	25	32	26	35	45	33
% of live foetuses	5·2	25·0***	22·7	29·8***	34·4***	40·6***	50·7***	56·3***	40·7***
no. with cleft palate	1	0	3*	4**	7***	1	2	21***	10***
no. with skeletal defects‖	10	21	18	28	31	25	36	39	41
Foetal sex ratio (M:F)	119:93	39:45	41:47	49:35	56:37	27:37	37:32	43:37	41:40

†Controls injected on day 8, 10, 12 or 14 are all treated as a single group.
‡Between day 1 and day 19 of gestation.
§Values are means for groups of eight mice (or 20 controls) ± SEM.
‖Minor skeletal defects including incomplete ossification of dorsal bones (frontals and parietals) of the skull and irregularities of the sternum. This incidence was not compared statistically with controls.
Values marked with asterisks differ significantly (see p. 331) from those of the control group: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table 2. *Distribution of radioactivity in maternal and foetal tissues after ip administration of [^{14}C]CTAB in a dose of 10·5 mg/kg body weight (about 1 μCi/mouse) to pregnant mice on day 14 of gestation*

Tissue	Time after injection (hr)...	Amount of radioactivity (% of administered dose) 1	8	24
Maternal liver		39·25 ± 2·55	12·76 ± 1·52	2·36 ± 0·09
Total placentae		3·01 ± 0·10	3·20 ± 0·08	1·47 ± 0·10
Total foetuses		0·15 ± 0·04	0·49 ± 0·08	0·43 ± 0·03
Total foetal livers		0·05 ± 0·01	0·25 ± 0·03	0·14 ± 0·01

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

in a liquid scintillation spectrometer (LKB-Wallac, Turku) and the results were corrected for quenching by internal standardization.

RESULTS

Toxicity

The acute ip LD_{50} for female mice was found to be 106 ± 21 mg/kg. Death occurred generally within 12 hr of administration. The animals that did not die recovered within 24 hr, although some delayed deaths did occur. Signs of toxicity included convulsions and respiratory depression.

Embryotoxicity and teratogenicity

Observations on the foetuses are summarized in Table 1. Within the control group, no significant differences were observed between foetuses from mothers injected on different days of gestation. The controls were therefore treated as a single group in the statistical evaluations. As seen from Table 1, CTAB treatment increased the number of dead implantations, compared with control animals, when administered at the higher dose (35·0 mg/kg) but not at the lower dose (10·5 mg/kg). At both dose levels, CTAB increased the number of malformed foetuses throughout the developmental period studied, and the incidence of malformations was dose-dependent. The principal anomalies found were cleft palate and minor skeletal defects, including incomplete ossification of the dorsal bones (frontals and parietals) of the skull and irregularities in the sternum. The frequency of cleft palate was highest when the surfactant was administered on either day 12 or day 14 of gestation. Foetuses from mothers given CTAB on day 8 of gestation had a higher average body weight than the controls. However, administration of CTAB on day 12 (35·0 mg/kg) or day 14 (10·5 or 35·0 mg/kg) caused a decrease in foetal body weight. No significant differences between controls and treated groups were found in respect of maternal weight gain.

Distribution of radioactivity

Only small amounts of radioactivity were found in the foetuses after ip administration of [^{14}C]CTAB to pregnant mice on day 14 of gestation. Some 8 hr after the injection, a total of about 0·5% of the administered radioactivity was found in the foetuses from each dam (Table 2), about half of this amount being present in the foetal livers. About 3% of the administered dose was present in the placentae 1 hr after injection. The concentration of radioactivity in homogenized foetuses (Fig. 1) was less than 10% of that found in placental tissues, suggesting poor placental transfer of CTAB. The decline of radioactivity in the placentae was slower than that in maternal blood plasma and liver, and in both the placentae and foetal tissues the peak levels of radioactivity occurred later than in the maternal liver and blood plasma. Insufficient radioactive material was present in the foetal tissues for the radioactive compounds to be satisfactorily identified.

DISCUSSION

CTAB is metabolized to some extent in rats and rabbits (Hughes, Millburn & Williams, 1973) and it is possible that the low levels of unidentified radioactive compounds found in the foetuses represented metabolized CTAB. It is also possible, however, that it represented radioactive impurities with a greater ability than CTAB to penetrate the placental barrier.

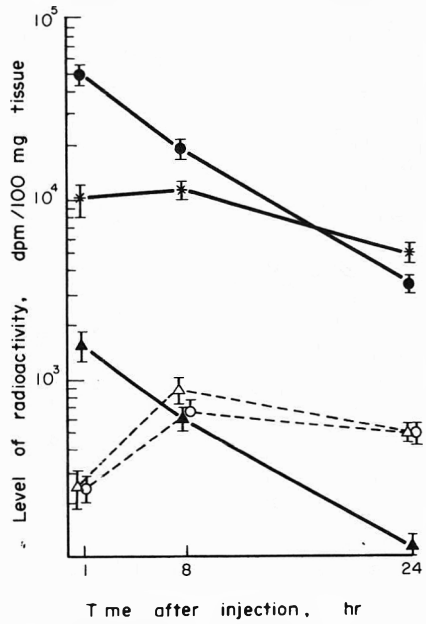

Fig. 1. Concentrations of radioactivity in maternal liver (●) and blood plasma (▲), in the placenta (*) and in foetal liver (△) and homogenized foetuses (○), after ip administration of [^{14}C]CTAB (10·5 mg CTAB/kg body weight and about 1 μCi/mouse) to pregnant mice on day 14 of gestation. Values are expressed as means ± SEM for groups of three or four mice.

Administration of CTAB to pregnant mice by ip injection in a dose corresponding to 10 or 33% of the LD_{50} (10·5 or 35·0 mg/kg) increased the incidence of dead implantations and malformations. The results indicate that CTAB may be considered a "classical" teratogen because at the lower dose the surfactant interfered with embryonic development without being embryolethal. At the higher dose, CTAB increased foetal mortality. The principal anomalies found were cleft palate, incomplete ossification of the dorsal skull bones and irregularities in the sternum.

The surfactant was found to affect foetal body weight in a rather puzzling way. CTAB administered on day 8 of gestation increased foetal body weight, but when the dose was given on day 12 or 14, foetal body weight was reduced. However, quaternary ammonium compounds are known to be poorly transferred across biological membranes. While the very small levels of radioactive compounds found in foetuses after ip administration of [^{14}C]CTAB to pregnant mice were probably too small to cause teratogenic and toxic effects, an appreciable amount of radioactivity accumulated in the placentae. Normal growth of the foetus is dependent upon the functional integrity of the placenta and the maintenance of adequate foetomaternal exchange. Cationic surfactants have been found by several workers to alter the permeability of cells and tissues (Moore *et al.* 1971; Salt & Wiseman, 1968; Penzotti & Mattocks, 1968; Ulitzur, 1970), and it is possible that the embryotoxicity and teratogenicity of CTAB is due to a disturbance of the functional integrity of the placenta.

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EXPERIMENTAL MODIFICATION OF PHOTOCARCINOGENESIS. I. FLUORESCENT WHITENING AGENTS AND SHORT-WAVE UVR

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(*Received 8 December 1974*)

Abstract—Erythema was produced on the skin of hairless mutant mice by a single exposure to low-pressure mercury-vapour (germicidal) ultraviolet lamps. The acute reaction was not affected by pretreatment of the skin with 20 μg of a fluorescent whitening agent (FWA). disodium 4,4'-bis-(4,6-dianilino-1,3,5-triazin-2-yl)-aminostilbene-2,2'-disulphonate, applied topically in 20 μl methanol. Skin tumours were produced in hairless mice during several months of daily exposure to the same lamps. Slightly fewer tumours, with a slightly longer latent period, were produced in mice similarly irradiated but pretreated daily with FWA as above. Thus, under the test conditions used, the FWA was not phototoxic, nor did it enhance photocarcinogenesis.

INTRODUCTION

The association of skin cancer and long-term exposure to ultraviolet radiation (UVR) has long been recognized (Blum, 1959; Emmett, 1973; Epstein, 1970; Johnson, Daniels & Magnus, 1968 Kopf & Gordon, 1969). The degree of acute skin damage as well as the chronic changes can be modified by certain chemicals which are phototoxic.

Concepts of chemical interaction with UVR-photocarcinogenesis are of more recent origin. Blum (1959) and Emmett (1973) reviewed a number of reports dealing with the influence of phototoxic substances on photocarcinogenesis. The results frequently appear to be in disagreement, a situation possibly reflecting differences in technique, including solvents, routes of administration, light sources and criteria for tumour recognition, and in statistical evaluation (Blum, 1959). In addition, characteristics of some compounds (toxicity, carcinogenicity, instability) render their interactions with light complex and their analysis difficult.

Interest in the photobiology of furocoumarins grew rapidly in the 1950s (*Journal of Investigative Dermatology, 1959*). A member of this family of compounds, 8-methoxypsoralen (8-MOP) was shown to be phototoxic and to enhance the ability of UVR to produce skin tumours in mice (Hakim, Griffin & Knox, 1960; Urbach, 1959).

Photocarcinogenesis can also involve non-photobiological promotion. Epstein & Roth (1968) and Pound (1970) exposed mice to a single dose of UVR which by itself did not produce tumours; subsequent repeated painting with croton oil elicited tumours in the irradiated mice.

Fluorescent whitening agents (FWAs) have been produced in increasing amounts for many years. Extensive toxicological testing in man and animals has shown most of these materials to be innocuous and non-sensitizing (Keplinger, Fancher, Lyman & Calandra, 1974; Snyder, Opdyke & Rubenkoenig, 1963; Stensby, 1967; Swedish Natural Science Research Council, 1973). However, other investigators have concluded that FWAs enhanced the carcinogenic potency of ultraviolet light (Bingham & Falk, 1970; Falk & Bingham, 1973). Bingham (1972 & 1973) has confirmed these observations. In view of the fact that FWAs are added to a variety of domestic products and have become a widely distributed component of the environment, any photobiological or promoting effect of FWAs, including the enhancement of photocarcinogenesis, could have significant impact.

Photochemical sensitization of human skin is a clinical problem of considerable significance (Harber & Baer, 1972). Phototoxicity and photoallergy can be produced experimentally in human and laboratory animals by the use of natural or simulated sunlight (Harber & Baer, 1972). However, the laboratory conditions of studies cited above (Bingham & Falk, 1970) were qualitatively different from any naturally occurring conditions. For example, the FWAs were suspended in a photoactive solvent (dimethylsulphoxide), applied to the densely-haired back of mice, and the animals were exposed to a germicidal lamp whose principal ultraviolet emission (UV-C; $\lambda < 280$ nm) is not found in earth-level sunlight. The potential significance of the results made it imperative to study FWAs under circumstances where it was possible to reduce the sources of ambiguity, while employing conditions reflecting those of human exposure to chemicals and light. Since the activation spectrum of an unknown photosensitizer cannot be predicted, light sources used for photobiological studies should contain the UVR spectrum in ratios approximating those found in sunlight. Conversely, there is no obvious reason for including portions of the solar spectrum that do not ordinarily reach the surface of the earth (UV-C, X-ray etc.).

This is the first of a series of studies reporting the alteration of photocarcinogenesis in mice. The purpose of the series is twofold. First, it documents the development of methods for evaluating the interaction of UVR and chemicals in skin carcinogenesis, utilizing methods that incorporate several recent developments in the production and measurement of light. Second, the series compares the ability of repre-

sentatives of two classes of compounds (FWAs and psoralens) to influence UVR photocarcinogenesis.

EXPERIMENTAL

Hairless mutant (HRS/J) mice were purchased from the Jackson Laboratory, Bar Harbor, Maine. Animals were housed throughout the experiment in special radiation cages (Forbes & Urbach, 1969) with free access to mouse chow and tap-water. The light source was a bank of three germicidal (G15T8) low-pressure mercury lamps, suspended 40 cm above the irradiated surface. The UVR flux was $4 W/m^2$ (J-225 meter, Ultraviolet Products, San Gabriel, Cal.). Chemical treatment consisted of pipetting 20 μl reagent-grade methanol, with or without 20 μg FWA on to 2 cm^2 of dorsal skin. The FWA, disodium 4,4′-bis-(4,6-dianilino-1,3,5-triazin-2-yl)-aminostilbene-2,2′-disulphonate (Fig. 1), from CIBA-GEIGY, Inc., Ardsley, New York, was approximately 90% pure, as supplied to detergent manufacturers. UVR exposure began 30 min after chemical treatment.

For phototoxicity testing, ten animals pretreated with FWA and an equal number pretreated with vehicle only were immobilized under the light source. A 1-cm diameter circular area of skin centred in each treated area was exposed, the remainder of the animal being covered with aluminium foil. These animals received a 5-min exposure once (1200 J/m^2) and were examined at 4, 8, 24, 48 and 72 hr after expsoure.

For the carcinogenesis experiment, the mice were exposed without restraint in the cages described above (Forbes & Urbach, 1969). Twelve female mice were treated with FWA and an equal number of mice were treated with vehicle alone and half of the mice in each group were exposed daily, on Mondays to Fridays, to a flux of $4 W/m^2$, starting 30 min after chemical treatment. The animals were irradiated for 5 min (1200 J/m^2) for each of 45 exposures, then for 10 min for each of the next 30 exposures and for 20 min for each of the final 125 exposures. The largest single possible dose was thus $72{\cdot}5 \times 10^6$ J/m^2. With each mouse free to move about in an 8 × 8 × 8 cm cubicle, the actual dose received by each area of skin varied somewhat with the animal's orientation during exposure, but the two UVR-exposed groups were subject to the same variability.

Each animal was examined weekly. One sheet of paper was made up for each mouse, the sheet bearing images of the mouse viewed dorsally, ventrally and from each side. Any change in skin appearance was noted at the appropriate location on the animal diagram, physical features and tattoo spots being used as landmarks. A tumour could be distinguished from cysts and other surface features by the time the growth was approximately 0·5 mm in height or diameter.

Tumours and other lesions were removed for histological examination from mice dying during the experiment, and from other animals at the end of the study (40 wk).

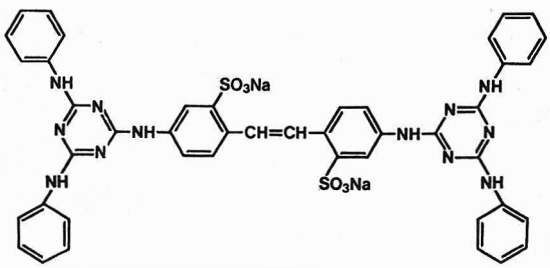

Fig. 1. Structure of fluorescent whitening agent used in the study.

RESULTS

Phototoxicity experiment

By itself, neither solution provoked a visible change in the skin. Areas exposed only to the vehicle and UVR developed barely perceptible erythema by 8 hr after exposure. The erythema had disappeared from some mice by 24 hr and from the remainder by 48 hr. The reaction of FWA-treated areas did not differ from the reaction of areas pretreated with vehicle only. Thus, the FWA was neither an irritant nor a phototoxic agent under the test conditions.

Carcinogenesis experiment

Half of each irradiated group (three mice out of six) survived to the end of the 40-wk observation period. No tumours appeared in the unirradiated mice. A total of 40 tumours appeared in the irradiated mice. Most tumours tended to enlarge and become increasingly cornified or necrotic, or both; very few proved to be invasive or metastatic. Of the 40 tumours analysed histologically, one was diagnosed as a haemangioma and two were fibrosarcomas; the remainder were squamous-cell carcinomas. Tumour production in irradiated mice was measured by several parameters (Table 1). Cumulative incidence refers to the proportion of mice that developed one or more tumours during the stated observation period. Latent period is a measure of tumour development time, and tumour yield is expressed both as total tumours produced and as the average tumours per affected animal. On each count, carcinogenesis was slightly greater in the vehicle-treated group than in the FWA-treated group. There is thus no evidence that this FWA enhances the carcinogenic potency of short-wave UVR in hairless mice.

Table 1. *Tumour induction in mice exposed to G15T8 (germicidal) UV lamps following topical pretreatment with FWA in methanol or with methanol only*

Tumour-induction parameter	Values for mice treated with FWA + UV-C	Values for mice treated with Vehicle + UV-C
Latent period to first tumour (wk)	17	16
Latent period to 50% incidence (wk)	19	17
Mean latent period for all tumours (wk)	27	25
Cumulative incidence at 24 wk* (%)	50 (3/6)	83 (5/6)
Cumulative incidence at 40 wk (%)	67 (4/6)	83 (5/6)
Total tumours at 40 wk	16	24
Mean no. of tumours/affected mouse	4·0	4·8
Survival at 40 wk (%)	50 (3/6)	50 (3/6)

*Time of first death.

DISCUSSION

Erythema and skin carcinomas were induced on hairless mice by exposure to UV-C. This radiation treatment did not cause skin ulceration. Neither the erythemal nor the carcinogenic potency of UV-C was enhanced by the presence of the FWA. The circumstances of testing, although not technically relevant to a potential human health problem, were similar to those used by Bingham & Falk (1970); the FWA was applied topically in an organic solvent, and the animals were then exposed to a germicidal lamp. In this system, we found no evidence that the FWA was photobiologically active or cocarcinogenic.

In order to relate laboratory data to realistic conditions of human exposure, we have developed test systems utilizing more appropriate light sources (Forbes & Urbach, 1974). Experiments based on these test systems are presented in companion reports (Forbes & Urbach, 1975a,b) and elsewhere (Forbes & Urbach, 1975c).

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EXPERIMENTAL MODIFICATION OF PHOTOCARCINOGENESIS. II. FLUORESCENT WHITENING AGENTS AND SIMULATED SOLAR UVR

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Abstract—Skin tumours were produced in hairless mice after several months of daily exposure to ultraviolet radiation from a solar simulator. The carcinogenic effect was enhanced by pretreating mice with 8-methoxypsoralen (tumours appearing sooner and in larger numbers). Other mice were pretreated with one of two substituted-stilbene fluorescent whitening agents (FWAs). Neither FWA produced phototoxicity or enhanced photocarcinogenesis.

INTRODUCTION

Several chemical agents have been reported to influence the course of photocarcinogenesis, but the mechanism of their action and even the direction of their influence are subject to debate (Blum, 1959; Emmett, 1973). Bingham and Falk (1970) concluded that three fluorescent whitening agents (FWAs) could enhance the carcinogenic potency of short-wave (254 nm) ultraviolet radiation (UVR). Their light source was a low-pressure mercury-vapour (germicidal) lamp.

Photobiological activity in as widely used a class of compounds as the FWAs would be of considerable importance. Consequently, we have examined a number of substituted-stilbene FWAs for such photobiological activities as phototoxicity, photoallergy and enhanced photocarcinogenesis (Forbes & Urbach, 1975c). The experiments were addressed to the questions of whether FWAs had demonstrable photobiological activity in human or animal skin, whether the Bingham & Falk (1970) findings could be confirmed in another strain of mice and whether photocarcinogenesis in mice could be enhanced by FWAs applied in a manner realistically representing the conditions of use.

The first report in this series presented evidence that one FWA was not phototoxic and did not enhance photocarcinogenesis induced by UVR in the 254 nm spectral region (Forbes & Urbach, 1975a). This report describes a study in which FWAs were applied in methanol to the backs of hairless mice prior to the exposure of animals to the UV portion of simulated sunlight.

EXPERIMENTAL

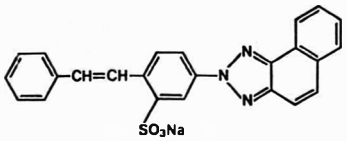
Animals. Hairless mice were initially purchased from Sandra Biological, Inc. (New York), were propagated in our animal colony over several generations and were listed (Skh:hairless) according to standard nomenclature (Festing, Kondo, Looslie, Poiley & Spiegel, 1972; Institute of Laboratory Animal Resources, Committee on Nomenclature, 1970). This outbred stock of animals is homozygous at the *hr* locus, with all offspring phenotypically hairless. Heterogenicity was maintained at the c (albinism) locus, resulting in the segregation of albino and pigmented animals. Only the albino hairless (Skh:hairless–1) were used in the work reported here. Throughout the experiments all animals had free access to mouse chow and tap-water. Mice were put into the experiments at 6–8 wk of age.

Light source. For convenience in discussion, the UV range of wavelengths is divided into three regions (modified from Meyer & Seitz, 1949): UV-A ('long'-wave UVR) 400–320 nm, UV-B ('sunburn' UVR) 320–280 nm and UV-C ('short'-wave UVR) 280 nm and shorter. The solar simulator was a modification of the Berger design (Berger, 1969). It consisted of a 1 kW xenon compact arc lamp with an optical system built to deliver the beam to six mice simultaneously. Radiation from the lamp was directed to a dichronic mirror through which most of the infra-red and visible portion of the spectrum was diverted to a heat sink; the UV-B and UV-A portions, together with some visible light, radiated on to the skin. A Schott WG320-2 filter was inserted into the beam to eliminate UV-C and to shape the UV-B to conform to a distribution found in noon summer sunlight. Mice were both housed and irradiated in specially designed isolation cages (Forbes & Urbach, 1969), with one mouse moving about freely in each cubicle.

Light measurement. UV-B flux was calculated as a function of current flow from a Westinghouse WL767 zirconium phototube (Brackett, Kuper & Eichor, 1941) measured with a Keithley picoammeter. Within the solar UV-B region, the WL767 phototube has response characteristics that closely parallel the action spectrum for 'minimal erythema' of untanned, white human skin. This apparatus yields information on the 'erythema effective energy' (EEE) of appropriate light sources (Koller, 1968).

Chemical agents. The structural formulae of the FWAs (CIBA-GEIGY, Inc., Ardsley, NY) used in this study are shown in Fig. 1. The compounds were sodium 2-(4-styryl-3-sulphophenyl)-2*H*-naphtho[1,2-*d*]-triazole (FWA-I) and disodium 4,4′-bis-([4-anilino-6-(*N*-methyl-2-hydroxyethylamino)-1,3,5-triazin-2-yl]-amino)stilbene-2,2′-disulphonate (FWA-III). They

FWA I



FWA III

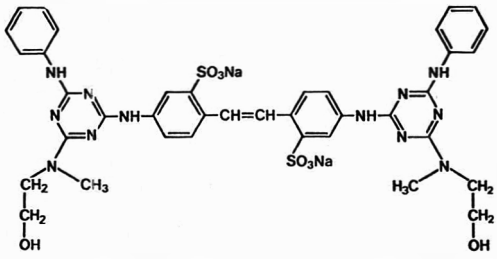


Fig. 1. Structure of fluorescent whitening agents used in the study.

were approximately 90% pure, as supplied to detergent manufacturers. The phototoxic agent 8-methoxypsoralen (8-MOP; Thomas B. Elder Co., Bryan, Ohio) was used as the positive control. Reagent-grade methanol was used as the negative control and vehicle.

Experimental design. For phototoxicity testing, mice were divided into groups of ten, and each group was pretreated with one of the four solutions (see below). The animals were then immobilized under the UVR source. A circular area of skin, 1 cm in diameter and centred in each chemically treated area, was exposed, the remainder of the animal being covered with aluminimum foil. The animals received one 55-min exposure (500 J/m^2 EEE) and were examined for skin erythema 4, 8, 24, 48, 72 and 96 hr after exposure.

For the carcinogenesis experiment, 120 mice were distributed into four groups of 30 each, with each group receiving one of the following treatments: methanol, 8-MOP (0·01%) or FWA-I or FWA-III (each 0·1% in methanol). All animals were treated with the test solution in a volume of 40 μl over an area of approximately 20 cm^2 of skin. This left a residue of 0·2 mg 8-MOP/cm^2 or 2 mg FWA/cm^2. The UV flux at the surface of the cage was 0·15 W/m^2 (EEE). The animals were exposed for 10 min daily for a maximum surface dose of 90 J/m^2 (EEE). The actual dose received by each area of skin varied somewhat with the position of the animal during the irradiation period. Radiation exposure began 30–60 min after application of the solution and both were repeated daily, on Mondays to Fridays.

Data and analysis. Mice were numbered consecutively by toe clipping and were randomized into isolation cages by age and sex. After the beginning of the experiment, each mouse was weighed once weekly and the distribution of FWA was noted under an F4OT12BLB blacklight; the skin of each mouse was then examined in detail under room light. One sheet of paper was made up for each mouse, the sheet bearing images of the mouse viewed dorsally, ventrally and from each side. Any change in skin appearance was noted at the appropriate location on the diagram, physical features and tattoo spots being used as landmarks. A tumour could be distinguished from cysts and other surface features by the time the growth was approximately 0·5 mm in height or diameter.

Biopsy specimens were taken from animals during the course of the project. Tumours and other lesions were removed at various stages of development for histological analysis. In reporting results, "tumour prevalence" is used to mean the proportion of surviving animals bearing one or more tumours at the time of observation. In this parameter, no weighting factor is given for animals bearing more than one tumour, or for the length of time that a tumour had been visible. A measure of development time for tumours first appearing on animals is available from prevalence figures. Differences in prevalence figures were evaluated by the chi-square test. Tumour yield is shown as the number of tumours present divided by the number of surviving mice (i.e. average number of tumours/mouse). At each observation period, the groups were compared by means of the Wilcoxon rank sum test (Wilcoxon & Wilcox, 1964). Survival is expressed as the absolute number of mice alive at the time of observation, 30 being the initial number in each group.

RESULTS

Phototoxicity experiment

Without subsequent irradiation, none of the solutions provoked a visible change in the skin. Areas exposed only to the vehicle and UVR developed barely perceptible oedema and erythema by 24 hr after exposure and the affected skin returned to a normal appearance by 72 hr. Skin pretreated with 8-MOP developed a more severe response, with the greatest intensity of oedema and inflammation occurring between 48 and 72 hr after exposure. In contrast, the reaction of FWA-treated areas did not differ from that of areas pretreated with vehicle only. Thus, 8-MOP was a phototoxic agent while the FWAs were neither primary irritants nor phototoxic agents in the presence of 300–400 nm UVR.

Carcinogenesis experiment

The UVR-exposed skin treated with 8-MOP became distinguishable from the surrounding skin after exposure for about 5 wk, the 8-MOP-treated areas having more erythema, hyperplasia and dry desquamation. This effect was not seen in FWA-treated areas. In fact, FWA-treated areas were distinguishable only by their fluorescence under 'black light'.

These short-term effects were mirrored in the late effects, 8-MOP markedly enhancing photocarcinogenesis, while the FWAs did not (Fig. 2). The 8-MOP group reached 50% prevalence before tumours appeared in the other groups (at wk 14). By wk 30, the vehicle-treated group had reached 50% prevalence (12/24 affected) and the FWA-III group had a significantly lower proportion of affected animals (6/27; $P < 0·01$). The vehicle and FWA-I groups did not differ significantly from each other (Fig. 2).

Tumour yield was significantly greater in the 8-MOP group than in any other group. After 32 wk, the FWA groups had a lower tumour yield than the vehicle control group ($P < 0·01$). Most of the tumours developed near the midline of the back. Very few de-

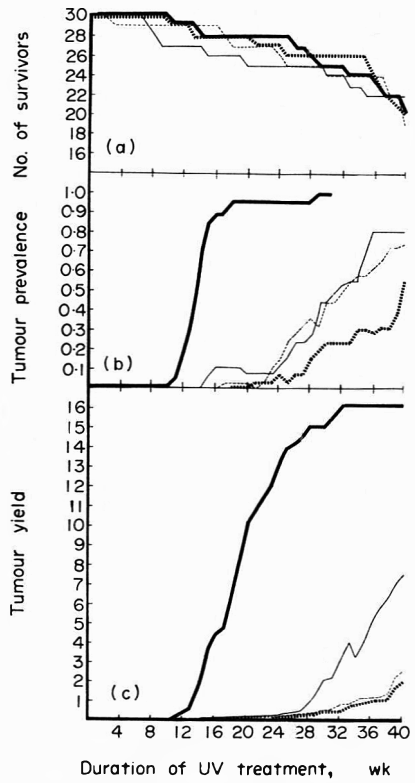


Fig. 2. Effects of pretreatment with 8-MOP (——), FWA-I (– – –), FWA-III (·····) or the detergent-solution vehicle (——) on (a) the survival, (b) the proportion of mice with tumours at any given time and (c) the average no. of tumours/survivor in groups of irradiated mice.

veloped on the head, snout, ears, extremities or abdomen.

Of the 123 tumours analysed histologically, 111 were squamous-cell carcinomas. The remainder were fibrosarcomas, lymphosarcomas, sebaceous adenomas and haemangiomas. Most tumours when left intact tended to enlarge and become increasingly cornified or necrotic or both. Very few proved to be aggressively invasive or metastatic. Tumours were rarely lost spontaneously.

Survival of the mice was similar in the four groups (Fig. 2).

DISCUSSION

Because of the extensive use of FWAs in industry and their introduction into products for domestic use, a great deal of toxicological information is available on these compounds. They have been considered safe for the uses and exposure conditions for which they are intended. However, Bingham & Falk (1970) reported that three FWAs could augment photocarcinogenesis in mice under the conditions of their experiment. Briefly, their procedure involved applying FWAs in dimethylsulphoxide (DMSO) to the backs of C3H mice and then irradiating them with UV-C from a low-pressure mercury (germicidal) lamp. They observed 16–24 tumours in groups of mice treated with FWAs plus UV-C, two tumours in mice treated with DMSO plus UV-C, and no tumours in mice treated with DMSO alone. No animals were treated with UV-C alone.

In order to evaluate the possible photobiological activity of some currently used FWAs, we have undertaken a number of studies on hairless mice utilizing a variety of UVR sources and exposure techniques. This is one of a series of reports resulting from these studies. The first report (Forbes & Urbach, 1975a) showed that one FWA, used under circumstances similar to those described by Bingham & Falk (1970), did not enhance photocarcinogenesis. We have now shown that FWAs do not enhance photocarcinogenesis induced by UVR in the spectral region 300–400 nm. A subsequent report (Forbes & Urbach, 1975b) deals with animals treated with FWAs in a laundry-type solution, followed by exposure to full-spectrum simulated earth-level sunlight.

With the light source used in this study, the 8-MOP solution was phototoxic, as indicated by the single-dose response as well as by the results observed after exposure for 5 wk in the longterm experiment. The same solution enhanced photocarcinogenesis. The FWAs tested were not phototoxic, nor did they enhance photocarcinogenesis.

Acknowledgement—This work was supported in part by NIH Grant ES 00269 from the National Institute of Environmental Health Sciences.

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EXPERIMENTAL MODIFICATION OF PHOTOCARCINOGENESIS. III. SIMULATION OF EXPOSURE TO SUNLIGHT AND FLUORESCENT WHITENING AGENTS

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Abstract—Skin tumours were produced in hairless mice after several months of daily exposure to ultraviolet, visible and infra-red radiation from a long-arc xenon-lamp solar simulator. Mice were bathed in an aqueous detergent suspension of a test compound (or the vehicle alone) prior to each light exposure. The carcinogenic effect was enhanced by pretreatment of mice with 8-methoxypsoralen (tumours appearing sooner and in larger numbers). Pretreatment of mice with any one of four substituted-stilbene fluorescent whitening agents did not produce phototoxicity or enhance photocarcinogenesis.

INTRODUCTION

It was recently reported (Bingham & Falk, 1970; Falk & Bingham, 1973) that three fluorescent whitening agents (FWAs) enhanced the carcinogenic potency of germicidal ultraviolet radiation (UVR) in mice. In contrast, one of the same FWAs was tested by Forbes & Urbach (1975a), and it neither produced phototoxicity nor enhanced photocarcinogenesis in hairless mice. Other FWAs were found to have no phototoxic effect in human skin or in the skin of miniature pigs or hairless mice (Forbes & Urbach, 1975c). When applied in methanol prior to irradiation, the FWAs that were tested did not enhance photocarcinogenesis in hairless mice (Forbes & Urbach, 1975a,b).

More recently, we have developed techniques for more closely simulating or appropriately exaggerating the conditions under which man is exposed to chemicals and solar radiation. This paper reports the results of bathing mice daily in aqueous solutions of test chemicals, followed by exposure to the UV, visible and infra-red (IR) component of simulated sunlight. Two of the tested FWAs had not previously been evaluated photobiologically; the other two had been tested in a different system (Forbes & Urbach, 1975b).

EXPERIMENTAL

The FWAs used, approximately 90% pure as supplied to detergent manufacturers, were sodium 2-(4-styryl-3-sulphophenyl)-2*H*-naphtho[1,2-*d*]triazole (FWA–I; Tinopal® RBS), disodium 4,4′-bis-[(4-anilino-6-morpholino-1,3,5-triazin-2-yl)amino]stilbene-2,2′-disulphonate (FWA–II; Tinopal AMS), disodium 4,4′-bis-([4-anilino-6-(*N*-methyl-2-hydroxyethylamino)-1,3,5-triazin-2-yl]amino)stilbene-2,2′-disulphonate (FWA–III; Tinopal 5BM) and disodium 4,4′-bis-(2-sulphostyryl)biphenyl (FWA–IV; Tinopal CBS). All were products of CIBA–GEIGY Inc., Ardsley, N.Y., and their structures are indicated in Fig. 1.

Hairless mutant mice (Skh:hairless-1) (Forbes & Urbach, 1975b) were housed in custom-built stainless-steel irradiation cage units (Figs 2a,b). The animals were put into the cages when 6–8 wk old and had free access to laboratory chow and tap-water

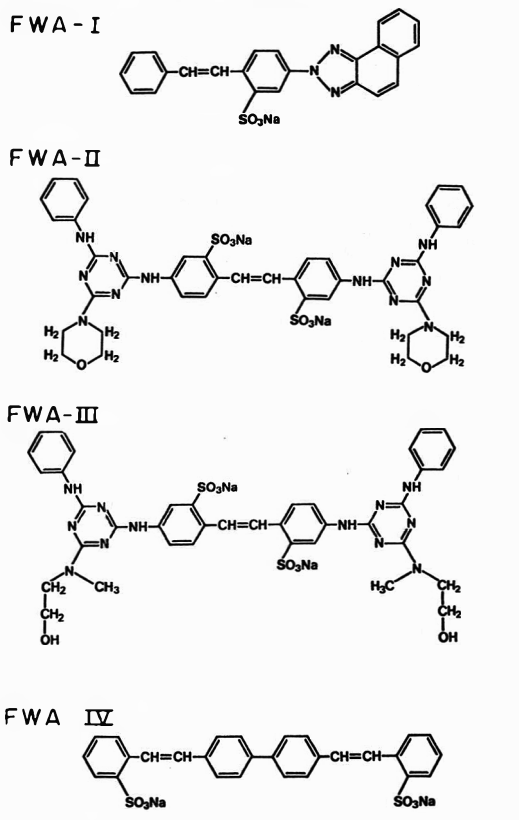

Fig. 1. Structure of the four fluorescent whitening agents used in the study.

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throughout the period of study. The light source was a 6-kW long-arc xenon burner (Osram XBF 6000) with a filter to eliminate UV shorter than 290 nm and to attenuate the IR (Atlas Electric, Chicago, Ill.). The output curve was a good match for a mid-latitude sea-level solar spectrum. The long axis of the lamp was hung vertically and animal housing units were arranged around the lamp. The UV flux at the side (exposed surface) of the cage was monitored with a zirconium WL 767 phototube (Forbes & Urbach, 1975b). The average flux was 0·042 W/m^2 erythema effective energy (EEE) during a daily (Monday to Friday) 2-hr exposure period for a maximum surface dose of 300 J/m^2/day (EEE). The actual skin dose was influenced by the position of the mice during exposure. The cages on each rack were rotated daily to minimize differences related to cage position. Mice were moved to clean cages once weekly.

All animals were bathed for 2 min in a test solution 30–60 min prior to light treatment and those in the carcinogenicity study were also treated daily with the solution for 2 wk prior to the first light treatment. The solution was at treatment-room temperature (27°C) and was contained in a glass tray. Cage units with six mice in individual cubicles were set into the glass trays, bringing the fluid level to within 1 cm of the top of the unit (Fig. 2c). The mice could swim or cling to the unit, with all but the head bathed. The animals adapted quickly to this mode of treatment; their growth and longevity compared favourably with those of untreated conventionally-housed mice (P. D. Forbes, in preparation).

All treatment solutions contained XTA-154 brightener-free detergent (Procter and Gamble Co, Cincinnati, Ohio) in distilled water in a concentration of 1 g/litre. One solution contained only detergent and was used as the vehicle control. A second solution contained the detergent and 0·1 g 8-methoxypsoralen (8-MOP)/litre, suspended by heating. The four FWAs were tested separately, each at 0·1 g/litre detergent solution. Fresh solutions were made daily.

For the study of phototoxicity, groups of ten mice were pretreated once as described above with one of the test solutions, and after an interval of 30 min were exposed for 2 hr to the long-arc xenon lamp. For the photocarcinogenesis study, 24 of the 48 mice treated with each solution were exposed to the xenon lamp daily, 5 days/wk for 40 wk. The other 24 mice in each group were kept as unirradiated controls. Details on the examination of mice and the recording of data have been described previously (Forbes & Urbach, 1975a,b).

RESULTS

Phototoxicity experiment

A single 2-hr exposure to the xenon lamp produced a moderately severe phototoxic response in mice pretreated with 8-MOP, but only a barely perceptible erythema in all others.

Carcinogenesis experiment

The fluorescence of the skin of FWA-treated mice increased during the first 2 wk of treatment. No direct determination was made of the amount of FWA on the skin, but the bathed mice were compared under 'black' light with mice that had received a single application of various concentrations of FWAs dissolved in methanol to controlled areas of skin. The FWA residue on bathed mice was estimated to be approximately 1 μg/cm^2 skin. Xenon-lamp exposure did not alter the skin fluorescence appreciably.

Except for skin fluorescence, the groups of unirradiated mice were indistinguishable. All irradiated mice had transitory erythema during wk 2–3 of UV treatment. By wk 10, the mice exposed to 8-MOP were distinguishable from all the others. They showed areas of more severe erythema, hyperplasia and dry desquamation, and later exhibited a marked tendency to develop multiple tumours (Figs 2d, e). The 8-MOP-treated mice had a higher tumour yield and prevalence than the irradiated controls treated only with detergent (Figs 3 & 4), but the FWA-treated groups had lower tumour yields and prevalence than the controls. Most of the tumours developed on the animals' sides, with very few on the midline of the back or on the head, ears, snout or abdomen.

DISCUSSION

Two distinct mechanisms for enhancement of photocarcinogenesis are suggested by currently available evidence. Photochemical interaction requires the presence of a photoactive agent during light exposure, e.g. 8-MOP plus UVR (Hakim, Griffin & Knox, 1960; Urbach, 1959). Promotion involves a sub-carcinogenic light exposure, followed by application of a

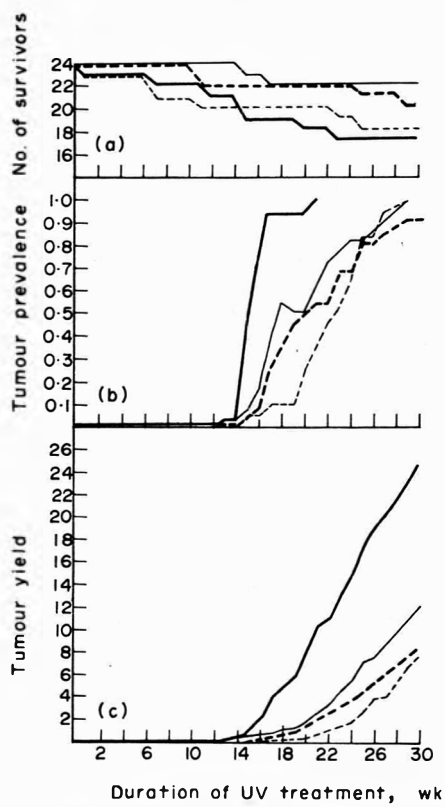

Fig. 3. Effects of pretreatment with 8-MOP (——), FWA-I (– – – –), FWA-III (- - - -) or the detergent-solution vehicle (——) on (a) the survival, (b) the proportion of mice with tumours at any given time and (c) the average no. of tumours/survivor in groups of irradiated mice.

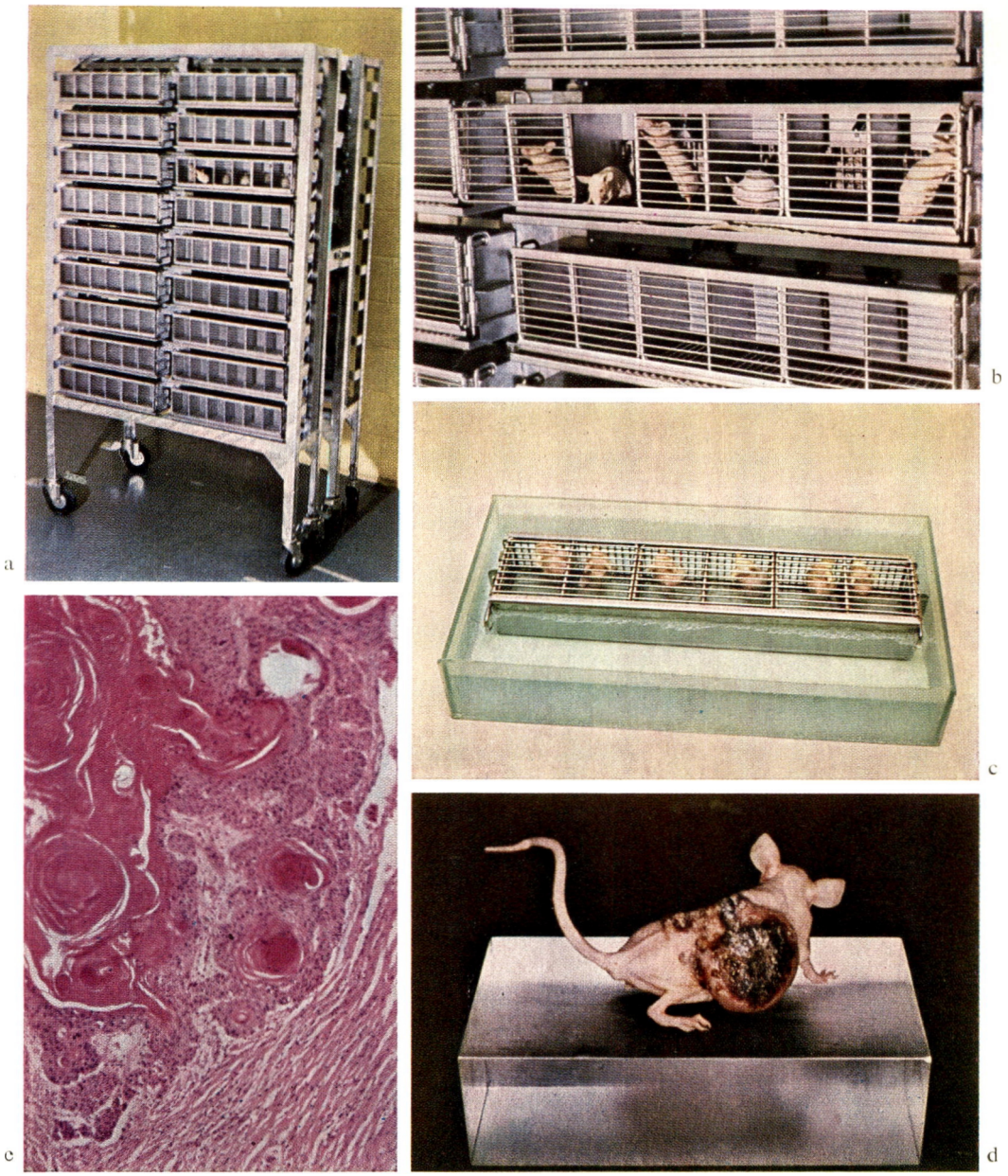

Fig. 2. (a) Cages on racks facing long-arc xenon solar simulator. (b) Cage unit detail. (c) Cage unit with mice in treatment solution. (d) Mouse with multiple tumours. (e) Histology of UV-induced carcinoma; keratin pearls are seen in upper left; tumour mass is invading musculature towards lower right. Haematoxylin and eosin × 100.

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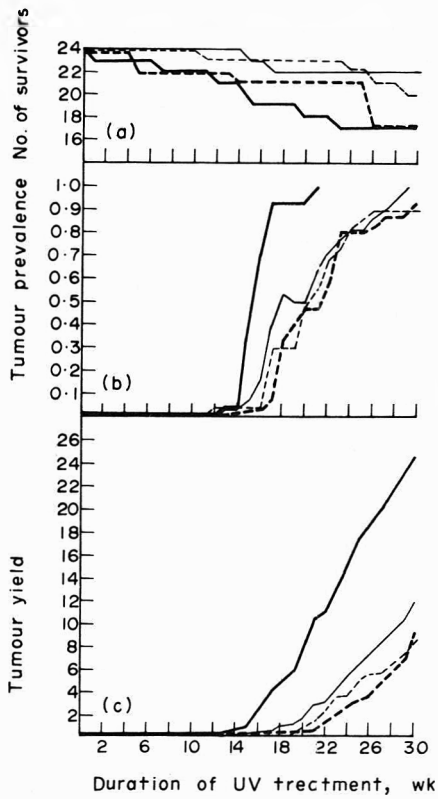

Fig. 4. Effect of pretreatment with 8-MOP (——), FWA-II (– – – –), FWA-IV (- - - - -) or the detergent-solution vehicle (——) on (a) the survival, (b) the proportion of mice with tumours at any given time and (c) the average no. of tumours/survivor in groups of irradiated mice.

compound capable of eliciting tumours only in irradiated skin; this is exemplified by studies involving UVR and croton oil (Epstein & Roth, 1968; Pound, 1970).

An adequate test system for evaluating light and chemical interaction in carcinogenesis should ensure that adequate light and chemical agent are available to an appropriate biological tissue, under circumstances designed to maximize the possibility of interaction.

This series of studies demonstrated that chemical enhancement of photocarcinogenesis by 8-MOP can readily be detected. In the same experiments, there was no evidence that the FWAs tested were phototoxic, nor that they were capable of enhancing photocarcinogenesis.

This stands in contrast to the reported finding of Bingham & Falk (1970) in haired mice. They pointed out that ulcerations developed in the region of application of the test material and that the ulcers progressed to tumours. Their report does not indicate what part was played by the components of treatment (UVR, dimethylsulphoxide, FWA, hair removal etc.) in the induction of ulcers or in the promotion of the ulcers to neoplasia. In a recent review (Falk & Bingham, 1973) the same authors stressed the possible influence of solvent on the expression of carcinogenesis, but the influence of ulceration (trauma, granulation and hyperplasia) was not discussed. In our study, neoplasia was not preceded by ulceration. Whether FWA-enhanced photocarcinogenesis can occur under any circumstances other than those existing in the original study (Bingham & Falk, 1970) has not been shown, nor can these authors' observations be generalized to conditions of known or reasonably predictable use (or misuse) of FWAs.

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ABSENCE OF TOXIC AND CARCINOGENIC EFFECTS AFTER ADMINISTRATION OF HIGH DOSES OF CHROMIC OXIDE PIGMENT IN SUBACUTE AND LONG-TERM FEEDING EXPERIMENTS IN RATS

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Abstract—The pigment chromic oxide (Cr_2O_3), which as chromium oxide green (C-green 9) or pigment green 17 is used as a cosmetics colouring, was tested in BD rats for subacute toxicity (90-day test) and for long-term toxicity and carcinogenicity (2-yr study). Administration of 2 or 5% in the feed for 90 days produced no signs of toxic effect and no detectable differences from untreated controls. Fertility of the animals was normal during treatment, and the young showed no malformations. Feeding of 1, 2 or 5% Cr_2O_3 in the feed for 2 yr was well tolerated. The animals were observed throughout life and there was no reduction in the average life expectancy of the experimental animals. Even with the very high oral doses of Cr_2O_3 given, no carcinogenic action was detected.

INTRODUCTION

The inorganic pigment, chromic oxide (chromium-(III) oxide; dichromium trioxide; Cr_2O_3) is used industrially, and as chromium oxide green (C-green 9) or pigment green 17 it is also incorporated into eye make-up, particularly as eye-shadow. In the classification in the Third Communication of the Deutsche Forschungsgemeinschaft, Farbstoff-Kommission (1959), the dye is placed in the C group of colourings, since it is applied on or near mucous membranes. According to the results of toxicological studies, C-green 9 is rated in this document as "provisionally permissible" in category Ca, data being available only on acute toxicity and local tolerance following application to the skin and eye.

A Russian paper (Dvizkov & Fedorova, 1967), described the formation of lung and pleural tumours after parenteral administration of an ill-defined chromium oxide to rats. The known carcinogenic actions of various chromium compounds in man and experimental animals have recently been summarized (International Agency for Research on Cancer, 1973). Since further investigation of the long-term toxicity of the insoluble pigment appeared to be necessary, chromium oxide green was fed to rats in the diet in a 90-day test and in a long-term feeding study lasting 2 yr.

EXPERIMENTAL

Materials. The substance used in the studies was generously made available by Dr. H. Eich, Besigheim, who also provided the following information:

> Chromium oxide green (Schultz no. 1451; Colour Index no. 77288; pigment green 17) was obtained by reduction of chromate at about 600°C. The product, which was washed until all soluble components, particularly chromate, had disappeared, was a fine green powder of pure Cr_2O_3, insoluble in water, alkali and mineral acids. The product was in a non-hydrated form. Precise analysis of the preparation used in the study gave a maximum content of 3 ppm Cr^{3+} with one of three investigators. The preparation was free from chromate (detection limits of the method of determination) 1 μg CrO_4^{2-}). With respect to heavy-metal impurities (arsenic, lead, mercury, antimony, soluble barium, copper and zinc), the product complied with the specifications of the Deutsche Forschungsgemeinschaft, Farbstoff-Kommission (1959).

Animals and diets. The studies were conducted on inbred BD rats of both sexes (Druckrey, Dannenberg, Dischler & Steinhoff, 1963), about 100 days old and weighing on average about 200 g at the start of the tests. They were housed in groups of four in Makrolon cages and both test animals and controls were given tap-water *ad lib.* Untreated controls were fed Altromin®, while the test pigment was administered in bread, which was made twice weekly by incorporating 1, 2 or 5% Cr_2O_3 into a dough made from 2835 g flour, 150 g milk powder, 150 g mixed salts (NaCl, $CaHPO_4$ and Cu, Zn, Co, Mn and K), 150 g cooking oil, 150 g cod-liver oil, 300 g malt extract, 600 g sugar, 80 g yeast and 900 ml water and subsequently baked. Uneaten bread was weighed in order to determine the amount consumed. There were only small losses from crumbs.

Experimental design and conduct

90-Day test. The test pigment was given to two groups on 5 days/wk, one group of 14 males and five females receiving 2% in the feed and a second of five males and ten females being given 5%. At weekends, the test animals received the control diet with a vegetable supplement. The untreated control group consisted of six males and six females. Uptake

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of food was determined weekly and body weight fortnightly. The blood picture was determined before the beginning of the experiment and monthly during feeding. Random samples were taken during the course of the study and from all animals in the last week of the experiment for urinary determinations of protein, sugar, bilirubin, blood and sediment. During the last 30 days of treatment, males and females from the same group were paired to test fertility, and the number of young in each litter was recorded.

At the end of the feeding period, the animals were fasted for 24 hr. Blood samples were taken from the retrobulbar plexus for determinations of blood sugar, serum protein and serum bilirubin, and from the tail vein for counts of erythrocytes and total and specific types of leucocytes and for haemoglobin estimations. Blood sugar was determined enzymatically, serum protein by biuret and serum bilirubin with diazotized sulphanilic acid. The animals were killed with ether and autopsied, the liver, spleen and kidney being weighed in all animals and the brain and ovaries in random samples. Samples of these organs and of lung, heart, pancreas, stomach, small intestine and urinary bladder were fixed in 4% buffered formalin and sections were embedded in paraffin wax and stained with haematoxylin and eosin.

Carcinogenicity (2-yr feeding) study. Groups of 60 male and female rats were fed 1, 2 or 5% Cr_2O_3 baked in bread on 5 days/wk for 2 yr (600 feeding days), the control diet with a vegetable supplement being given each weekend. A further group of 60 male and female rats served as untreated controls. Intake of the test pigment was calculated weekly and the animals were weighed monthly. At the end of the feeding period, surviving animals were maintained on the control diet until they died or became moribund, death being induced with ether in the latter case. At autopsy, all the important organs, including the brain and nervous system, were fixed in 4% formalin solution and studied histologically.

RESULTS

90-Day test

The treatment was well tolerated at both dose levels. One male rat fed the 5% Cr_2O_3 diet died 70 days after the start of the experiment from acute pneumonia. Food intake was normal throughout the study, averaging 20 g/day for males and 15 g/day for females. The total amount of Cr_2O_3 consumed during the whole of the experiment in which 2% was fed was 75 g/kg body weight (25 g/animal) for males, and 72 g/kg for females (18 g/animal). In the experiment in which 5% was fed, 180 g/kg was consumed by males, and 160 g/kg by females. No significant differences in body weight were evident between the two experimental groups and the controls (Fig. 1).

The faeces of the treated animals showed an intense green coloration throughout the study, indicating significant excretion of the administered pigment. In a subsidiary experiment in which 5 g Cr_2O_3/kg was given in a single dose to four rats, excretion was evident from the green coloration of the faeces about 16 hr after administration and ended after about 4 days. A total of 5·95 g Cr_2O_3 was given to these animals and some 4·9–5·0 g Cr_2O_3 was excreted unchanged in the faeces*.

Haemoglobin determinations and counts of erythrocytes and total and differential leucocytes in blood samples from the Cr_2O_3-treated groups showed no significant deviations from the corresponding control values (Table 1) and there were no adverse findings in determinations of serum protein, serum bilirubin and blood sugar (Table 2). These estimations were carried out at the end of the Cr_2O_3 feeding and shortly before the death of the animals. Urine analyses carried out during the experiment and at termination showed no significant differences between the treated and control animals.

Altogether nine females were paired with males from the same dosage group 60 days after the start of feeding. All the females became pregnant in due course, the gestation period was normal (23 days) and the young showed no malformations or other adverse effects. Litter sizes were in the normal range, averaging eight pups. Some of the progeny were retained for lifetime observation and so far (600 days) no tumours have been detected. It was thus shown that no toxic or teratogenic effects were associated with Cr_2O_3 treatment throughout the gestation period and fertility was not adversely affected.

All the experimental animals except those from the fertility study, which were still suckling, were killed within 1 wk of the cessation of Cr_2O_3 feeding. All the major organs were investigated at autopsy. With the exception of the weights of the spleen and liver, which showed some reduction in the treated animals

* These determinations were carried out by Dr. H. Eich.

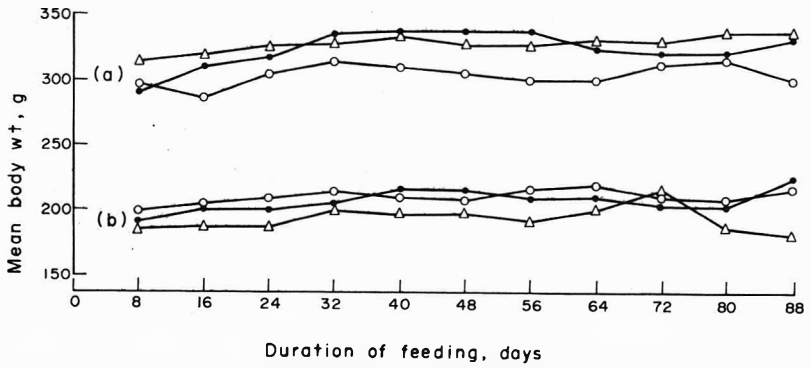

Fig. 1. Body-weight curves for (a) male rats and (b) female rats fed Cr_2O_3 at dietary levels of 0 (control; ●), 2 (△) or 5% (○) on 5 days/wk for a 90-day period.

Table 1. *Haematological findings in BD rats fed diets containing 0, 2 or 5% Cr_2O_3 for 90 days*

Sex and dietary level (%)	No. of rats	Haemoglobin (g/100 ml)	Erythrocytes (10^6/mm^3)	Leucocytes: Total (10^3/mm^3)	Leucocytes: Differential (%) N	L	M	E
Males								
0	6	16·0 ± 0·80	6·2 ± 0·76	12·3 ± 2·30	27·2 ± 1·8	70·3 ± 2·1	1·1 ± 0·3	1·4 ± 0·70
2	14	15·9 ± 0·77	6·0 ± 0·64	14·7 ± 1·80	25·7 ± 1·3	71·4 ± 0·8	1·0 ± 0·2	1·9 ± 0·65
5	4	15·1 ± 0·60	5·7 ± 0·59	10·8 ± 1·10	23·6 ± 2·0	72·2 ± 0·7	1·8 ± 0·4	2·4 ± 0·76
Females								
0	6	14·8 ± 0·50	5·9 ± 0·60	11·7 ± 2·00	25·5 ± 1·1	68·4 ± 2·0	3·1 ± 0·2	3·0 ± 0·88
2	5	13·9 ± 0·66	5·5 ± 0·63	14·4 ± 1·30	30·2 ± 1·4	67·1 ± 1·8	1·1 ± 0·3	1·6 ± 0·70
5	10	15·1 ± 0·55	5·9 ± 0·80	11·9 ± 1·60	27·0 ± 1·6	70·4 ± 2·2	0·9 ± 0·44	1·5 ± 0·80

N = Neutrophils L = Lymphocytes M = Monocytes E = Eosinophils

Values are means for the numbers of animals stated ± SEM. Those recorded for all three groups initially and at the end of months 1 and 2 of the study were closely similar to those reproduced in the table.

(Table 3), there were no macroscopic or histological changes. Compared with sections of organs from control animals, no changes could be detected that could be attributed to the Cr_2O_3 treatment.

Carcinogenicity study

Feeding of Cr_2O_3 at all three dose levels was well tolerated. As in the 90-day test, the body-weight curves for the treated animals did not differ from those for the controls. The 60 control rats reached an average life expectancy of 890^{+45}_{-30} days. Two mammary fibroadenomas, one mammary carcinoma and two hypophyseal adenomas were observed in this group. Of the 60 rats given 1% Cr_2O_3, the first died from a lung infection after 130 days and the last 1315 days from the start of the experiment. The average life expectancy was 870^{+30}_{-15} days. The total dose of Cr_2O_3 consumed was about 120 g/rat, or about 360 g/kg body weight, in 600 days of feeding. Only three mammary fibroadenomas were detected in this group. The remaining animals showed no macroscopic or microscopic signs of benign or malignant growths. The first death in the group given 2% Cr_2O_3 occurred after 127 days and the last 1230 days from the start of feeding. The average survival period was 880^{+30}_{-20} days. The total dose of Cr_2O_3 consumed in 600 days of feeding was 240 g/rat, or 720 g/kg body weight. One mammary fibroadenoma was the only tumour observed. In the group given 5% Cr_2O_3, the first rat died after 113 days and the last after 1295 days. The average survival was 860^{+40}_{-25} days. The total dose of Cr_2O_3 consumed in 600 days of feeding was about 600 g/animal or 1800 g/kg body weight. Three mammary fibroadenomas and one hypophyseal adenoma were found, but no other benign or malignant tumours appeared.

DISCUSSION

This investigation of the pigment Cr_2O_3 (C-green 9) showed no toxic effects in BD rats given high oral doses over 90 days or 2 yr. This result is the more convincing because the doses used (up to 5% of the feed) were substantially higher than the 1% in feed recommended by national (Deutsche Forschungsgemeinschaft) and international (WHO) authorities.

In the 90-day test, there were no statistically significant differences from the values found in control animals for the haematological determinations (total and differential leucocytes, erythrocytes and haemoglobin) or for the biochemical measurements of serum protein and blood sugar and of blood, sugar, protein, bilirubin and sediment in the urine. The slightly reduced total bilirubin content in serum was within the normal limits for the strain of rat used.

The only striking observation in the subacute study was the dose-dependent reduction in the organ weights of the liver and spleen. No differences were noticed in the other organs. However, no pathological changes, either macroscopic or histological, were

Table 2. *Blood analyses in BD rats fed diets containing 0, 2 or 5% Cr_2O_3 for 90 days*

Sex and dietary level (%)	No. of rats	Sugar (mg/100 ml blood)	Protein (g/100 ml serum)	Total bilirubin (mg/100 ml serum)
Male/female				
0	10	74·1 ± 4·8	6·4 ± 0·30	0·57 ± 0·28
Male				
2	13	72·9 ± 5·1	6·2 ± 0·26	0·19 ± 0·11
5	4	75·5 ± 4·8	5·7 ± 0·32	0·23 ± 0·15
Female				
2	4	77·2 ± 4·4	6·2 ± 0·30	0·36 ± 0·20
5	7	73·4 ± 5·0	6·2 ± 0·28	0·34 ± 0·11

Values are means for the stated numbers of rats ± SEM.

Table 3. *Weights of organs from BD rats fed diets containing 0, 2 or 5% Cr_2O_3 for 90 days*

Sex and dietary level (%)	No. of rats	Organ weights (g)		
		Liver	Spleen	Kidneys
Males				
0	6	8·9 ± 0·7	0·82 ± 0·08	1·6 ± 0·10
2	14	8·8 ± 0·6	0·61 ± 0·15	2·2 ± 0·10
5	4	7·7 ± 0·5	0·52 ± 0·12	2·1 ± 0·08
Females				
0	6	7·3 ± 0·5	0·55 ± 0·10	1·3 ± 0·08
2	5	6·7 ± 0·7	0·48 ± 0·16	1·8 ± 0·09
5	10	5·1 ± 0·6	0·43 ± 0·15	1·2 ± 0·08

Values are means for the stated numbers of rats ± SEM.

found in the liver or spleen or any other organ studied. The reduced weights of the liver and spleen could therefore not be evaluated as a serious toxic effect without further investigation.

This interpretation is supported by the result of the long-term feeding study. Concentrations of 1, 2 and 5% Cr_2O_3 in the feed over a period of 2 yr (600 treatment days) with a total consumption between 360 and 1800 g Cr_2O_3/kg body weight were tolerated without signs of chronic toxicity. The body-weight curves of the treated animals showed no differences from those of the untreated controls and the median survival times in all three dosage groups were comparable with that of the controls. No macroscopic or histological post-mortem findings could be causally related to the Cr_2O_3 treatment.

The lack of any carcinogenic action of Cr_2O_3 is of particular importance. The type and frequency of the tumours appearing in the experimental and control animals were comparable; mammary fibroadenomas and the hypophyseal adenomas are 'spontaneous' tumours characteristic of strains of BD rats. Orally administered Cr_2O_3 has no carcinogenic action in rats even when excessive doses are given.

In contrast, Dvizkov & Fedorova (1967) described the development of malignant tumours (mainly at the site of application) after intratracheal, intrapleural and ip administration of single doses of 10–50 mg Cr_2O_3/rat. These divergent results can easily be explained by the different route of administration, since our own studies have shown that most of an orally administered dose of Cr_2O_3 is excreted unchanged in the faeces. It is known that various chromium compounds are very poorly absorbed from the gastro-intestinal tract of experimental animals (Akatsuda & Fairhall, 1934; Aronson & Rogerson, 1972). Similar observations have been made in man (Schroeder, Balassa & Tipton, 1962). On the other hand, parenterally administered chromium (either in ionized or insoluble form) is easily transported and distributed, and can be accumulated in the lungs and kidneys (Baetjer, Damron & Budacz, 1959; Grogan, 1957; Kovalchuk, 1966; Visek, Whitney, Kuhn & Comar, 1953). The occupationally conditioned susceptibility to lung cancer in workers in the chromium-processing industry may be attributed almost entirely to inhalation of chromium-containing dusts, as has been established by epidemiological studies from Germany (Gross & Kölsch, 1943; Letterer, Neidhardt & Klett, 1944), the USA (Baetjer, 1950; Brinton, Frasier & Koven, 1952; Machle & Gregorius, 1948; Mancuso & Hueper, 1951) and Great Britain (Bidstrup & Case, 1956).

A possible defect in our studies is that the oral route is an inappropriate mode of application for a cosmetic product which is applied to the skin, but this may be refuted on the grounds of the poor resorptive capacity of the skin. It is recognized that substances shown to be non-toxic when administered orally are generally non-toxic when applied to the skin. Far more extensive absorptive surfaces are available in the gastro-intestinal tract, and experience such as that reported by Gloxhuber (1970) has shown that the skin absorbs less readily than the mucous membranes of the gastro-intestinal tract.

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LONG-TERM TOXICITY STUDIES ON OXIDATION HAIR DYES

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Abstract—Three oxidation hair dye formulations, mixed with hydrogen peroxide as in use, were tested for long-term toxicity and carcinogenic activity by topical application to groups of 100 mice weekly or every alternate week for 18 months. Chemical intermediates present in the various formulations were *p*-phenylenediamine, 2,5-toluenediamine sulphate, resorcinol, *m*-phenylenediamine, 2,4-diaminoanisole sulphate and 2,4-toluenediamine. The latter compound has been shown to produce malignant hepatomas when fed to rats in a sub-optimal diet at a level of 0·1% for 35 wk. In this study none of the formulations produced evidence of systemic toxicity or carcinogenicity.

INTRODUCTION

The natural vegetable and mineral substances used for centuries to colour the hair have largely been replaced by "permanent" synthetic oxidation dyes. As a result of many technical improvements, the use of oxidation hair dyes has increased dramatically in the last 20 yr, accounting for 75% of the hair colouring products sold today.

The dyes are formed inside the hair by the oxidation of colourless intermediates, principally aromatic diamines and aminophenols, to imines which react very rapidly with colour modifiers or couplers to form indo dyes (Corbett & Menkart, 1973). Although these reactions occur rapidly, it has been shown (Kiese & Rauscher, 1968) that in small amounts (less than 0·2% of the applied dose) unreacted intermediates may penetrate the skin during the dyeing of human hair.

Heavy industrial exposure to certain aromatic amines has caused bladder cancer in the past (Clayson & Cooper, 1970). Hueper (1963) has shown that although these tumours arose from prolonged skin absorption, there was no evidence of any effect of these chemicals on the skin itself. The reason for this now seems obvious in view of studies showing that aromatic amines appear to require metabolic conversion to the *N*-hydroxy derivative to induce cancer (Miller & Miller, 1969). We have found no published data demonstrating that *N*-hydroxylation occurs when the aromatic diamines used in permanent hair dyes are oxidized and there is no evidence indicating an increase in malignancies in the likely target organs in women using these dyes regularly for many years (National Cancer Institute, 1971).

Evidence that the use of hair dyes is not a significant factor in the production of scalp malignancies has been provided by Dr. F. Urbach, Skin and Cancer Hospital of the Temple University School of Medicine (personal communication 1973). Over a period of 15 yr, Dr. Urbach found only 16 persons (eight men and eight women) with scalp tumours out of 1672 consecutive patients with biopsy-proven cancer of the skin. This incidence of primarily sunlight-induced basal-cell carcinomas of the haired scalp area has not changed since the condition was first reported in 1878 (MacDonald & Bubendorf, 1964).

The purpose of this study was to determine whether any toxicity is associated with long-term cutaneous exposure to important ingredients or reaction products in oxidation hair dyes. Included in the study was one compound, *m*-toluene diamine, which has been shown to produce hepatoma in rats when fed at a level of 0·1% in a sub-optimal diet for 35 wk (Ito, Hiasa, Konishi & Marugami, 1969). This compound, which is no longer used in hair dyeing in the United States, was used for a number of years at a concentration of less than 1% in oxidation dye formulations. The extent of its skin penetration and metabolism has not been determined. Oxidation dyes, which are applied to the hair as a reacting system, can only be meaningfully evaluated from the toxicological viewpoint by the same route of administration as occurs in use, namely skin application.

In hair dyeing, the user is exposed to unreacted *para* components (*p*-phenylenediamine, *p*-aminophenol) and couplers (*m*-phenylenediamine, 2,4-diaminoanisole, *m*-aminophenol, resorcinol, hydroquinone), as well as to reactive intermediates, particularly quinone imines and the various indo dyes. The oxidation of the *para* components by hydrogen peroxide is relatively slow and has been shown to be incomplete even after 24 hr. However, the coupling reactions are extremely fast and thus there is no significant build-up in the concentration of the intermediate quinone imines. Nevertheless, it is important that the testing protocol should involve exposure to these extremely reactive species. This cannot be done by testing with the intermediates themselves since, in aqueous media, they undergo rapid polymerization and/or hydrolysis (depending on the pH) to give products that do not arise in the oxidation dyeing process. Similarly, if aged reaction mixtures were used,

there would be little or no exposure to the quinone imines since they are no longer formed once the hydrogen peroxide is depleted.

The feeding of aged reaction mixtures may, at first sight, appear to be an attractive compromise. However, under conditions prevailing in the stomach (a pH around 2 and a temperature of about 38°C), the indo dyes would undergo rapid hydrolysis to give a mixture of *para* components, hydroxybenzoquinones and reaction products thereof. The quinone products would not arise in other metabolic pathways and the ingestion route is thus unrepresentative of the metabolism of indo dyes. Furthermore, no intermediate quinone imines would be involved in this mode of testing. Thus, from the chemical standpoint, topical application of fresh reaction mixtures is the only protocol involving exposure to all the materials that are inherent in the application of oxidation hair dyes to human subjects. This can only be achieved by careful choice of composites not by the use of individual precursors in the presence of peroxide. On the basis of these considerations, the protocols and test compositions used in this study were selected.

EXPERIMENTAL

Materials. The hair-dye intermediates used in these studies were obtained from several suppliers and either met standards as purchased or were subjected to further purification. *p*-Phenylenediamine (lot 1143) and resorcinol (lot 312), purchased from Nyanza, Inc., Lawrence, Mass., were over 99% pure. *p*-Toluenediamine (lot K38671) from Naftone, Inc., New York, assayed at 98·5%. The 2,4-toluenediamine and *m*-phenylenediamine from Eastman Kodak, Kingston, Tenn., were recrystallized from ethanol and isopropanol to a final purity of over 98%. On a moisture-free basis, the 2,4-diaminoanisole sulphate (lot A56442) from Lowenstein Inc., Brooklyn, New York, assayed at 96–97%.

Animals and diet. Random-bred Swiss Webster mice of both sexes, 6–8 wk old, from Charles River Laboratories were housed individually and were given Ralston Purina Laboratory Chow and water *ad lib.*

Experimental design and procedure

The mice were randomly allotted to nine groups each of 100 mice and one group of 250 mice. There were equal numbers of each sex in each group. The experimental design is shown in Table 1. Three oxidation hair-dye formulations were tested (Table 2), each formulation being mixed with an equal volume of 6% hydrogen peroxide just prior to use. Once weekly or once every other week for 18 months, 0·05 ml of the mixture was applied to the skin of the mid-scapular area, which was kept free of hair by shaving with an electric clipper. The dose was spread just enough to prevent run-off. After each application all unused material was discarded. The vehicle-control group received the base with no added dye intermediate. The positive-control group was housed in a separate room and received weekly applications of 0·05 ml of a solution of 7,12-dimethylbenz[*a*]anthracene (DMBA) in acetone.

It is not always possible in a topical study to select the single dose of the positive-control carcinogen that will give the best indication of the sensitivity of the test animal. One may simply apply an overwhelming dose producing a high incidence of tumours in a short period of time, or it may be more meaningful in terms of the usual human exposure to apply small doses over longer periods and thus achieve the best estimate of the minimal tumorigenic dose. Poel (1956) has shown that 125 μg benzo[*a*]pyrene applied topically to mice as a single dose is not carcinogenic in the normal life span of the mouse, while 1 μg administered three times weekly for 40 wk (total 120 μg) produced tumours in nine of 42 mice by wk 40. Klein (1956) has shown that as little as 1·2 μg DMBA applied topically to Swiss mice over a 10-month period caused a 13% incidence of tumours. In the study reported here, a split-dose schedule was used, with mice receiving 50 ng DMBA/wk for 6 months, followed by 10 μg/wk for 4 months and finally 50 μg/wk for the remainder of the study.

The mice were observed daily for signs of toxicity. Each week they were weighed, the skin graded for irritation and papillomas and other gross lesions were noted. The progress of papillomas and other lesions

Table 1. *Experimental design of 18-month carcinogenicity study of hair-dye formulations, involving topical application to mice*

Group	Test material*	No. of mice/group†	Dose‡ (ml)	Dosing frequency
UC	None	250	—	—
PC	DMBA	100	0·05	W
A	PP-7588	100	0·05	W
B	PP-7586	100	0·05	W
C	PP-7585	100	0·05	W
D	PP-7588	100	0·05	F
E	PP-7586	100	0·05	F
F	PP-7585	100	0·05	F
G	Vehicle	100	0·05	W
H	Vehicle	100	0·05	F

UC = Untreated control PC = Positive control
DMBA = 7,12-dimethylbenz[*a*]anthracene
W = Weekly F = Fortnightly

*For identification of 'PP' samples, see Table 2.

†Each group consisted of equal numbers of males and females.

‡The applied dose of the hair-dye was 0·05 ml of a 1:1 mixture of the formulation and 6% hydrogen peroxide. The concentration of the positive control (DMBA in acetone) was adjusted so that 0·05 ml provided a weekly dosage of 50 ng DMBA for 6 months, 10 μg for 4 months and 50 μg for 8 months.

Table 2. *Experimental hair-dye formulations tested in an 18-month carcinogenicity study in mice*

Ingredient	Level (%) in experimental formulation		
	PP-7588	PP-7586	PP-7585
Oleic Acid	5·00	5·00	5·00
Isopropanol	3·00	3·00	3·00
Sodium sulphite	0·20	0·20	0·20
Ammonia (29%)	6·00	6·00	6·00
2,5-Toluenediamine sulphate	3·00	3·00	3·00
p-Phenylenediamine	1·50	1·50	1·50
Resorcinol	0·40	0·40	0·40
2,4-Toluenediamine base	0·20	—	—
2,4-Diaminoanisole sulphate	—	0·38	—
m-Phenylenediamine base	—	—	0·17
Deionized water	80·70	80·70	80·70

was charted monthly. Animals that died or that were killed because of general debility were autopsied and examined histopathologically when possible.

At the termination of the study all survivors were weighed and killed and a gross autopsy was performed. A sample of blood taken from a random selection of at least four mice of each sex in each group, except those groups treated only every other week, was analysed for haematocrit and haemoglobin, and mean corpuscular volume and mean corpuscular haemoglobin were calculated. A complete blood count, including differential, platelet and reticulocyte counts, was done. The livers of ten animals in each group were weighed and liver/body weight ratios were calulated. From each animal were taken sections of skin, thyroid, lung, gastro-intestinal tract (three sections), spleen, pancreas, liver, kidneys, adrenals, urinary bladder, ureter, mesenteric lymph nodes, sternal bone marrow and gonads. These tissues were fixed in 10% buffered formalin. Slides were prepared from haematoxylin/eosin-stained tissues of 13 male and 13 female mice in each test, vehicle-control and positive-control group and of 32 mice of each sex in the untreated control group and were examined microscopically for evidence of hair dye-induced toxicity. Also, every gross tumour-like lesion, regardless of location, was examined microscopically and classified.

RESULTS AND DISCUSSION

There were no overt signs of systemic toxicity in any of the dye-treated groups. The survival varied from 58 to 80%, except in the positive controls in which only 21% of the mice were alive after 18 months. Average body weights were comparable in all groups throughout the study. The absolute and relative liver weights were in the range of normal values for all groups and none of the differences were statistically significant by analysis of variance (Snedecor, 1966). All of the haematological data recorded were within normal limits. Table 3 shows mean terminal body weights, liver/body weight ratios and the percentage survival for each group.

Disease processes commonly seen in aged mice of this random-bred strain were observed in all groups with essentially the same frequency. These included chronic murine pneumonia, amyloidosis, chronic focal nephritis, focal lymphoid infiltration in lungs, liver and kidneys and testicular atrophy. The incidence of alveologenic adenomas and adenocarcinomas (Table 4) was comparable among the various control and test groups and was within the range of control values for this strain of mice (Percy & Jonas, 1971). The type and distribution of all other tumours in the groups treated weekly and in the untreated, positive-control and vehicle-control groups are shown in Table 5.

Table 3. *Survival, mean terminal body weights and liver weight/body weight ratios in mice treated for 18 months with topically applied hair-dye formulations*

Group*	Survival of 18 months (%)	Terminal body weight (g)†		Liver weight/ body weight ratio†
		Males	Females	
UC	78	37·8	34·5	0·060
PC	21	40·6	34·5	0·058
A	62	36·0	31·3	0·066
B	65	34·8	31·1	0·067
C	66	37·1	33·0	0·060
D	68	37·7	32·7	0·056
E	69	37·4	35·0	0·053
F	80	36·2	32·5	0·062
G	59	37·4	30·8	0·064
H	62	36·8	33·1	0·062

*For identification of groups, see Table 1.
†Values for body weights are means for all survivors and those for liver weight expressed relative to body weight are means for ten mice in each group.

Table 4. *Incidence of alveologenic adenomas and carcinomas in mice treated topically with hair-dye formulations for 18 months*

Group*	No. of mice/group	Sex	No. of mice with lung tumours	
			Alveologenic adenoma	Alveologenic carcinoma
UC	125	M	23	4
	125	F	14	2
PC	50	M	2	1
	50	F	5	2
A	50	M	4	1
	50	F	8	1
B	50	M	6	0
	50	F	5	2
C	50	M	8	1
	50	F	7	1
D	50	M	6	1
	50	F	3	1
E	50	M	9	1
	50	F	13	0
F	50	M	18	2
	50	F	9	1
G	50	M	12	1
	50	F	8	1
H	50	M	11	2
	50	F	8	4

*For identification of groups, see Table 1.

For the first 5 months of the study, moderate alopecia occurred in about 50% of the animals of each sex in each of the three groups receiving the hair dyes once weekly. This was not seen in the groups treated every other week or in the vehicle or positive controls. The condition diminshed with time and after 11 months hair growth appeared normal. Microscopic examination of sections taken at the final autopsy showed that the skin and its appendages were normal in all mice in these groups. There were no papillomas or carcinomas of the skin except those in the positive control group. The other tumours of the skin, in whatever groups they occurred, were all outside the treated area.

The skins of the mice in the positive control group showed no response following the weekly application of 50 ng of the carcinogen for 6 months followed by 10 μg/wk for an additional 4 months. However, when the dose was increased to 50 μg/wk, there was an almost immediate response and tumours appeared on six animals. This dose proved to be irritating to the skin and mortality increased sharply from 18 to 46% in 3 months. Although the response was somewhat less than expected, it did serve to demonstrate the susceptibility of the animals to the action of the carcinogen.

No evidence of carcinogenic activity was seen in the hair dyes tested in this study. The results of a companion study which was carried out by the US Food and Drug Administration and which included groups of mice receiving topical applications of 3% *m*-toluenediamine in water/isopropanol, were also

Table 5. *Distribution of tumours other than alveologenic adenomas and carcinomas in untreated and positive controls and in mice treated once weekly with hair dyes*

Type of tumour	No. of mice/group...	No. of mice affected and location of tumours in group*					
		UC	PC	A	B	C	G
		250	100†	100	100	100	100
Lymphosarcoma		1 M, liver 1 M, kidney 1 F, lung	1 F, spleen 2 F, general				
Fibrosarcoma		1 M, dermis 1 F, dermis	1 M, dermis			1 F, dermis	
Fibroma		1 F, dermis 1 F, cervix 1 F, body wall		1 F, body wall		1, prostate 1 F, dermis	
Adenocarcinoma		2, mammary gland				2, mammary gland	3, mammary gland
Granulosa-cell tumour		1, ovary					
Leiomyosarcoma		1, uterus	1, uterus				
Reticular-cell sarcoma			1 M, kidney 1, prostate	1 F, liver, spleen, lung, lymph node 1 F, liver, lymph node			1 M, liver
Myxofibroma			1, uterus				
Squamous carcinoma			1 F, skin and lung				
Papilloma			2 M, skin				
Adenoacanthoma						1, mammary gland	
Osteogenic sarcoma							1 F, lung
Serosal fibroma							1 F, stomach

*For identification of groups, see Table 1.
†Two positive-control mice with skin papillomas in the treated area were lost as a result of death and autolysis.

negative for carcinogenicity and will soon be published (H. Blumenthal, personal communication 1974).

Included in these studies was a compound previously shown to be a potent hepatocarcinogen when fed to rats. This raises the question again of the relevance of testing topical products by oral administration and of the significance of studies showing the toxic effects of prolonged oral dosing with high levels of ingredients of products that have been shown to be safe when tested under reasonably exaggerated conditions of use. It is often argued that oral administration is the means of choice to achieve the exaggerated dosage levels necessary to detect, in small numbers of test animals, effects that may occur in a few exposed individuals in a large population. This oral dosing frequently assumes massive proportions bearing no reasonable relationship to normal exposure and is often limited only by the amount the animal can tolerate while remaining compensated and apparently normal. This kind of testing may give false and misleading information for a number of reasons: (1) Blood levels will undoubtedly occur that would never be seen in product use, and may result in abnormal metabolism and pathology; (2) due to the effects of pH and gut bacteria, there could be absorption of molecular species that would never occur with topical application; (3) metabolic conversion to a more (or less) toxic substance may occur, or may occur at a rate quite different from that following topical application; (4) chemical reaction with essential nutrients in the ingesta can produce toxicity as a result of deficiencies and imbalances that bear no relationship to any inherent toxicity of the compound tested; (5) the normal gut flora may be drastically altered causing additional nutritional problems; (6) the target organs and excretory patterns may not be representative of those occurring during normal exposure. As indicated in the Introduction, these considerations have particular relevance in the case of oxidative hair dyes. They are, however, valid also for other systems.

Conclusions

No evidence of toxicity, or carcinogenicity, was seen when several oxidative hair-dye formulations were tested in an 18-month topical study in mice. Included in the formulations were 2,5-toluenediamine sulphate, *p*-phenylenediamine, resorcinol, 2,4-diaminoanisole sulphate, *m*-phenylenediamine and 2,4-toluenediamine. The latter compound has produced liver cancer when fed to rats in a sub-optimal diet. Safety evaluation studies of products must involve actual conditions of use to produce meaningful results.

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SHORT PAPERS

ABSORPTION GASTRO-INTESTINALE, CHEZ LE RAT, DE L'ANISOLE, DU *TRANS*-ANÉTHOLE, DU BUTYLHYDROXYANISOLE ET DU SAFROLE

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Résumé—Il a été étudié, par une méthode de perfusion *in situ* chez le rat, l'absorption gastro-intestinale de quatre additifs alimentaires dérivés du méthoxybenzène: l'anisole, le *trans*-anéthole, le butylhydroxyanisole et le safrole. Ces composés sont largement absorbés au niveau du tractus digestif par un mécanisme de diffusion passive mais la cinétique d'absorption varie d'un produit à un autre. Ces variations sont expliquées, au moins partiellement, par des différences du coefficient de liposolubilité.

Abstract—A method of *in situ* perfusion in the rat was used to study the gastro-intestinal absorption of four food additives derived from methoxybenzene, namely anisole, *trans*-anethole, butylated hydroxyanisole and safrole. These compounds are largely absorbed from the digestive tract by a mechanism of passive diffusion, but the absorption kinetics vary from one product to another. These variations are explained, at least partially, by differences in the coefficients of liposolubility.

Introduction

La série chimique des dérivés du méthoxybenzène groupe un certain nombre d'additifs alimentaires comme des arômes (anéthole, vanilline), un antioxydant (butylhydroxyanisole) et des corps utilisés aussi en thérapeutique (trithioanéthole, safrole, gaïacole).

Nous avons fait porter notre étude sur quatre de ces dérivés intéressants à différents points de vue: le méthoxybenzène, ou anisole, origine de la série chimique, fait partie de la liste des matières aromatisantes artificielles provisoirement admissibles (Conseil de l'Europe, 1970; Le Moan, 1973); le *p*-propényl-1-méthoxybenzène (dérivé *trans*), ou anéthole, est le principe naturel responsable du goût anisé; le butylhydroxyanisole (BHA), mélange de 3- et 2-butyl-*tert*-4-hydroxyanisole, est un antioxydant des plus employés dans la conservation des huiles et des corps gras alimentaires; enfin, le 4-allyl-1,2-méthylènedioxybenzène, ou safrole, est un exemple d'un arôme naturel, longtemps utilisé en confiserie et dans la préparation de certaines boissons, qui est désormais interdit depuis que Long, Nelson, Fitzhugh et Hansen (1963) ont découvert qu'il était un agent cancérigène hépatique chez le rat.

La DL_{50} (*per os*) de ces dérivés est de l'ordre de 2–3 g/kg chez le rat et la souris (Jenner, Hagan, Taylor, Cook et Fitzhugh, 1964); mais des effets hépatotoxiques ont été observés pour la plupart de ces corps, chez le rat (Creaven, Davies et Williams, 1966; Daly, 1970).

Il faut admettre que ces additifs alimentaires sont absorbés au niveau du tractus digestif car ils sont excrétés de façon importante par voie urinaire; ainsi l'anisole et le BHA sont éliminés à des taux de 80–90% (Astill, Mills, Fasset, Roudabush et Terhaar, 1962; Bray, James, Thorpe et Wasdell, 1953), l'anéthole à plus de 50% (Le Bourhis, 1970). Certes, comme tous les composés liposolubles, ces quatre dérivés méthoxybenzène doivent traverser les barrières gastro-intestinales de façon passive, comme beaucoup de médicaments par exemple. Néanmoins, le problème des processus et des sites d'absorption de ces composés reste entier; aussi, avons-nous pensé qu'il était nécessaire de préciser la cinétique d'absorption de ces dérivés au niveau de l'estomac et de l'intestin. En outre, pour tenter d'élucider leurs modalités d'absorption, nous avons évalué leurs coefficients de liposolubilité et nous avons recherché leur éventuelle dissociation en fonction du pH.

Methodes experimentales

Perfusion gastro-intestinale. Parmi les nombreuses méthodes d'étude de l'absorption digestive d'un composé, nous avons choisi la perfusion *in situ*, en circuit fermé, avec recyclages continus du perfusat (Doluisio, Billups, Dittert, Sugita et Swintosky, 1969). Nous avons utilisé des rats Wistar, mâles, d'un poids moyen de 300 g, mis au jeûne 17 h avant l'opération; après l'anesthésie à l'éthyl-uréthane et la pose d'une canule intratrachéale, nous avons préparé les animaux pour une perfusion simultanée de la cavité gastrique et de la cavité intestinale; pour la perfusion gastrique, nous avons placé le cathéter d'entrée au niveau du cardia et celui de sortie au niveau du pylore; le cathéter d'entrée plonge dans une enceinte thermostatée à 37°C contenant le liquide de perfusion qui est entraîné par une pompe péristaltique réglée sur un débit de 1 ml/min. Le cathéter de sortie draine le liquide de perfusion qui s'écoule par gravité dans l'enceinte thermostatée; la perfusion intestinale a été effectuée de la même manière et nous avons placé le cathéter d'entrée dans la région duodénale post-

* Avec la collaboration technique de M.T. Canal.

Tableau 1. *Absorption gastrique et intestinale de l'anisole, de l'anéthole, du BHA et du safrole chez le rat*

Produit†	Absorption (%*) au temps (min)							
	15	30	45	60	75	90	105	120
				Gastrique				
Anisole	24,06 ± 2,10 (8)	35,68 ± 1,81 (8)	43,78 ± 1,54 (8)	52,26 ± 0,81 (10)	60,48 ± 0,77 (4)	59,57 ± 1,23 (4)	60,21 ± 1,27 (4)	64,06 ± 0,62 (4)
Anéthole	4,47 ± 1,82 (6)	16,63 ± 1,82 (6)	23,91 ± 3,80 (6)	40,71 ± 2,21 (10)	42,38 ± 1,75 (4)	41,88 ± 4,08 (4)	48,46 ± 0,66 (4)	54,05 ± 4,25 (4)
BHA	3,77 ± 0,54 (7)	4,33 ± 0,41 (7)	8,25 ± 1,16 (7)	15,05 ± 0,55 (7)	15,35 ± 3,31 (4)	18,21 ± 4,72 (4)	13,27 ± 1,80 (4)	20,04 ± 3,81 (4)
Safrole	9,95 ± 1,01 (8)	20,13 ± 0,90 (8)	32,21 ± 0,74 (8)	44,33 ± 1,47 (8)	43,57 ± 3,38 (4)	46,97 ± 1,36 (4)	56,43 ± 0,85 (4)	61,55 ± 1,11 (4)
				Intestinale				
Anisole	32,53 ± 1,32 (8)	46,37 ± 1,41 (8)	56,82 ± 0,90 (8)	59,84 ± 1,02 (10)	71,61 ± 1,10 (4)	72,33 ± 1,11 (4)	73,95 ± 0,53 (4)	76,70 ± 0,45 (4)
Anéthole	20,96 ± 3,07 (6)	33,33 ± 3,67 (6)	44,13 ± 3,42 (6)	56,71 ± 1,59 (10)	66,56 ± 2,30 (4)	67,31 ± 1,15 (4)	72,96 ± 3,20 (4)	75,66 ± 3,00 (4)
BHA	15,51 ± 1,08 (7)	19,69 ± 1,00 (7)	27,38 ± 1,81 (7)	38,82 ± 0,97 (7)	42,90 ± 1,87 (4)	42,50 ± 1,26 (4)	43,50 ± 1,73 (4)	52,92 ± 0,53 (4)
Safrole	18,99 ± 0,76 (8)	32,76 ± 1,87 (8)	41,92 ± 1,07 (8)	54,98 ± 0,82 (8)	58,29 ± 3,14 (4)	61,05 ± 1,90 (4)	68,62 ± 3,04 (4)	74,12 ± 2,77 (4)

*Les valeurs moyennes avec l'erreur-type (et le nombre d'expériences effectuées).
†Chaque produit est à la dose de 2 mg/ml de liquide de perfusion.

pylorique et le cathéter de sortie dans la région iléale précaecale. Après avoir lavé l'estomac et l'intestin avec une solution de NaCl à 0·9% à 37°C, on perfuse la cavité gastrique avec 30 ml et la cavité intestinale avec 50 ml d'une solution de Ringer-bicarbonate, à pH 7,4, pendant 1 h, pour équilibrer le montage. L'expérience est ensuite réalisée dans les mêmes conditions, mais avec addition du produit étudié au milieu de perfusion, à la dose de 2 mg/ml. L'osmolarité de la solution de perfusion est de 327 mOSM au temps zéro. L'anisole, l'anéthole et le safrole ont été mis en suspension dans du Tween 20 à 1% selon la méthode de Regdon-Kiss et Mester (1966) et le BHA dans le Tween 80, également à 1% en concentration finale. Afin d'établir une cinétique de l'absorption, nous avons effectué des perfusions durant 15, 30, 45, 60, 75, 90, 105 et 120 min.

Méthode de dosage. Pour chacune des périodes expérimentales, nous avons évalué la quantité du produit restant dans le milieu de perfusion de l'estomac et de l'intestin, puis par différence avec la dose mise au temps zéro, nous avons estimé la quantité absorbée. A ce propos, nous avons élaboré une méthode d'extraction et de dosage en fluorescence de l'anisole, de l'anéthole, du BHA et du safrole: 1 ml de la solution contenant de 1–10 μg de produit est agité mécaniquement pendant 10 min, en présence de 1 g de NaCl, de 1 ml de NaOH-2N et de 10 ml d'heptane redistillé; après centrifugation, la phase surnageante heptanique, éventuellement diluée, est dosée au spectrofluorimètre en même temps que des essais étalons. Les longueurs d'onde optimales d'excitation et de fluorescence pour les quatre dérivés du méthoxybenzène étudiés sont les suivantes: Anisole, 275 nm (excitation) et 300 nm (fluorescence); anéthole, 299 et 330 nm; BHA, 295 et 320 nm; safrole, 296 et 322 nm.

Mesure des coefficients de liposolubilité. Nous avons mis en présence 10 ml d'une phase aqueuse constituée par la solution de perfusion précédemment décrite et 30 ml d'une phase organique contenant 97% de cyclohexane et 3% d'alcool octylique primaire. Cette phase organique tente de reproduire la légère polarité des phospholipides de la muqueuse intestinale (Bastide, Rouffiac et Stanislas, 1972). Après agitation et décantation, le dosage du dérivé méthoxybenzène a été effectué sur une prise aliquote de la phase aqueuse; par différence avec la quantité initiale, il a été estimé la concentration dans la phase organique. Le coefficient de liposolubilité (CL) est le rapport entre la concentration de la phase organique et celle de la phase aqueuse. Pour étudier la dissociation éventuelle en fonction du pH, nous avons mesuré les CL aux quatre valeurs de pH (2, 5, 7,4 et 9).

Resultats

Absorption gastro-intestinale

Les résultats ont été exprimés, pour chaque temps de perfusion, en pourcentage, afin de faciliter la comparaison des degrés d'absorption. Le Tableau 1 représente les valeurs moyennes des pourcentages de l'absorption gastrique et de l'absorption intestinale des dérivés du méthoxybenzène étudiés. Dans le but de clarifier ces résultats et surtout de faciliter la comparaison des taux d'absorption dans nos différentes conditions expérimentales, nous avons cherché à déterminer une relation linéaire entre le taux d'absorption y et le logarithme du temps de perfusion x. Le Tableau 2 regroupe l'ensemble des équations des droites de régression relatives à la cinétique de l'absorption des dérivés étudiés, pour l'estomac et pour l'intestin. Il faut noter que, pour le F de la régression et le test de la pente, le degré de signification est toujours de l'ordre de $P < 0{,}001$.

Comparaison des taux d'absorption gastro-intestinale

Toutes les comparaisons des cinétiques d'absorption ont été effectuées à partir des équations des droites de régression en utilisant le test dit de "comparaison des pentes" (Schwartz, 1972). Pour chaque organe perfusé (estomac ou intestin), nous avons donc comparé le taux d'absorption des quatre dérivés méthoxybenzène étudiés, par groupe de deux. Pour chaque couple de pentes des droites de régression relatives aux dérivés correspondants, nous avons porté sur le Tableau 3 la valeur algébrique du t de comparaison des pentes, le degré de liberté ($n - 2$) et la limite de signification.

Coefficients de liposolubilité

Les valeurs des CL ne sont pas statistiquement modifiées en fonction du pH, quel que soit le corps

Tableau 2. *Relations entre le pourcentage d'absorption et le logarithme du temps au cours de l'absorption des dérivés du méthoxybenzène à la dose de 2 mg/ml de liquide de perfusion*

Produits	Equation de la droite de regréssion [$y = (a \pm Sma).x - (b \pm Smb)$]	n	r*	Test de F de la regression*	Test de la pente*
	Estomac				
Anisole	$y = (46{,}94 \pm 2{,}05).x - (32{,}08 \pm 3{,}49)$	50	0,950	522,51	22,90
Anéthole	$y = (56{,}18 \pm 4{,}34).x - (63{,}17 \pm 7{,}35)$	44	0,930	167,79	12,95
BHA	$y = (18{,}28 \pm 2{,}49).x - (19{,}74 \pm 4{,}16)$	44	0,766	53,90	7,34
Safrole	$y = (56{,}09 \pm 2{,}18).x - (58{,}87 \pm 3{,}14)$	48	0,968	663,33	25.75
	Intestin				
Anisole	$y = (50{,}25 \pm 1{,}93).x - (27{,}49 \pm 3{,}32)$	50	0,964	679,64	26,04
Anéthole	$y = (63{,}91 \pm 4{,}92).x - (57{,}51 \pm 8{,}33)$	44	0,928	168,83	12,99
BHA	$y = (40{,}33 \pm 2{,}43).x - (35{,}47 \pm 4{,}05)$	44	0,937	276,13	16,62
Safrole	$y = (60{,}10 \pm 2{,}32).x - (54{,}08 \pm 3{,}87)$	48	0,969	671,22	25,90

y = pourcentage d'absorption x = log du temps (en min)
n = nombre d'expériences effectuées r = coefficient de corrélation
*Limites de signification: toujours $P < 0{,}001$.

Tableau 3. *Comparaison des pentes des droites de régression representatives de l'absorption*

Produits	Paramètres: t (pente)	Degré de liberté ($n-2$)	Limite de signification (P)
	Estomac		
Anisole/anéthole	2,129	42	<0,05
Anisole/BHA	11,510	42	<0,001
Anisole/safrole	4,197	46	<0,001
Anéthole/BHA	15,221	42	<0,001
Anéthole/safrole	0,041	46	NS
BHA/safrole	17,344	46	<0,001
	Intestin		
Anisole/anéthole	2,776	42	<0,01
Anisole/BHA	4,082	42	<0,001
Anisole/safrole	4,246	46	<0,001
Anéthole/BHA	9,704	42	<0,001
Anéthole/safrole	1,642	46	NS
BHA/safrole	8,521	46	<0,001

NS = non-significatif

étudié; aussi ne présentons-nous que les moyennes ($n = 8$): Anisole, 104,53 ± 3,23; anéthole, 17,22 ± 1,39; BHA, 1,72 ± 0,04; safrole, 15,37 ± 1,06.

Discussion

La méthode de perfusion employée nous a permis de mesurer l'absorption *in situ* chez l'animal entier sans provoquer de grandes perturbations sur la circulation ou la motricité gastro-intestinale; l'anesthésie reste le principal inconvénient d'une telle technique mais ce genre de méthode est souvent utilisé pour l'étude de l'absorption de drogues chez la souris (Mitjavila, de Saint-Blanquat et Derache, 1970) ou le rat (Koizumi, Arita et Kakemi, 1964; Miller et Schedl, 1970). Nous avons également maintenu le pH de la solution à une valeur de 7,4 qui se situe dans les limites indiquées par Rousseau et Sladen (1971) pour favoriser l'absorption.

Les dérivés méthoxybenzène, insolubles dans l'eau, ont été mis en suspension grâce à un agent tensioactif de la série des Tweens; certes, la présence de ces détergents peut modifier les caractéristiques cellulaires de l'épithélium gastro-intestinal et par là même les potentialités d'absorption; cependant, dans nos conditions, la dose utilisée ne paraît pas exercer d'effets toxiques (Krantz, Carr, Bird et Cook, 1948).

Si on analyse les résultats proprement dits, on peut tout d'abord noter que l'estomac est le siège d'une absorption relativement élevée de ces dérivés méthoxybenzène. Par ailleurs, l'anisole est beaucoup plus rapidement absorbé que les autres composés et cela aux deux niveaux gastrique et intestinal (Tableau 1). Le BHA, quant à lui, possède l'absorption la plus faible, surtout au niveau de l'estomac (20% après 2 h de perfusion). Ceci se retrouve dans les tests de comparaison des pentes relatives aux "couples" où le BHA est impliqué (Tableau 3), tests qui sont tous hautement significatifs ($P < 0,001$). Enfin, si on analyse les taux d'absorption de l'anéthole et du safrole, on doit souligner que les pentes des droites de régression correspondantes sont très proches les unes des autres (Tableau 2). La non-signification du test des pentes (Tableau 3) confirme cette analyse.

Les mesures des CL permettent, pour une grande part, d'expliquer les différences observées dans l'absorption gastro-intestinale des dérivés étudiés. L'inconvénient majeur de ces mesures reste que le mélange (liquide de perfusion-phase organique) est un ensemble statique, en équilibre pour un produit donné, dans des conditions bien déterminées de volume, de concentration, de température etc.; par contre, au cours de nos perfusions, l'ensemble est dans un état dynamique permanent. Dans ces conditions, il est impossible de rechercher une corrélation stricte entre les valeurs brutes des CL et celles de l'absorption effective des mêmes composés au niveau gastro-intestinal. Cependant, les valeurs d'absorption ou de liposolubilité vont dans le même sens, à savoir, par ordre croissant: BHA, anéthole et safrole (voisins) et anisole. Par ailleurs, comme les mesures des CL ne varient pas en fonction du pH, la constante de dissociation ne peut être invoquée ici (Wagner et Sedman, 1973). Le caractère exclusivement liposoluble de ces composés nous apparaît donc comme étant une des clés de l'explication des taux d'absorption observés, ceci étant valable pour l'estomac comme pour l'intestin: en effet, pour les composés liposolubles insensibles aux variations de pH, l'absorption aux niveaux gastrique (Hogben, Schanker, Tocco et Brodie, 1957) et intestinal (Schanker, Tocco, Brodie et Hogben, 1958; Wagner, 1968) s'effectue selon le principe de la diffusion passive.

En conclusion, il ne fait aucun doute que ces quatre corps étudiés sont largement absorbés et au niveau gastrique et au niveau intestinal. Cela pose un problème nutritionnel et toxicologique pour les composés liposolubles largement utilisés comme additifs, dans l'alimentation courante. Une étude corrélative entre le taux d'absorption des composés et leur poids moléculaire n'a donné aucune signification. En outre, le fait qu'ils appartiennent à une même famille chimique n'empêche pas qu'ils soient très différents vis-à-vis des phénomènes étudiés; on peut simplement noter une certaine similitude de l'absorption entre l'anéthole et le safrole. En définitive, les différences d'absorption observées entre les produits sont difficilement explicables, mais deux caractéristiques essentielles apparentent néanmoins, de façon indiscutable, ces quatre dérivés: d'une part, ils sont tous à caractère franchement liposoluble; d'autre part et corrélativement, ils sont absorbés, par diffusion passive, au travers des muqueuses gastrique et intestinale, si bien qu'une étude approfondie de leur devenir métabolique et de leurs actions physiopathologiques apparaît donc indispensable.

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THE REACTION OF NITRITE WITH PESTICIDES. II. FORMATION, CHEMICAL PROPERTIES AND CARCINOGENIC ACTIVITY OF THE *N*-NITROSO DERIVATIVE OF *N*-METHYL-1-NAPHTHYL CARBAMATE (CARBARYL)

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Summary—Nitrosation of the insecticide *N*-methyl-1-napthyl carbamate (carbaryl) with a fivefold molar excess of nitrite was measured at carbaryl concentrations of 10^{-3}–10^{-4} M. The maximum yields of the *N*-nitroso product were about 2 and 1% of the theoretical at the 10^{-3} and 10^{-4} M levels respectively. Dependence of the rate of nitrosation on pH was investigated and the highest yields were found to be obtained in 1 N-HCl. The chemical and physicochemical properties of synthesized *N*-nitrosocarbaryl were determined. When administered in a single sc injection of 1000 mg/kg to Wistar rats, *N*-nitrosocarbaryl induced local sarcomas at the site of injection. Single oral doses of 200–1500 mg/kg produced no tumours in 21 months in a continuing experiment.

Introduction

Formation of carcinogenic *N*-nitroso compounds from nitrite and nitrosatable substances has been demonstrated under *in vitro* conditions simulating the human stomach and also in animal experiments. These problems and their implications have been reviewed by Sander & Schweinsberg (1972) and Preussmann (1974). Numerous pesticides (including carbamate, dithiocarbamate, *sym*-triazine and phenylurea derivatives) contain structures that can undergo *N*-nitrosation. Residues of such substances in food could therefore form *N*-nitroso compounds by reaction with nitrite under suitable conditions (Eisenbrand, Ungerer & Preussmann, 1974 & 1975; Elespuru & Lijinsky, 1973; Sen, Donaldson & Charbonneau, 1975; Siebert & Eisenbrand, 1974). In part I of this series, we described the formation, chemical properties and carcinogenic action of the *N*-nitroso derivative of the herbicide *N*-methyl-*N'*-(2-benzothiazolyl)-urea, known as benzthiazuron (Ungerer, Eisenbrand & Preussmann, 1974).

Following up this work, we studied the capacity of the insecticidal carbamate derivative, carbaryl (Sevin®)for nitrosation at low concentration in an aqueous medium and under conditions similar to those in the stomach. *N*-Nitrosocarbaryl was tested for carcinogenicity in Wistar rats.

Experimental

Materials. Carbaryl (99·9% pure; m.p. 139–140°C) was obtained from Union Carbide Corp., New York. *N*-Methyl-*N*-nitroso-1-naphthyl carbamate (nitrosocarbaryl) was synthesized according to the method of White (1955):

> A solution of 0·8 mols dinitrogen tetroxide in 600 ml dichloromethane at −50°C was mixed with 1·6 mols anhydrous sodium acetate. A solution of 0·4 mols carbaryl in 350 ml dichloromethane was added dropwise and the mixture was stirred for 18 hr at −6°C. After the solution had been washed with ice-water, the dichloromethane phase was reduced on a rotation evaporator. The residue was taken up in 30 ml acetone and precipitated by addition of 60 ml water. The yellow solid was recrystallized from dimethylsulphoxide/water, forming light-yellow needles. The *N*-nitrosocarbaryl, obtained in 81% yield, was homogeneous on a thin-layer chromatogram and was characterized as follows: m.p. 66–67°C; found: C—62·69, H—4·55 and N—12·07% (calculated for $C_{12}H_{10}N_2O_3$ (mol wt 230·22): C—62·61, H—4·38 and N—12·16%); λ max in methanol: 219, 268, 277 and 402 nm (log ϵ = 4·97, 3·85, 3·87 and 2·13, respectively); infrared (KBr pellet): 1760 cm^{-1} (CO) and 1520 cm^{-1} (NO).

The mass spectrum agrees with the postulated structure, showing a parent peak at *m/e* 230. The other prominent signals in the upper mass scale (at *m/e* 144, 143, 127 and 115) are attributable to a fragmentation pattern similar in many ways to the reported breakdown of other 1-naphthyl carbamates (Durden, Stollings, Casida & Slade, 1970). The transition *m/e* 143 to *m/e* 115, which gives a distinct metastable signal at *m/e* 92·5, is a particularly common fragmentation pathway of this class of compound.

Nitrosation of carbaryl in vitro. Carbaryl was incubated in dilute aqueous solution (10^{-3} or 10^{-4} M containing 10 or 2% acetic acid, respectively) with a fivefold molar excess of nitrite at 37°C in a closed vessel. The desired pH values were attained by addition of HCl or NaOH in a pH-stat and were kept constant. The reaction was stopped after 15 or 60 min by addition of amidosulphonic acid. The reaction mixture was extracted with dichloromethane and the organic phase was reduced to dryness on a rotation evaporator after drying over Na_2SO_4. To measure the conversion quantitatively, the residue was taken up in glacial acetic acid and the *N*-nitrosocarbaryl that had been formed was determined colorimetrically

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(Eisenbrand & Preussmann, 1970). Standard curves prepared with known amounts of *N*-nitrosocarbaryl that had been subjected to the process described were linear at both 10^{-3} and 10^{-4} M concentrations.

Carcinogenicity studies. *N*-Nitrosocarbaryl in oil suspension (10% in Livio oil) was administered to eight male and eight female Wistar rats in a single sc injection of 1000 mg/kg. The same amount of oil containing no *N*-nitrosocarbaryl was injected into eight animals, which served as controls. A further 37 Wistar rats of both sexes (about 90 days old at the start of the experiment) were given single oral doses of *N*-nitrosocarbaryl ranging from 200 to 1500 mg/kg body weight. The substance was suspended in 10% starch paste and administered by stomach tube to animals that had been fasted for the previous 24 hr. Twenty-two untreated male and female Wistar rats served as controls. All the animals were fed Altromin® and given water *ad lib.*

Results and Discussion

Chemical studies

Carbaryl reacted with nitrite in acidic aqueous solution (under conditions similar to those in the stomach) to form the corresponding *N*-nitroso compound. Nitrosation in approximately 0·1 N-HCl at a constant pH of 1 and with a carbaryl concentration of 10^{-3} M led, after 15 and 60 min respectively, to the formation of 1·2 and 1·7% of the maximum possible conversion to nitrosocarbaryl. An increase in pH greatly reduced the yield, the corresponding yields after 15 and 60 min at pH 2 being 0·1 and 0·2% and at pH 3, 0·05 and 0·1% of the theoretical. Higher yields were obtained from nitrosation in stronger acid; in a 15-min nitrosation of 10^{-3} M carbaryl in 1 N-HCl, the yield was 3·2% of the theoretical. Reaction for 1 hr under the same conditions produced a yield of only 2·2%, however. This smaller yield despite longer incubation may be explained by the decreased stability of the compound in strongly acid medium. Maximal stability of the *N*-nitroso compound is found between pH 3 and 5 and the compound is considerably more unstable at pH 1. As would be expected from an *N*-nitroso-amide, it decomposes very rapidly under alkaline conditions. The actual half-lives of the compound determined in unbuffered aqueous media containing 20% methanol were 62, 320, 360, 67 and 1·5 min at pH 1, 3, 5, 7·2 and 9, respectively.

Lowering the concentrations of the two reactants, carbaryl and sodium nitrite, by a factor of 10 reduced the percentage yield by about half. Nitrosation of 10^{-4} M-carbaryl (21 ppm) with 23 ppm nitrite at pH 1 gave 0·9% of the theoretical yield after reaction for 60 min.

Carcinogenic action of N-*nitrosocarbaryl*

Of the 16 rats given a single sc dose of 1000 mg *N*-nitrosocarbaryl/kg, 14 had died by day 450. All 14 had developed local tumours at the injection site (Fig. 1). The neoplasms were shown histologically to be polymorphic-cell sarcomas. A spindle-cell sarcoma was diagnosed in one case. No tumours were observed in other organs and neither autopsy nor histopathological investigation yielded evidence of any other toxic effects. Of the two surviving animals, one has a palpable plum-sized growth at the injection site and the other has no tumours so far (630 days). No control animals have any tumours.

The findings following sc administration of *N*-nitrosocarbaryl are thus unequivocal (15/16 animals with local tumours). Although the doses applied were relatively high, it must be noted that this high tumour yield resulted from only a single injection. On the other hand, orally administered single doses of 200–1500 mg/kg have shown no toxic effects and have so far induced no tumours (21 months after treatment). Investigations are at present being carried out on the long-term oral administration of this compound (130 mg/kg twice weekly). The first animals to die in this study showed all stages of malignant transformation in the fore-stomach, from hyperplasia to squamouscell carcinoma. These studies, carried out with D. Schmähl, will be reported separately when completed.

The yields obtained by interaction of nitrite with carbaryl under the conditions described are low and the potential health risk of this and similar reactions is difficult to evaluate at present. It should be borne in mind, however, that *N*-nitrosocarbaryl is a potent carcinogen as well as an extremely effective mutagen, as has been shown by Siebert & Eisenbrand (1974) and Elespuru, Lijinsky & Setlow (1974).

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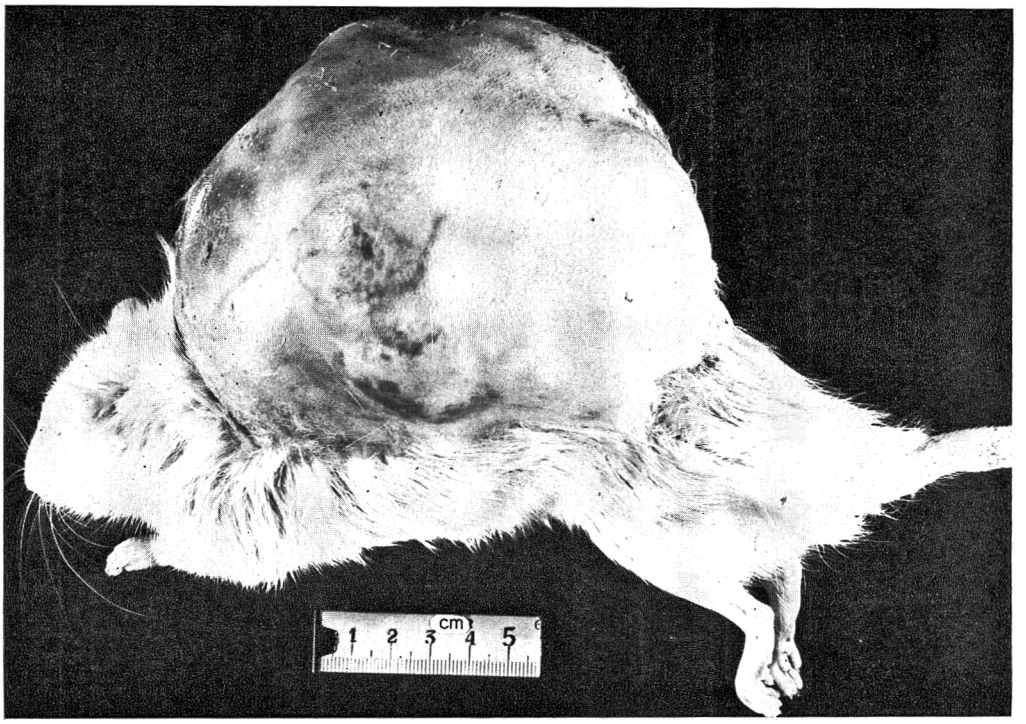

Fig. 1. Rhabdomyosarcoma developing after an induction period of 280 days at the site of injection in a Wistar rat given a single sc dose of 1000 mg N-nitrosocarbaryl/kg. The tumour weighed 450 g.

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Review Section

A CASE FOR USING INBRED STRAINS OF LABORATORY ANIMALS IN EVALUATING THE SAFETY OF DRUGS

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Summary—The case for evaluating the safety of drugs by using several inbred strains of a laboratory animal species arranged in a factorial design of experiment rather than a single outbred stock is discussed. It is concluded that the factorial design should give a more powerful experiment, produce more information and have a more valid basis for extrapolation to man. This extra information will be obtained at the expense of a more complex statistical analysis and will present some practical problems in obtaining a supply of several different inbred strains, although the total size of the experiment should not increase. It is shown that the differences between inbred strains may be a valuable aid in interpreting the results of toxicity tests.

Introduction

Little serious consideration has been given to genetic aspects of the type of laboratory animal most suitable for use in studying drug toxicity, although it is well established that there are important genetic differences in responses to a number of drugs (Kalo, 1962; Meier, 1963). Boyland (1958) considered that pure-line (i.e. inbred strain) mice might be more suitable for chronic toxicity tests than stock mice (i.e. outbred ones), although a possible drawback was that "the strain chosen might be specifically resistant to the carcinogenic action of a particular compound. By using stock mice in which there is some genetic variation, the chance of all members of the group being resistant is reduced." Strasser (1973) emphasized the importance of using genetically defined animals in drug testing, without specifically favouring inbred strains or outbred stocks. Most of the animals used in toxicity testing by a contract research organization are outbred (Mawdesley-Thomas, 1973—see particularly unpublished appendices), though in some specific instances, inbred strains are preferred. Falconer & Bloom (1962) showed that genetic factors accounted for over 80% of the variation in susceptibility to urethane-induced lung tumours in two outbred stocks of mice.

Types of experiment

Festing (1971) recognized the following three distinct types of animal experiment, each of which required a genetically different type of animal:

Biological assay. In this case the animal is used as a measuring tool to assess the potency of a substance (such as insulin) with a known effect on the animals. The animals are of no direct interest, and the experimenter is free to choose those that give the most economical results after conducting pilot trials in different strains and species. In some cases, advances in analytical technique may supersede the need for animals. This could not happen in evaluating the safety of a drug, although in some cases drugs might be rejected as unlikely to be safe on the basis of *in vitro* tests. Outbred stocks may be justified in biological assay largely because their low cost may more than compensate for the larger numbers likely to be required than would be the case if an inbred strain or F_1 hybrid were to be used.

Experiments in which the target population is available. In this case, the research worker is interested in the effect of a treatment on a particular group of animals, the 'target population'. Samples of this population (which could be a single strain, several strains or a whole species) are available, and no attempt is made to extrapolate the results rigorously to another population. Fundamental research may come into this category, since the scientist is often content to be able to make some authoritative statement about a particular strain of rat, for example, rather than attempt to extrapolate to man or other species. A single inbred strain of laboratory animal may be chosen as the target population in this type of research.

Experiments in which the target population is not available for research. In toxicity testing the aim is usually to make some statement about the safety of a compound or 'drug' in man (the target population) but the experiments needed to establish safety levels of the drug cannot be carried out in man for obvious reasons. The usual procedure is to extrapolate to man from the results of experiments conducted on at least two different species of laboratory animal.

The purpose of this paper is to consider genetic aspects of the choice of animals within a species. It must be emphasized that the first and second categories of experiment listed above are excluded from further consideration in this paper.

Table 1. *Primaquinone sensitivity in man**

Group	No. tested	Percentage reactors
Negro	144	14·6
Oriental	51	2·0
European, white	30	0·0
Ashkenazi Jews	203	0·0
Sephardic Jews	267	11·2
Sardinian	61	13·1
Peruvian Indians	238	0·0

*Data from Kalo (1962).

Strains and stocks

Definition of 'inbred strains' and 'outbred stocks'

Inbreeding may be defined as the probability that the two genes at a single locus in an individual are alike by descent (Falconer, 1960). But by internationally agreed convention (Festing & Staats, 1973; Staats, 1968), an inbred strain is one that has been maintained by brother × sister mating (or its genetic equivalent) for more than 20 generations with all animals being descended from a single pair in generation 20.

In such strains all animals are virtually genetically identical (isogenic) and the probability of the two genes at a locus being alike by descent is more than 0·986.

Rules for the nomenclature of outbred stocks (the term now used in preference to 'random-bred strains' or 'random-bred stock') have only recently been formulated (Festing, Konda, Loosli, Poiley & Spiegel, 1972). These rules are designed to ensure that such stocks should be closed colonies maintained so as to have less than 1% inbreeding per generation, with no directional selection. The aim is to maintain the stock without any genetic change for as long as possible. No statement is made about the level of inbreeding in the stock; it is a mating system that is defined and the genetic consequences of outbreeding depend on the previous genetic history. Some stocks are genetically variable, while others are nearly isogenic due to previous inbreeding. For example, two commonly used outbred mouse stocks, CFW (also called LACA) and CF-1, have a history of more than 20 generations of brother × sister mating (Carworth Handbook, Anglia Laboratory Animals Ltd., Alconbury, Huntingdon; p. 1. 4).

Unsuitability of using a single strain or stock

The use of a single outbred stock in drug testing is sometimes justified on the grounds that humans are random bred, so in order to model humans realistically, an outbred stock of laboratory animals should be used. However, humans are not random bred. Not only is the human race divided geographically and culturally into a large number of distinct races and ethnic groups, but even within a single ethnic group, mating does not occur at random, there being a high positive correlation between mates in a range of personality and physical characteristics (Vandenberg, 1972). Racial groups can differ markedly in their response to drugs. Table 1 shows the incidence of primaquinone sensitivity among males in various populations (Kalo, 1962). European whites, for example, are by no means typical of the human race. Kalo (1962) also recorded racial differences in the metabolism of isoniazid, the pupillary reaction to sympathomimetic drugs and the effects of atropine on pulse rate. Inbreeding, selection and assortative mating all act on man and are known to increase overall genetic variability compared with true random mating, so no random-bred laboratory animal population can logically be compared with the human race, or even with a single ethnic group.

Table 2. *Immunological response to dextran in guinea-pigs**

Strain or breed	No. tested	Percentage reactors
Abyssinian	19	58
Pirbright	14	7
Hartley	16	0
Peruvian	9	0
Strain 13	2	0

*Data from Battisto *et al.* (1968).

Similarly, there are important stock and strain differences among laboratory animals. Table 2 shows the immunological response of a number of strains or breeds of guinea-pig to dextran (Battisto, Chiappetta & Hixon, 1968). The proportion of reactors in the Abyssinian breed was high, but it was low in all other colonies tested. The commonly used outbred Hartley and Pirbright stocks have a low proportion of animals capable of reacting to this antigen. A similar type of result was obtained by Battisto (1960) with regard to a naturally occurring serum factor, showing that races or breeds of laboratory animals often do not contain many of the genotypes typical of the species as a whole.

Wide within-group variability may, in any case, be a disadvantage since it may reduce the precision of the statistical analysis. This can be seen from an examination of the formula for Student's t test, a statistic commonly used to evaluate the difference between the means of two groups of animals, where the data is normally distributed:

$$t = \frac{\bar{X}_1 - \bar{X}_2}{S_{\bar{x}_1 - \bar{x}_2}}$$

when $\bar{X}_1$ and $\bar{X}_2$ are the means of the treatment and control groups, and $S_{\bar{x}_1 - \bar{x}_2}$ is the standard error of the difference between the means.

If an outbred stock did have greater within-group variability, the result would be a reduction in the power of the experiment to detect a true difference between treated and control groups, since $S_{\bar{x}_1 - \bar{x}_2}$ would be larger and therefore Student's t would be smaller.

Thus, a single outbred stock may not contain a wide range of types, and even if it did, such within-group variability may be undesirable. Fortunately, both these problems may be overcome by using several inbred strains in a factorially designed experiment. Different strains of animal provide a substantially wider range of genotypes than are present in a single outbred stock, and high statistical precision is obtained by using strains with a low within-group variability. The total number of animals used in the experiment remains the same.

Single factor experiment

Treatment	N	Total
1	N	N
2	N	N
.	.	.
.	.	.
.	.	.
K	N	$\frac{N}{KN}$

Factorial experiment

Treatment	1	2	Strain	X	Total
1	n	n	..	n	N
2	n	n	..	n	N
.	.	.	..	.	.
.	.	.	..	.	.
.	.	.	..	.	.
K	n	n	..	n	$\frac{N}{KN}$

Fig. 1. The single-factor experiment using a single stock with N animals per treatment and K treatments, therefore using a total of KN animals, and the factorial design using X different strains with n animals per treatment-by-strain subclass, such that $Xn = N$ and, with K treatments, giving a grand total of KN animals altogether.

Factorial experiments

The principle of a factorial design of experiment (Cochran & Cox, 1957) is illustrated in Fig. 1. In a typical single factor toxicity test there might be four treatments with, say, 20–50 or more animals per treatment, depending on the type of test (Kirkby, Salmond & Davies, 1972; Mawdesley-Thomas, 1973). With a factorial design the same total size of experiment would be used, but the sub-group would be split up so that more than one strain or stock would be used. Treatment means are estimated by averaging over strains, strain means by averaging over the treatments, and a strain by treatment interaction effect is estimated which would give an indication of whether all strains behave uniformly with respect to the treatment. The simplest possible example of a factorial design employing two treatments (in this case diets) and two strains is given in Table 3. It will be noted that had only a single strain been used it might have been concluded incorrectly that one diet was better than the others. As it is, it was concluded that different strains have different dietary requirements for maximum growth.

Table 3. *Growth rate of two strains of mice (inbred CBA and outbred LACA) on two diets designated FFG and CDD**

Strain	Growth rate (g/wk) on diet		Strain mean†
	FFG	CDD	
LACA	3·88	3·72	3·80
CBA	3·37	3·67	3·52
Diet mean† ...	3·63	3·70	

*Data from Porter & Festing (1969).

†Statistical analysis indicated that there was no significant difference between diets, but a highly significant difference between strains, and a highly significant strain by diet interaction. Thus, LACA grew best on diet FFG whereas CBA grew best on CDD.

Fisher (1960) emphasized that factorial experiments have three main advantages over single-factor experiments; in relation to drug testing these may be interpreted as follows:

(1) Greater efficiency, in that if the drug were to be tested in one strain first and then in another strain, instead of in a factorial design, the experiment would have to be twice as large.

(2) Greater comprehensiveness, in that the experiment gives information on the strain by treatment interactions, which may be of great importance in the response to drugs.

(3) The conclusions reached as a result of the experiment have a wider inductive basis than would be the case with a single factor experiment.

In toxicity testing there is usually no direct interest in the differences between strains, but a conclusion reached on the basis of the response to a drug in a number of different strains would be more valuable than one based on only a single stock. The advantage of the factorial design is that the experiment does not have to be any larger to obtain this additional information.

The main disadvantages of the factorial design are the logistical difficulties of using several additional strains and the statistical analysis of the results. The latter is relatively straightforward when all subgroups are the same size, but in the case of unequal numbers an electronic computer will usually be required to carry out the more sophisticated statistical analysis, though standard computer programmes are readily available. A statistician may become an essential member of any team working on drug testing.

Inbred strains

The advantages of strains compared with stocks

The main objection to the use of inbred strains is that the strain chosen may be resistant to the drug being examined (Boyland, 1958). However, if the experimental design includes a number of inbred strains the chance of them all being resistant is low, and it becomes worthwhile to consider some of the advantages of inbred strains compared with outbred stocks.

Isogenicity. All individuals of a properly maintained inbred strain will be genetically identical. This means that the genetic characteristics of the strain may be determined by examining a single or a very small sample of individuals. In contrast, a large sample is required in an outbred stock since the frequency of the different genotypes would have to be determined. Many inbred strains have been characterized for important Mendelian traits, such as blood groups and isozymes. These are discussed later. All individuals of an inbred strain will react in the same way to any drug in which the reaction is dependent on a single Mendelian gene. For example, Kouri, Salerno & Whitmire (1973) and Thomas, Hutton & Taylor (1973) have shown that the inducibility of the liver enzyme aryl hydrocarbon hydroxylase depends on a single Mendelian gene. All individuals of an inbred strain react identically to the enzyme inducer (within the limits of experimental error). This is shown in Fig. 2 where the 14 strains fell into two

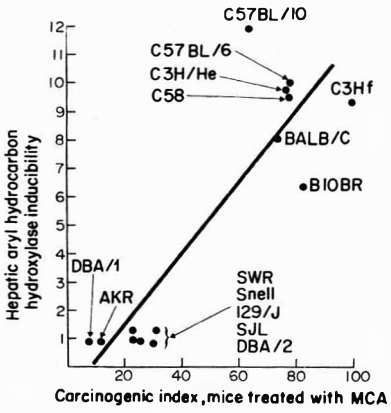

Fig. 2. Correlation between susceptibility to 3-methylcholanthrene (MCA) and the inducibility of hepatic aryl hydrocarbon hydroxylase in different mouse strains (Kouri *et al.* 1973).

non-overlapping groups with either a non-inducible or an inducible type of enzyme.

In contrast, any level of reaction between 0 and 100% may be observed, depending on gene frequency in an outbred stock, as shown in Tables 1 and 2. Outbred stocks may also contain rare genotypes leading to low repeatability of experiments if sample sizes are small.

In the case of polygenically determined characteristics of low heritability, inbred strains may be more sensitive to the environment than outbred stocks (Lerner, 1954) leading to increased within-strain variability. However, the factors that make a strain sensitive to minor environmental effects may also increase their sensitivity to drugs, so that on balance there is no good evidence that for this type of character inbred strains would be less useful than outbred stocks.

In the case of highly heritable characters, inbred strains will be more uniform than outbred stocks, and therefore inbred strains should be more powerful tools in detecting a true adverse effect.

Identifiability. Until recently (Festing, 1973) there has been no practical way of identifying outbred stocks, which had to be taken on trust. Festing (1974) has shown that such trust cannot always be justified. In the past, stocks have clearly become muddled together, and it is doubtful whether generic names, such as 'Wistar', 'Sprague-Dawley' or 'Swiss' have any meaning in the UK. In contrast, inbred strains can be identified by their biochemical and immunological characteristics (Moutier, 1971), and undetected low levels of genetic contamination are improbable in the long run provided the strain is maintained by continued brother × sister mating. This is because all breeding stock in a given generation traces back to a common ancestral breeding pair within about 5–10 generations. If that pair were illegitimate, there would be a substantial genetic change in the whole colony, which would almost certainly be detected. Thus, the literature on the genetic characteristics of inbred strains is substantially more reliable than that of outbred stocks. This is particularly so in the case of single gene characters, and in cases where the inbred strain has an unusual coat colour and may therefore be recognized easily.

Long-term genetic stability. Inbred strains can only change genetically as a result of mutation with subsequent fixation of the mutant alleles (Falconer, 1960). The chance of a mutation occurring and becoming fixed in the population is independent of the population size, and hence change due to mutation is likely to be about equal in strains and stocks. However, stocks are also subject to genetic changes due to selection, genetic sampling at transfer to new laboratories, inbreeding and possibly genetic contamination at a low level as noted above. Hence, stocks are likely to be less stable than strains. Unfortunately, no data are available to show the long-term stability of stocks, particularly in view of their genetic unreliability. The stability of strains is demonstrated for a behavioural characteristic in Fig. 3. The correlation in open-field exploration activity between two investigations using the same strains but working in different laboratories and separated by at least 13 yr was 0·86. Moreover, the immediate history of these strains is not known, and they may well have been separated for a considerably longer period. Relatively high long-term stability in strains was also demonstrated by Festing (1973) and Taylor (1972). However,

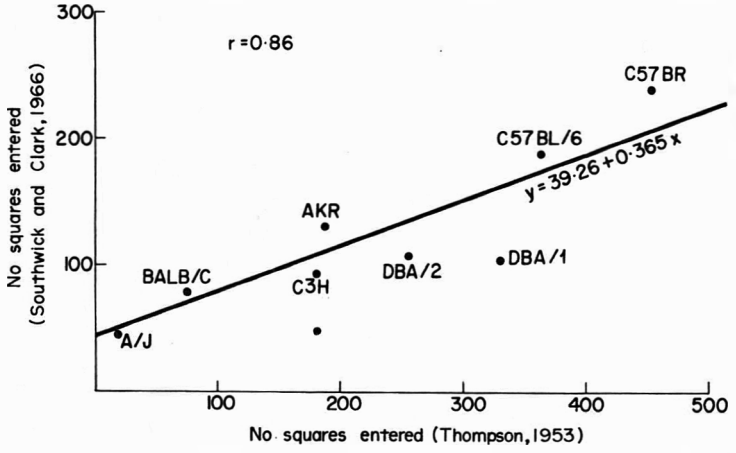

Fig. 3. Correlation between mouse strain means for the behaviour trait 'open-field activity' (number of squares entered in a fixed time) in two studies separated by a period of at least 13 yr.

some characters also depend on environmental factors, which may alter. Thus, the incidence of spontaneous tumours is not constant (Mühlbock & Tengbergen, 1971). This can be attributed to changes in the environment and genetic changes in oncogenic viruses. Genetic constancy is important since it makes it possible to collect background information for future use.

Background information on strain characteristics. A great deal of information is available on the characteristics of inbred strains. Many strains have been characterized for single gene loci, e.g. erythrocyte and leucocyte antigens, immunoglobulin allotypes, haemoglobin, immune response genes, isozymes and abnormalities such as retinal degeneration in mice (Staats, 1972). Such data are not available for outbred stocks. Many quantitative characters have also been studied in mice (Green, 1966), rats (Robinson, 1963) and hamsters (Hoffman, Robinson & Magalhaes, 1968), and strain differences have been noted.

Advantage of stocks

Outbred stocks are usually more prolific breeders and are therefore cheaper. In some cases, considerable background information is also available. In these circumstances a factorial experiment using both inbred strains and the most valuable outbred stocks may be used. Outbred stocks may also have advantages over inbred strains in biological assay (Festing, 1971), in which they are used as a measuring tool.

F_1 hybrids

F_1 (i.e. first generation) crosses between two inbred strains have most of the advantages of inbred strains, with the added advantage of increased vigour. This extra vigour could be used in studies where a high breeding performance is needed (such as in teratogenesis).

Choice of specific strains

At present, a single outbred stock is usually chosen for drug testing on the basis of availability and known incidence of spontaneous disease of various types. In the case of a new drug, which may have entirely unsuspected adverse effects, there appears to be no rational basis for choosing any particular strains with which to set up the factorial experiment, so strains should be chosen at random and according to availability. In practice, although many strains are known, most research involves relatively few strains (Festing, 1969). Background information is available on lifespan and cause of death of a number of inbred strains of mice (Festing & Blackmore, 1971; Smith, Walford & Mickey, 1973; Storer, 1966), although little information is available on rat strains. Strains with a high incidence of a particular type of disease or abnormality at an early age will usually be rejected for long-term studies. Coat colour may be used in some cases as a practical method of identifying the different strains, though it should be emphasized that there is usually no correlation between coat colour and strain characteristics. The number of different strains to be included in the study will depend on the size and complexity of the experiment, but it is suggested that for a typical experiment up to ten different strains should be included. Strains of mice may be located through Staats (1972), of rats through Festing & Staats (1973) and of all species through Festing & Conn (1971).

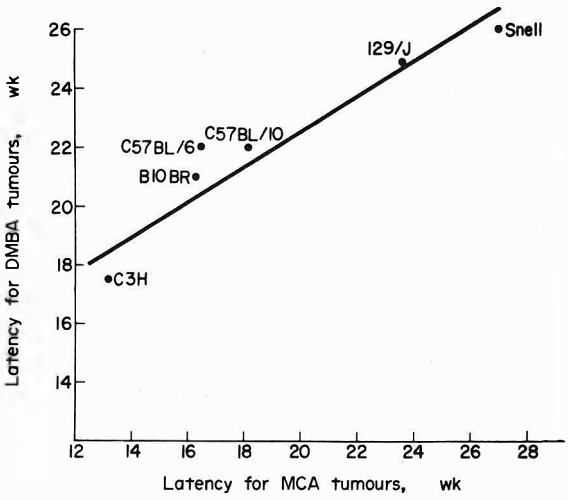

Fig. 4. Correlation between strain means in the latency of tumour development caused by 3-methylcholanthrene (MCA) and 7,12-dimethylbenz[*a*]anthracene (Kouri *et al.* 1973).

Strain differences as an aid to interpreting the results of an experiment

If an experiment can be performed with five or more different strains it may be possible to use the strain differences in the interpretation of the results. The main ways in which this might be done are:

(1) *Common mode of resistance to two drugs.* Figure 4 shows that there is a high correlation between mouse strains in their resistance (as judged by latency) to 3-methylcholanthrene and 7,12-dimethylbenz-[*a*]anthracene (Kouri *et al.* 1973), suggesting that the mechanism of resistance to these two carcinogens is similar. If the strain pattern of susceptibility to a new drug follows that of one previously tested, this suggests a common mode of absorption and/or metabolism, and thus may give a lot of information very quickly. The observation that the strains susceptible to a drug not previously investigated are also susceptible to a drug with a known strain-pattern of susceptibility and *vice versa* could be helpful in the interpretation of results.

(2) *As an aid in extrapolating to man.* Suppose strains differ in the observed severity of an adverse effect, and this can be correlated with the rate of metabolism of the drug (say, the blood levels 24 hr after administration of the drug), then a possible causal relationship might be established. In such a case, it may be possible to obtain data on the metabolism of the same drug in man using very low dose levels, and also in the other species tested. The likely adverse effect in man might then be predicted from a knowledge of the rate of metabolism in man and the predicted and observed sensitivity in the other test species could be calculated. If the predicted adverse effect in the other test species is in good agreement with the observed adverse effect, a safer extrapolation to man should be possible. Although there would still be considerable

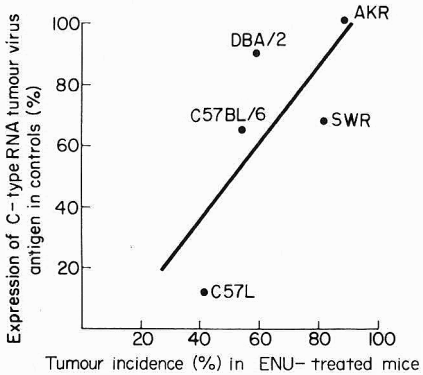

Fig. 5. Correlation between susceptibility to tumour induction with 1-ethyl-1-nitrosourea (ENU) and the expression of C-type RNA tumour-virus antigen in controls (Diwan *et al.* 1973).

hazards in extrapolating from animals to man in this way, at least such extrapolation would be based on a more rational method than is used at present. These techniques might be refined by using multiple correlations (Cooley & Lohnes, 1971) if data on other relevant strain characteristics are available. Kouri *et al.* (1973) have shown that the susceptibility to tumour induction with 3-methylcholanthrene is correlated with the inducibility of the enzyme aryl hydrocarbon hydroxylase (Fig. 2). In this case, the inducibility of this enzyme in man might be relevant in deciding how dangerous 3-methylcholanthrene is to man.

(3) *As an aid in interpreting results.* In some cases, an adverse effect may be associated with a condition that is not relevant to man. Suppose that in testing a drug a high tumour incidence was observed in some strains, but these strains were known to have high titres for oncogenic viruses. It might be argued that the drug itself was not causing the tumours but was acting as a co-carcinogen since in the absence of the virus, the drug had no adverse effect. Data of this type were obtained by Diwan, Meier & Huebner (1973), who showed a strong correlation between the tumour incidence in mouse strains treated transplacentally with 1-ethyl-1-nitrosourea and the expression of C-type RNA tumour-virus antigen in control mice (Fig. 5).

Practical difficulties

Some practical difficulties associated with these proposals clearly prevent their immediate implementation. Although a number of inbred strains of mice of category 3 or 4 health status (Medical Research Council, 1974) are readily available in the UK, inbred rats, hamsters and guinea-pigs are not yet commercially available on a large scale, a situation due largely to the low current demand for them. Inbred strains of other species are not generally available, and in all cases inbred animals are three or four times as expensive as outbred ones, although again this is largely a function of the sporadic demand.

Users wishing to breed their own animals would have to become involved in breeding a much wider range of strains, and there may be logistical problems in supplying uniform groups of animals from several strains rather than from one outbred stock, as is done at present. However, these logistical problems may not be as great as those involved in conducting within-litter experiments (Kirby *et al.* 1972), which may no longer be necessary if inbred strains are used.

There are other, semi-philosophical, problems. As each strain will grow at a different rate the animals cannot be matched for both age and weight, though this is not a serious problem. In some cases the high dose level is set at the maximum tolerated dose. If strains differ in this, there may be some difficulties in deciding what dose levels should be given. In practice this might have to be the maximum tolerated dose for the most susceptible strain, to avoid introducing the complication of different doses for each strain. This might require discussion with Government authorities.

None of these problems is insuperable, given time, and the potential benefits are likely far to outweigh the practical difficulties.

Other problems in interpreting results

If a very low incidence of an adverse effect (such as a tumour) is noted in the treated group, but not in the controls, this can pose severe problems if only a single outbred stock is used. The appearance of one or two tumours in the treated group is not 'statistically significant' however large the experiment, yet it is difficult to claim that the experiment has shown the test compound to be safe. A good compound may thus be rejected even though the adverse effect may have been a spontaneous condition depending on a rare genetic combination occurring in the outbred stock. The trial can never be repeated since it will be impossible to produce animals with exactly the same genotype as the affected animals.

If a factorial design is used and one or two tumours are noted in the treated group in one particular strain, further experiments can be conducted on that particular strain in order to clarify the situation. Thus, the possibility of identifying and repeating the genotype of an animal showing an adverse effect is an important additional advantage of the factorial design of experiment.

Conclusion

Most studies on the safety of drugs are performed on a single outbred stock on the (probably false) grounds that such a stock contains a wide range of genetically different individuals, thus increasing the chance of detecting an adverse effect. However, the variation within a stock is considerably smaller than the variation between different inbred strains. Thus, the inclusion of a range of different strains gives an even better chance of detecting an adverse effect and a much broader basis for extrapolating to man than the use of a single stock. The use of a factorial design makes it possible to include a number of different strains without enlarging the total size of the experiment. The main difficulties are the logistical ones of having to obtain animals from a number of strains, and the increased complexity of the statistical analysis. These practical problems may prevent the immediate adoption of these proposals, but are certainly not insuperable in the long term.

Inbred strains are genetically more stable than outbred stocks, they can be characterized more easily,

and more effective use can be made of published information about them. The inclusion of a number of different strains in one study gives a broader base for extrapolation to man and makes it possible to study the pattern of response to the drug, and to correlate this with biochemical or physiological data obtained in the same experiment or from the published literature. Where correlations are observed, it may be possible to develop a more rational basis for extrapolation to man. Thus, the case for using several different inbred strains rather than a single outbred stock in drug testing is overwhelming.

Acknowledgements—I should like to thank Dr. D. G. Davey, Dr. B. Leonard, Mrs. G. Wexler and Dr. J. Turton for valuable comments on a draft of the manuscript, although this does not in any way imply that they endorse the views expressed in the paper.

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BOOK REVIEWS

Biological Effects of Asbestos. IARC Scientific Publications No. 8. Edited by P. Bogovski, J. C. Gilson, V. Timbrell and J. C. Wagner. International Agency for Research on Cancer, Lyon, 1973. pp. xxi + 346. Sw fr. 80 (available from HMSO).

This volume represents the proceedings of a working conference on asbestos held at the International Agency for Research on Cancer in Lyon in 1972. The papers presented at the conference are drawn together under 13 headings dealing with the various aspects of the asbestos problem and providing in each case an assessment of some aspect of methodology or an area of research. For example, four sections forming a composite group review in turn the methodologies of clinical research, pathology, experimental pathology and environmental studies. Asbestosis and mesothelioma are considered in relation to the type, shape and nature of the asbestos particle, occupation and duration of exposure of the patients and various other factors.

This book assembles in a fairly logical and comprehensive manner a large amount of information hitherto to distributed widely over the scientific literature, and each section concludes with a discussion summary provided by the session rapporteur. The text certainly cannot be classified as bedtime reading, but for anyone with an interest in asbestos, it presents the bulk of the known relevant data, appears to be reasonably adequately referenced, and effectively demonstrates the complexities of the asbestos problem.

Toxic Constituents of Animal Foodstuffs. Edited by I. E. Liener. Academic Press, New York, 1974. pp. x + 222. £6.80.

In these days of concern about the intentional addition of a variety of chemicals to our diet, it is useful to be reminded that all that is natural is not safe. This book, a companion volume to one published in 1969 (*Cited in F.C.T.* 1971, **9**, 872) concerned with the toxins occurring in foods of plant origin, itself covers the toxins that occur in foods derived from animals—both those that occur naturally and those that are introduced either intentionally or as an inadvertent result of man's activities.

The second, third and fouth chapters provide a comprehensive review of naturally-occurring animal toxins that are likely to occur in food, dealing in turn with the toxic components of avian eggs, fish eggs, and shellfish, fish and algae. References to plant toxins at various points in this volume may appear at first to conflict with the title, but because many of our food animals are herbivorous, there is often the problem of the animal acting as a vehicle for bringing plant toxins and contaminants (such as pesticides and radionuclides) to the consumer. This point is particularly underlined in the first chapter, which deals with a variety of toxins likely to be found in meat and dairy products.

The general approach of the final chapter of this book has little in common with those preceding it. The problem pinpointed in this contribution is that of the nitrosamines, but the justification for this choice is far from clear and the subject is reviewed without any consideration of the broader context of environmental carcinogens. Admittedly the nitrosamines, as the many current research programmes show, are a topic of great interest and importance. However, with the widespread investigations at present in hand, any review is likely to suffer premature ageing as new facts come to light and new ideas emerge. This is not necessarily a sound reason for refraining from writing reviews on this or any other subject, but in this particular context, the review —while reasonably comprehensive as it stands—is likely to become outdated long before the subject matter of most of the rest of the book shows any sign of change. As a whole, however, this book should make useful background reading for anyone with an interest in nutritional toxicology.

Residue Reviews. Residues of Pesticides and Other Contaminants in the Total Environment. Vol. 47. Edited by F. A. Gunther. Springer-Verlag, Berlin, 1973. pp. vii + 198. DM48.10.

This issue of a familiar series contains four reviews on various aspects of the use of pesticides or herbicides.

The first considers ways in which the bioactivity of pesticides may be affected by the properties of the soils with which they come in contact. Primarily the behaviour of a pesticide in the soil depends on the extent of its adsorption on soil colloids, which are acidic and therefore serve as base exchangers. This interaction is affected not only by the soil pH, but also by other soil properties such as the content of clay and organic matter, the temperature and the moisture capacity. The summary of this contribution makes the interesting point that, in view of the number of factors that may affect pesticide activity, restrictive regulations setting maximum usage levels for pesticides irrespective of the environmental conditions cannot be satisfactory and serve only to penalize the good producer, who could, when necessary, use more than the specified amounts without giving rise to the environmental problems that less efficient producers would probably cause even when using permitted levels. The general rate and dose limitations are seen as an oversimplified approach to a complex problem, ignoring much of the knowledge that has accumulated in the past 20 years.

A related contribution reviews the effect of a specific soil condition, namely flooding, on the microbial degradation of various insecticides. Some organochlorine insecticides, including lindane, DDT and

endrin, are known to degrade rapidly under the relatively anaerobic conditions in flooded soil, while others, such as TDE, dieldrin and aldrin, are less readily biodegradable in such a situation. Flooded rice culture usually involves first the flooding and then the drying of the soil during the production of one crop, so that compounds or metabolites stable at one stage may be broken down at the next and problems of pesticide persistence encountered under normal soil conditions may be less severe under the fluctuating conditions associated with rice growing.

A major part of this issue is occupied with a detailed paper on the degradation of vinyl phosphate insecticides in plants, soils, water and animals. The biodegradation pathways of six members of this class and their metabolic fate in animals are described with the aid of numerous schematic diagrams and formulae, which are clear and helpful. Metabolism is discussed in the light of the known toxicity of the compounds and information is also given on the stability and degradation of residues of the compounds during food processing.

The book concludes with a short review of electron-microscope studies on the effect of various herbicides on the ultrastructure of plant cells. This work, aimed at elucidating the mechanism by which herbicides modify or inhibit plant growth, demonstrated the similarity between the ultrastructural changes associated with herbicide-induced plant death and those encountered in death from senescence and other causes. These ultrastructural changes were always detectable before visible changes were apparent, and the authors offer some constructive suggestions about the design of future electron-microscope studies for investigating the primary sites and mechanism of herbicide action. The electron micrographs are a new departure for *Residue Reviews*, but their reproduction maintains the standard we have come to expect in other aspects of this series.

Residue Reviews. Residues of Pesticides and Other Contaminants in the Total Environment. Vol. 48. Edited by F. A. Gunther. Springer-Verlag, Berlin, 1973. pp. v + 168. DM38.20.

Volume 48 in this excellent series presents, once again, concise critical reviews of a high standard on a variety of subjects of topical interest.

The opening chapter surveys the literature relating, on a world-wide basis, to cadmium residues in the environment. Soil, water, vegetation and food are considered in detail. On the basis of these data the authors conclude that uncontrolled or only partially-controlled emissions from industrial operations provide the largest single source of environmental cadmium, and that the accumulation of residues in excessive amounts occurs largely in areas close to these sources of emission. Existing data relating to the extent to which environments adjacent to residue sources are contaminated are considered to be fragmentary and inadequate. To assess adequately potential problems in the field of public health, the authors suggest that more information is needed on the cycling of cadmium in the environment and on the effects of long-term low levels of exposure. A consideration of natural occurrence, world production and uses of cadmium and a summary of its toxic effects in man complete the chapter.

While an examination of the available literature suggests that most plants, animals and micro-organisms are likely to metabolize a foreign compound in the same general way, it is still necessary to investigate the metabolism of each new pesticide in several biological systems. Although certain predictions about metabolites and the metabolic route can be made, it is not yet possible to decide without specific investigation which metabolic products will be the significant terminal metabolites and which will be only transitory intermediates in a metabolic route. This is the conclusion drawn by the author of a contribution entitled "Biological oxidation and conjugation of pesticidal chemicals", on the basis of a consideration of oxidation and conjugation reactions involved in the metabolism of a representative selection of widely-used pesticides in animals and plants. Metabolism by glutathione-dependent enzyme systems is also considered in detail.

The complexity of the problems associated with the evaluation of carcinogenic hazards of pesticide residues are highlighted in a chapter entitled "The carcinogenicity of pesticides". This complexity is illustrated with reference to the organochlorine pesticides, particularly DDT, aldrin and dieldrin. The discussion ranges not only over the validity of extrapolating animal data to man but also over such aspects as the synergistic effects of pesticides as they relate to carcinogenicity, the carcinogenic potential of photodecomposition products of pesticides and the association between the induction of microsomal enzymes by pesticides and the development of liver tumours.

The Plant Protection Institute is the key authority in the implementation of the Pesticide Act in Finland. In addition to outlining the provisions of the Act and the procedure for obtaining approval for a product, this chapter describes the extent of use of pesticides in Finland and provides lists of safety intervals for pesticide use and the tolerances for these materials in foodstuffs. Finally, the remaining chapter in this issue presents a consideration of the apparently somewhat neglected subject of the stability of pesticides in foodstuffs and other substrates kept under conditions of cold storage prior to extraction or analysis.

Residue Reviews. Residues of Pesticides and Other Contaminants in the Total Environment. Vol. 49. Edited by F. A. Gunther. Springer-Verlag, Berlin, 1973. pp. vii + 158. DM32.90.

Editorial reference to the extensive and varied interests covered by *Residue Reviews* is well illustrated by the four chapters included in this 49th volume in the series.

Of topical interest, particularly in the light of the present publicity surrounding North Sea oil and its repercussions, is a chapter entitled "Fate and effects of polluting petroleum in the marine environment". This review attempts to assess the extent of the problem and define its impact on the marine environment. It is of interest to note that although the much-publicized major spills generally occur near the shore and cause dramatic and highly visible damage, chronic low-level pollution derived from so-called "normal"

operations contributes the bulk of the oil polluting the sea on a world-wide basis. Available evidence suggests that the largest source of marine oil pollution emanates from ships, and from oil tankers in particular, rather than from shore-based sources. The great complexity of crude oil, which consists of a mixture of several hundred identified and many unidentified compounds, has presented a serious obstacle to studies of the fate and effects of oil pollution. The roles of physical, chemical and biochemical processes in the elimination of oil from marine environments are considered in detail in this contribution, which also summarizes the pathways and products of the microbial degradation of various petroleum components. The possibility of oil removal by stimulated biodegradation is discussed in the context of other pollution abatement and prevention techniques.

Unlike some countries, New Zealand has not consolidated its pesticide legislation under one all-embracing regulation. Largely for historical reasons, several different Acts of Parliament are involved, among them The Agricultural Chemicals Act and The Animal Remedies Act. For ease of reference, the current pesticide legislation is reviewed in the final chapter of this volume under five headings, covering in turn the agricultural chemicals and animal remedies legislation, both of which are administered by the Ministry of Agriculture and Fisheries, the poisons and the food and drug legislation, both the responsibility of the Department of Health, and relevant aspects of more general legislation. This chapter should be a great help to those trying to find their way around the complexities of New Zealand's pesticide control.

Volatilization and vapour-phase transport play a significant part in the dissipation of pesticides from plant, water and soil surfaces. Evidence is presented in support of this view and the subject is reviewed in considerable detail in a chapter headed "Pesticide volatilization". This concludes that although considerable progress has been made in quantifying the factors affecting volatilization and in developing mathematical models for predicting its rate, much additional work is needed to predict more accurately the rates of volatilization under field conditions.

Finally a comparative study is made of the possibilities of using computation models in the quantitative study of pesticide transport in soil. The nature of the basic data available for a quantitative approach is discussed briefly, and augmented by reference to more detailed reviews.

Contact Dermatitis. 2nd ed. By A. A. Fisher. Lea & Febiger, Philadelphia, 1973. pp. xi + 448. £8.75.

A wealth of new material and extensive reshaping of the old, and the consequent 124 extra pages, 14 colour plates and three additional chapters, make this second edition of *Contact Dermatitis* substantially better than the first. To chapters on irritancy due to chemicals, excretions and secretions, on contact dermatitis due to clothing, shoes, rubber, ashesives and gums, metals, cosmetics, plants and spices, on contact dermatitis in childhood and in atopic subjects, on photodermatitis and on various other aspects have been added considerations of industrial dermatitis and the role of patch testing in this area, the use of patch-test series, dermatitic responses of all mucous membranes, and aquatic content dermatitis. The illustrations in the latter chapter would deter the most persistent beachcomber!

The new section on industrial dermatitis draws together notes on many of the compounds to which workers may be exposed, including various types of resin, rubber and plastics compounds and their additives, as well as solvents and pesticides. Patch-test procedures in industry are discussed in a useful fashion. Briefer consideration is given to patch testing of the mucous membranes in comparison with cutaneous reactions. However, the new section on mucous membranes is welcome, the former chapter on cheilitis and stomatitis having been extended to cover eye irritation and conjunctivitis, vaginal irritation and balanitis, as well as the effects of dentures and dentifrices. The practice of grouping references according to compound at the end of the chapters has been introduced in this volume where relevant, and is helpful for those who wish to look into some of the cases discussed in more detail.

The book largely retains its original format, dealing with related problems in a series of interlinked monographs on individual allergens. Outlines of standard patch-test trays and procedures will be of interest to the practising dermatologist, as will the various lists of products that contain or are free from certain potential sensitizers. Attention is also paid to the relief of cutaneous reactions.

Like its predecessor, this volume is well produced and referenced and includes an appendix referring to over 800 contact allergens, with details of their occurrence and any cross-sensitizers and suggestions for suitable vehicles and concentrations for patch-test procedures. Unfortunately, the practice of placing 'buzz words' and key phrases in black-outlined boxes scattered over the pages has been retained. While this may be helpful for the purpose of quick reference, it is an irritating eyesore in an otherwise well presented and eminently readable text.

Progress in Medicinal Chemistry. Vol. 10. Edited by G. P. Ellis and G. B. West. North-Holland Publishing Co., Amsterdam. pp. x + 294. $30.00.

The aim of this series is to present comprehensive reviews on topics related to the development, study and discovery of drugs. Emphasis is placed on new discoveries, so that the active research worker or teacher may keep abreast of current developments. The present volume contains six articles on these lines, covering a variety of subjects.

One review, by Dr. G. Feuer of the University of Toronto Clinical Biochemistry Department, deals with the metabolism and biological actions of compounds containing the coumarin moiety. Topics surveyed in its 58 pages include toxicity, carcinogenicity and anticarcinogenic activity, effects on plants, antibiotic and anticoagulant properties, effects on muscle and the central nervous system and photosensitization, involving the citation of 581 references dating from 1820 onwards. Work on coumarin metabolism

and on the effects of coumarin on liver enzymes receives due mention, but unfortunately the German rat study imputing hepatocarcinogenicity to coumarin (Bär & Griepentrog, *Medizin Ernähr.* 1967, **8**, 244) has been wrongly cited. It would also seem somewhat against the professed aim of the series that the review was apparently written in 1970–1971; areas of research which have expanded rapidly in the last few years, such as the effects of the coumarin-based mycotoxins, thus receive relatively scant attention, and recent epidemiological studies on the hepatotoxicity of aflatoxin in man are absent altogether.

Another interesting topic is the relationship between the structure and carcinogenicity of polycyclic hydrocarbons, an erudite survey of which is presented by two university chemists, Drs D. W. Jones and R. S. Matthews. Perhaps wisely, this contribution does not attempt to review the vast literature on carcinogenicity, but rather appraises current theories of mechanism and their interrelationships. It is somewhat disappointing that the role of aryl hydrocarbon hydroxylases (admittedly still uncertain) is not discussed. However, the paper is doubtless correct in its conclusion that "one cannot hope yet to provide unequivocal chemical criteria of carcinogenic potency".

Dr. W. G. Smith, of the Canadian Environmental Health Directorate, surveys the use of enzymology in pharmacological and toxicological investigations. Intended as a laboratory guide, this contribution terminates with a list of more than 100 enzymes of special significance to pharmacological studies, together with a basic bibliography for each one. It is daunting to realize that this list represents less than 10% of the enzymes discovered up to the end of 1971. Another paper, entitled "Linear free energy relationships and biological action", by Dr. K. C. James of the Welsh School of Pharmacy, aims to show how physical properties may be correlated with biological action, and to define and explain the free energy parameters that are used. Guidance is also given on the choice of the most suitable parameters and the recognition of their limitations. A short article at the beginning of the volume usefully reviews the MEDLARS computer information retrieval system and includes a sample search on the chemotherapy of allergy to demonstrate the use of this service, while the final paper is devoted to recent advances in the synthesis of nitriles.

As intended, the papers in this volume should be of value to anyone embarking on research in one of the relevant fields or to lecturers seeking comprehensive background information on a particular topic. However, the apparent delays between writing and publication, in one case as long as 3 years, must detract from the aim to provide in this series surveys of the current state of knowledge.

Pathobiology of Development—Or Ontogeny Revisited. American Association of Pathologists and Bacteriologists, Cincinnati, Ohio, 1972. Edited by E. V. D. Perrin and M. J. Finegold. The Williams & Wilkins Company, Baltimore, 1973. pp. xi + 151. $19.75.

It is not often that pathologists stray from their pots and their slides—so when they do there must be a very good reason, as was clearly the case when a recent excursion took them into the field of teratology. The malformations that can occur in the developing foetus offer a tempting hunting-ground for the morphologist versed in verbal descriptions of the abnormal, but—despite this temptation—pathologists, with a few brilliant exceptions, have taken only a limited interest in this area of study. One recalls here R. A. Willis, who attempted to define the borderline between embryology and pathology, and C. Taruffi who, as early as 1881, commenced his eight-volume description of the history of teratology, a task which apparently took him well into 1894.

The decision to invite speakers from disciplines other than pathology to present their views at a symposium on the pathobiology of development, organized by the Council of the American Association of Pathologists and Bacteriologists in conjunction with the Pediatric Pathology Club, was no doubt a reflection of the Council's recognition of the limitations of the art of pure description. The contributions to this symposium, which was held in Cincinnati in 1972, have been collected in a slim volume of under 150 pages. Despite the restrictions on space which this must have imposed on the authors, each has managed to present a clear summary of the problem of maldevelopment as seen from his particular viewpoint. Furthermore, the editors have succeeded in slotting in a discussion after some of the chapters.

The first chapter gives a brief but interesting account of the history of teratology, stressing the superstitious beliefs and misconceptions that have surrounded the aetiology of malformations in man and animals since the dawn of history. Despite the greater degree of enlightenment in our age, some of the less obvious superstitions apparently survive not only among laymen but in professional circles as well.

This contribution is followed by a lucid account of the principles underlying teratology testing, a chapter that will be of great value to toxicologists. It stresses the importance of knowing the precise day of fertilization and the period of organ development if teratological tests are to be interpreted with any validity. It also underlines the importance to teratology of other toxicological principles with a much wider application, namely the genotype of the species under examination (or, commonly, species specificity), the type of metabolic breakdown, the tissue levels of the test substance and the need to bear in mind the possibility that minor deviations may occur in addition to and sometimes without readily detectable gross abnormalities.

Two chapters deal with the more theoretical aspects of normal organ development and the possible derangement in this process that could lead to malformations. Then follow two chapters on the role of viruses and radiation in the production of malformations in infants. The latter pair are commendably well balanced and keep the risks and hazards well in perspective. Some contrast is offered by the following contribution, which deals with the teratogenic effects of chemicals and presents a picture painted in sombre colours. The unrelieved gloom is carried over into the discussion, during which the author, in eloquent and often emotive terms, appears to have vanquished any attempt at a sober appraisal of the hazards.

. The penultimate chapter tackles a subject of great interest to pathologists, discussing the histogenesis of those types of malformation most frequently linked with malignancy. While the author considers that there is a clear causal relationship between malformation and tumour in many cases, he regards the now familiar stilboestrol-associated adenocarcinoma of the vagina as an important exception. Although he acknowledges that this tumour originates in a malformation of the Mullerian duct, he still, oddly enough, appears to attribute its occurrence directly to the carcinogenic action of stilboestrol.

Taken as a whole, this little volume is interesting and informative and is free of the jargon that makes many publications on teratology almost unreadable. It does, however, reflect the immaturity of teratology as an applied science, justifying the Introduction's picturesque, though hardly scientific, description of teratology as "a patchwork of concepts from developmental biology and allied sciences, embroidered here and there with politics, sociology, and the history of ideas, splashed with the brilliant insights of genetics, threadbare in the many spots where conjecture and extrapolation are substituted for data".

Chemical Mutagens. Principles and Methods for Their Detection. Vol. 3. Edited by A. Hollaender. Plenum Press, New York, 1973. pp. xxii + 304. £10.35.

Rapid expansion in the field of mutation research in recent years has produced a myriad of potentially useful procedures for evaluating the mutagenic activity of environmental chemicals. The most important of these methods have been selected to form the basis of the excellent series of books on chemical mutagens sponsored by the Environmental Mutagen Society.

For most people, the primary value of these books will lie in their provision of a single source of reference for the detailed methodology of the various tests. However, there are chapters on other aspects of mutagenicity, such as the correlations between mutagenicity, carcinogenicity and teratogenicity, and this third volume carries an interesting introductory chapter by Dr. Charlotte Auerbach on the history of research on chemical mutagenesis. This volume also includes more detailed discussions of some of the topics covered in the earlier issues, including the techniques available for monitoring human populations for mutagenesis, and there is an excellent summary of the numerous methods developed for testing chemicals for mutagenic activity using yeast systems.

All in all, the contributors to Volume 3 have maintained the high standards set in its predecessors and the book can be thoroughly recommended.

Inborn Errors of Metabolism. Edited by F. A. Hommes and C. J. Van Den Berg. Academic Press, London, 1973. pp. xv + 375. £7.

This volume presents the proceedings of a symposium on the relationship between developmental biochemistry and inborn errors of metabolism, held at Warffum in the Netherlands in August 1972. The text includes 20 papers by 38 contributors ranging from biochemists to clinicians with particular experience of inborn errors associated with brain damage.

This book is not a comprehensive text on the subject, but is rather a collection of contributions describing a diversity of research projects in this field. Readers unfamiliar with the background to this important and complex subject are likely, therefore, to find the contents of limited value. However, workers actively involved should gain much useful and interesting information on these problems.

The discussion sections following the papers are particularly to be commended. Reproducing this part of a symposium always presents certain difficulties for editors, but the effort involved is often well worth while, and in this case the discussions presented are stimulating, wide-ranging and obviously of considerable merit.

The Striated Muscle. International Academy of Pathology Monograph. Edited by C. M. Pearson and F. K. Mostofi. The Williams & Wilkins Company, Baltimore, 1973. pp. xiii + 518. $32.00.

The rapidity with which the biological sciences are advancing makes it difficult for the scientist to keep abreast in all fields, and the task is made more difficult by the fact that the information on any one topic is scattered among a large number of journals. Fortunately most workers are aware of this and frequent attempts are made to collect together recently acquired information in the form of monographs.

The monograph on striated muscle named above represents one such effort to gather together up-to-date information and present it in a condensed manner. It is intended mainly for the diagnostic pathologist, and a substantial proportion of the chapters deals with current views on the pathological interpretation of muscle biopsies. Apart from histology, which is given due prominence, there are descriptions of ultrastructural and histochemical changes, which may supplement classical histology in the diagnosis of muscle disease. It is indeed refreshing to find that these two techniques are used widely and effectively in this field.

It has not been assumed, however, that all histologists are familiar with the histochemistry and ultrastructural appearance of striated muscle. Descriptions of these two aspects of mammalian striated muscle both under normal conditions and after experimental injury occupy the first half of the book. Methods of biopsy and fixation for ultrastructural studies are dealt with in detail as are some common artefacts. Histochemistry is also given detailed treatment, including a discussion on artefacts and a section on the rationale for the selection of the group of enzymes widely used in the diagnosis of muscle disease.

The great value of this book lies in the fact that histology is integrated with histochemistry and electron microscopy in all the chapters, so that the reader becomes fully aware of the potential value of these newer techniques without expecting too much from them. Too often in the past, inordinate claims have been made and subsequently shown to have little foundation, and this has tended to discredit these new procedures.

The experimental approach to an understanding of muscle pathology has not been neglected. In fact

this approach has been successfully blended in some contributions with the pathology of naturally occurring diseases in man, giving a more credible and secure foundation to the opinions and views expressed on these diseases. There is a lesson here that authors in other fields of human pathology could learn with profit.

The text is clearly written throughout, despite the fact that it consists of individual contributions from 28 specialists, and is adequately illustrated. It may be recommended to all those who are interested in muscle pathology, whether or not they are professional pathologists.

Biology Data Book. Vol. I. 2nd ed. Edited by P. L. Altman and Dorothy S. Dittmer. Federation of American Societies for Experimental Biology, 1972. pp. xvii + 606. $30.00.

Biology Data Book. Vol. II. 2nd ed. Edited by P. L. Altman and Dorothy S. Dittmer. Federation of American Societies for Experimental Biology, 1973. pp. xix + 826. $30.00.

The appearance of additional volumes or revised editions in the Biological Handbook series will always be welcome. This series has filled a real need as a source of biological data and the particular editions named above contain a great diversity of information.

The first volume presents data relating to genetics and cytology, reproduction, development and growth, and concludes with a section on materials and methods for a variety of studies, including *inter alia* details of animal diets and culture media. Appendices include lists relating common names of animals and plants to the corresponding scientific names, outlines of animal and plant classifications, conversion formulae and factors and physical constants. The volume should be of use to many practising biologists. Sections of particular interest to the reviewer were the survey of characteristics of specified mouse strains, the phenotypic expression of gene linkages and the composition of various culture media. In general, the section entitled "Materials and Methods" will be of wide use and may well obviate the necessity of resorting to several different publications for the range of information that may be required for a particular purpose.

The four main sections of Volume II are concerned with biological regulators and toxins, the environment and survival, parasitism and sensory and neurobiology. Appendices again provide cross-references between the scientific and common names of the animals and plants referred to in the volume—an admirable inclusion.

In general, the data in these volumes are clearly presented and easy to use, the arrangement of each section following the standardized tabular format adopted in the earlier publications in this series. When the facts given are predominantly numerical this format is ideal, but it has severe limitations when the information is qualitative or descriptive. In the subsection on animal toxins, for instance, the signs and symptoms of poisoning in man are presented in note form and must have involved the exercise of considerable personal choice on the part of the compiler. This is not a disadvantage if the original references are consulted, but the clarity with which data are presented may tempt some to neglect this course of action. There is an excellent and extensive reference system, however, and if put to good use, this will allow the reader to acquire a sound background to any of the data given only in outline.

Although the data presented are extensive, it would be unjust to assume that they are exhaustive. Thus a search for a particular fact should be made on the basis of expectation rather than certainty. Nevertheless in some 'test runs' information was found on a high proportion of randomly selected questions. There is also a possibility that the information required may be contained in a sister volume, and in this connexion it would be helpful to have the headings of the major sections printed on the spines of each volume and dust jacket for rapid location of the contents. Another possible improvement might be the publication of an index covering more than one volume. However, these suggested modifications would merely add to the already established value of this series. In their present form they should not only be invaluable to many working in the pure and applied fields of biology but they also represent very good value for money.

Drug Resistance and Selectivity. Biochemical and Cellular Basis. Edited by E. Mihich. Academic Press, New York, 1973. pp. xii + 529. £15.20.

This book gives a detailed account of the biological, pharmacological and biochemical aspects of drug resistance, selectivity of drug action, collateral sensitivity and drug dependence. Twelve separate sections, each with different authors, deal in turn with the dynamic multifactorial basis for the selectivity of anticancer agents, the genetics of drug resistance, cross-resistance and collateral sensitivity, resistance to antibiotics, the biochemical bases of drug resistance in protozoa, resistance and dependence in viral chemotherapy, enzyme changes in resistant tissues, the uptake of drugs and resistance, natural resistance of tumours to selective metabolic and hormonal imbalances, tumour immunogenicity in therapeutics, species and tissue differences in drug selectivity and clinical resistance to cancer chemotherapy.

Thus 'resistance' is the keyword of this book and one of the main objectives is to illustrate the relevance of the inter-related pharmacological and biochemical factors that affect this phenomenon. Drug resistance may be natural or acquired; resistance and drug sensitivity are inter-related. This book shows how this is particularly critical in cancer therapeutics, in which the selectivity of anti-tumour action depends largely on the quantitative differences in pharmacokinetics and cellular metabolism between tumours and normal tissues.

Drug resistance is considered at the cellular level in man, animals, protozoa and bacteria. The parts played by genetic changes, by enzyme changes in both anabolic and catabolic pathways and by differences in the antigenicity of the plasma membrane are discussed. The metabolic interaction between virus and host cell is also considered.

Several contributions are concerned with resistance and its relationship to the selectivity of effects, both

in man and in animals, particular reference being made to anti-cancer drugs. The reasons for species differences in drug selectivity and the phenomena involved in natural resistance to hormonal and metabolic imbalances are discussed. The last chapter is devoted to the clinical basis for resistance to cancer chemotherapy and, where possible, this is related to pre-clinical data.

This book is extremely well arranged. Each chapter is divided into numerous sections but always includes an introduction, a conclusion and a list of references, and the full text is rounded off with an author and a subject index. Although the book is both complicated and detailed, its clear arrangement greatly eases the task of locating any particular aspect of the subject. Because it embraces many disciplines, the text should be equally useful to biochemists, molecular biologists, immunologists, pharmaceologists and toxicologists.

The Hazards of Work: How to Fight Them. By P. Kinnersly. Workers' Handbook No. 1. Pluto Press, London, 1973. pp. 394. £0.90.

This book is not, as its title may suggest, a manual on work avoidance for the work-shy. On the contrary, it is a self-defence manual which should be in the pocket of every man on the shop floor. It is a directory of industrial safety intended not for the toxicologist but for those who may be at risk.

Industrial toxicology is an area in which the workman needs to be made more aware of some of the risks he may be facing, and this little book gives some sound advice towards this end. A useful directory of toxic substances indicates the particular hazards of individual chemicals commonly met in industry. Specific problems such as cancer risks, skin effects and effects on the lung are also dealt with fairly thoroughly. The writing is generally clear and straightforward, simple definitions being given for necessary medical terms where they occur, although the style of writing occasionally supplies some probably unintended humour. However, the commonsense approach of much of this paperback makes it a useful text for the layman concerned with industrial toxicology.

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Residue Reviews: Residues of Pesticides and Other Contaminants in the Total Environment. Vol. 51. Edited by F. A. Gunther. Springer-Verlag, New York, 1974. pp. ix + 189. $20.00.

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Symposium: Sweeteners. Edited by G. E. Inglett. The Avi Publishing Company, Inc., Westport, Conn., 1974. pp. ix + 240. $21.00.

The Structure of Mitochondria. Edited by E. A. Munn. Academic Press, London, 1974. pp. xiii + 465. £9.80.

Perinatal Pharmacology: Problems and Priorities. Edited by J. Dancis and J. C. Hwang. Raven Press, Publishers, New York; North-Holland Publishing Co., Amsterdam. pp. xii + 228. $23.10.

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Information Section

ARTICLES OF GENERAL INTEREST

COSMETICS LEGISLATION IN EUROPE—A PERSONAL VIEW*

by

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There are 36 countries in Europe with, in principle, as many different approaches to the regulation of cosmetics (in which I include toiletries). Official attitudes to the need for control vary from *laissez-faire* to detailed intervention. Some systems of regulation are old, some new. Some are simple almost to the point of non-existence, some complex and specific. Most have evolved over the years with, until quite recently, little or no regard to uniformity or the needs of international trade. Nevertheless, amidst this diversity there is a perceivable pattern. It would seem that there is at present within Europe a fairly widespread acceptance of a system of control based on the following elements:

(1) *A prohibition of the manufacture or sale of cosmetics that may be a danger to health.* As in the UK, which has no specific cosmetics law, this may stem from the common law duty that the seller owes to the buyer and that enables the consumer to recover damages for harm done. Alternatively it may be spelled out in cosmetics legislation, as for example in the Netherlands[1], Belgium[2] and (from January 1975) the Federal Republic of Germany[3]. In Switzerland[4] and Spain[5] an attempt has been made to express this concept in more concrete form by specifying minimum LD_{50} values for cosmetics.

(2) *A 'negative list' of substances that must not be used in cosmetics.* In the UK the regulations governing the retail sale of poisons, dangerous drugs and therapeutic substances are tantamount to a prohibition on the use in cosmetics of some 7000 substances, the vast majority of which, I hasten to say, would never be considered for use in consumer goods. In France and the Irish Republic the approach is similar in principle but quite different in detail. Elsewhere, a great variety of forbidden substances and classes of substances are set out in cosmetics legislation, for example in the Netherlands, Denmark, Spain, Belgium and Switzerland. There appears to be little agreement between countries on what substances should be prohibited.

(3) *A 'positive list' of substances that can be used only in specified maximum concentration and/or applications.* Examples of these positive lists, all different and usually quite short in contrast to the negative lists, are to be found in Dutch, Danish, Belgian, Swiss and Spanish legislation. Cosmetics containing these substances sometimes have to be specially labelled. Often there are special provisions for the control of colourings, for example in the Netherlands, Belgium, Spain and notably Germany.

The European Economic Community Cosmetics Directive[6], now before the Council of Ministers, follows these principles. A series of Articles sets out the general conditions for marketing cosmetics within the Community, including a requirement that cosmetics must not be capable of causing damage to health when applied as directed. These Articles are given more specific application in five Annexes. Annexe II is a 'negative list' of over 400 substances that cannot be used in cosmetics. Annexe III is a 'positive list' of 27 substances that can be used subject to various restrictions as to field of application, maximum concentration and special labelling requirements, plus some 84 colourings for use in products that come in contact with mucous membranes. Annexe IV is a further 'positive list' of 49 substances (including 27 colourings) provisionally admitted for a period of 3 years, again subject to restrictions as to field of application, concentration and labelling.

Application of the Directive within the Community at the national level should not require fundamental changes in the legislative framework now existing in the member States. The Directive would seem to be compatible in principle with the new German law and with the recently published Italian draft Cosmetics Law[7]. Only in France are there proposals for new legislation which must surely require modification in the interest of harmonization.

In my judgement, adoption of the Directive as at present drafted should cause few serious problems for the UK industry and this would seem also to be true elsewhere in Europe. Most manufacturers should surely welcome a step that would reduce the multiplicity of separate national laws that at present seriously hinder international trade. Indeed there are already signs that, unless the Directive is adopted without much further delay, existing plans for domestic legislation in a number of countries will go ahead and thus perpetuate and extend the differences that already exist. It would be unfortunate if the pattern of cosmetics legislation within the Community became as unco-ordinated as it now is with, for example,

*Paper read at the International Federation of Societies of Cosmetic Chemists 8th International Congress, London, 26 August 1974.

pharmaceuticals. Diversity in regulatory affairs, once established, is hard to rectify. The Joint FAO/WHO Codex Alimentarius Commission has been struggling for over a decade to bring some degree of international harmony into food legislation with not very much so far to show for its efforts.

It also seems to me that the consumer's interests have in practice been well looked after under the present system. Current cosmetics legislation should not be viewed in isolation but rather within the general framework of law relating to medicinal products, weights and measures, trade descriptions and advertising and not least the high degree of responsibility with which the vast majority of firms operating in the industry approach problems of product safety.

Nevertheless, there is growing pressure from various quarters inside and outside the EEC to develop the Directive into a wholly positive list form. In giving their opinions on the Directive, both the Economic and Social Committee[8] and the European Parliament[9] pressed for speedy action in this direction and similar views have subsequently been expressed in the Council of Ministers by certain of the member governments with the backing of consumer organizations. According to reports, the Commission has given an undertaking that if the Directive is adopted in its present form it will at once start work on the preparation of positive lists and will invite proposals from the member States. I understand that there is a move to write this undertaking into the Directive, with specified dates for completion of work on various categories of ingredients, and that draft lists of preservatives and sunscreens have already been submitted to the Council of Ministers. Parallel with this activity at the Community level, the Council of Europe Public Health Committee has set up a Working Party on the Possible Toxicity of Cosmetics which has started work along the same lines. While there is no formal link between the Community and the Council of Europe, any list prepared by the Council of Europe could easily form the basis for a revised Directive and it would of course in addition potentially apply over the wider membership of the Council of Europe which embraces eight States in addition to the members of the EEC.

It would be pointless as well as unwise to ignore the strong tide of opinion in Europe towards positive listing, but this is a prospect I view with some apprehension, for the following reasons:

(1) It has been estimated[10] that upwards of 13,000 ingredients are used in cosmetics. To name, list, characterize, specify and evaluate for safety this vast array of substances and, presumably, decide at what concentrations and even in what products they can be used, would impose on government and industry a severe work load over many years and use scarce and expensive resources without commensurate public benefit. In the UK, it has taken half a century to introduce positive lists for the control of less than 300 food additives, and flavouring substances have yet to be tackled. The process started[11] in 1925 and was only completed[12] this year.

(2) Any positive list, once compiled, would prove a bar to progress without the prior establishment of effective and speedy machinery for making additions. UK experience with food additives in this regard is not encouraging.

(3) The introduction of a positive list system would not significantly reduce the incidence of allergic reactions by individuals sensitive to specific cosmetics ingredients.

(4) The inclusion in a positive list of new compounds developed by a manufacturer or a supplier could directly or indirectly reveal to competitors the results of expensive research. This could discourage innovation.

(5) Whatever may be the theoretical objections to negative listing, I am unaware of any evidence that public health requirements have not been adequately met by the present system of control based primarily upon this system within the framework of general law. It is significant that no country at the present time has a comprehensive positive list system for cosmetics ingredients.

If governments, for political or other non-scientific considerations, decide that the present system must be overtly strengthened in the consumer interest, I very much hope, therefore, that all possible alternatives are carefully considered before any irrevocable commitment to comprehensive positive listing is made. The present system in the United States[13] is one such, combining mandatory ingredient declaration with voluntary registration of manufacturing establishments, submission of ingredient information and filing of product experience. Whether arrangements along these lines would be preferred by European industry to positive listing remains to be established. Product licensing as with medicinal products has also been put forward as an alternative. Powers to license products already exist in the UK in medicines legislation and, it would seem, in the new German cosmetics law. In my experience of the pharmaceutical industry, any such system would be quite incompatible with the high rate of product introduction that characterizes the cosmetics industry.

If, in spite of all, positive listing is to come, I think that, at the very minimum, the industry is entitled to demand—and must demand if it is to survive in its present form—the following:

(1) The lists must be introduced gradually in an orderly and logical way with proper regard to legitimate commercial considerations.

(2) The criteria for safety-in-use applied in compiling these lists must be reasonable and realistic and give due weight to past experience without adverse effects as well as to benefit.

(3) The lists must be prepared with close and real collaboration between government and industry, not by officials and academics working in isolation.

(4) There must be proper provision not only for quickly evaluating and approving new compounds but also—and this is by no means the same thing—for incorporating them in the official permitted list.

(5) There must be proper provision for the protection of industrial property arising from investment in innovation, to give those who develop new compounds an adequate period in which to profit from their inventions before these pass into the public domain.

The regulatory environment in which the European cosmetics industry operates is entering a period of rapid and even bewildering change at the national and international levels. Scientists in the industry must be prepared to participate in the work that will inevitably be generated in the difficult area where science, law and commerce meet. International liaison and co-operation will be essential if industry is to play its proper role in these developments, which could crucially affect commercial operations.

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MORE THOUGHTS FOR SMOKING MOTHERS

A great deal of publicity has been given to demonstrations that smoking during pregnancy results in a reduction in birth weight, and there is considerable support for the view that smoking during pregnancy should be actively discouraged. Nevertheless, some of the existing evidence is conflicting and much remains to be learnt about the mechanisms of the foetal response to maternal smoking as well as about the more favourable effects that have been reported, namely a reduction in the incidence of eclampsia and morning sickness among smoking women. More evidence on the effect of smoking on the foetus is constantly coming to light, and we review here some papers that have appeared since the publication of our previous comments on this subject (*Cited in F.C.T.* 1973, **11**, 671).

Further clinical studies

Hickey *et al.* (*Br. med. J.* 1973, **3**, 501) dispute the significance of a statistical association between lower mean birth weight and cigarette smoking. The possible hypotheses, they assert, are two: either cigarette smoking in the mother causes a lower birth weight, or the same individual genotype or constitution accounts for both the smoking behaviour and the lower birth weight of the children. The second hypothesis is supported by the findings that infants born to women before they become smokers are generally smaller than those of non-smokers, and that infants born to smokers who subsequently give up the smoking habit show a lower incidence of low birth weight than infants of habitual and continuing smokers. However, as far as the first of these pieces of supporting evidence is concerned, the relevant work by Yerushalmy (*Am. J. Obstet. Gynec.* 1972, **112**, 277) has been discounted by Ross *et al.* (*Br. med. J.* 1973, **4**, 51) on the grounds that the original study had certain methodological shortcomings.

Harlap & Davies (*Lancet* 1974, **I**, 529; *idem*, *Br. med. J.* 1974, **2**, 610) have demonstrated that the children of West Jerusalem mothers who smoked during pregnancy were more often admitted to hospital with bronchitis and pneumonia during the first year of life than were children of non-smokers. A dose-response effect could be shown between the infant morbidity and the number of cigarettes smoked by the mother. The authors attribute this effect, however, to passive inhalation of tobacco smoke by infants after birth, and urge caution against the assumption that long-term adverse effects among children of smoking women are necessarily or primarily due to prenatal exposure, since continuing exposure to tobacco smoke in the atmosphere may well have an effect on the child's health. James (*Br. med. J.* 1974, **2**, 610) points to the further possibility that the lifestyle and mental environment of children of smoking mothers may provide an explanation for the intellectual impairment that has sometimes been alleged to occur in such children (*Cited in F.C.T.* 1973, **11**, 674). Asserting that households of smokers are, in general, characterized by unrest and a search for novelty and excitement, with a consequent lack of emphasis upon the academic attainments of children, James (*loc. cit.*) comments that it would also be interesting to know whether mothers who smoke are in fact less intelligent than those who do not. In this connexion, he cites a report in the *Sunday Times* (28 April 1974, p. 32) indicating that smokers who discontinue the practice have been shown to possess higher than average intelligence, but one feels that this hypothesis is likely to provoke emotional rather than scientific argument.

On the question of child development subsequent to perinatal survival, Butler & Goldstein (*Br. med. J.* 1973, **4**, 573) have reported significant differences in height and reading ability between children of smokers and non-smokers at the age of 7 years. Tak-

ing into account the factors of the mother's age and height, social class by father's occupation, the number of younger and older children in the household and the sex of the child, there was a deficit in both height and reading ability among the offspring of smokers. Between children of non-smokers and those of smokers of ten or more cigarettes daily, there was an average difference of 1 cm in height and 4 months in reading attainment, although such effects were small in comparison with those associated with some of the other factors. Repetition of the analysis when the same children reached 11 years showed a mean difference of 1 cm in height (1·6 cm before adjustment for the same associated factors as were considered at age 7), 3 months in general scholastic ability (8 months before adjusment), 4 months in reading ability (9 months before adjustment) and 5 months for mathematical ability (8 months before adjustment). The authors interpret their findings as establishing that smoking during pregnancy has a continuing effect on the offspring, though without any widening of the difference between the smokers' and non-smokers' children between the ages of 7 and 11 years. The results fail to support the hypothesis that it is the type of mother rather than the smoking which is responsible, but it is important to remember that personality factors were not among the relevant influences for which allowance was made. This study was based on a sample of several thousand children, but tests based on much smaller groups, such as that on only 88 pairs reported earlier by Hardy & Mellits (*Cited in F.C.T.* 1973, **11,** 674) would probably fail to detect any existing cause-and-effect relationship since, as Donovan (*Lancet* 1973, **I,** 376) has pointed out, the effect of maternal smoking on development would, if it exists at all, be very small in comparison with individual variations. Whether it exists at all, however, is a much-disputed question (Burch, *Br. med. J.* 1974, **1,** 40; *Nature, London* 1973, **245,** 61).

Perinatal mortality and toxaemia

Goldstein (*ibid* 1973, **246,** 540) has shown that an increase in mortality associated with lower birth weights among the infants of smokers does not hold true if only those infants weighing 2500 g or less are considered. However, the evidence that the low-birth-weight infants of smoking mothers have a better survival rate than those of non-smoking mothers may indicate not that maternal smoking causes fewer perinatal deaths but that such smoking is less lethal to infants than other causes of low birth weight, which, where weights of 2500 g or less are concerned, may carry a 16-fold increase in liability to perinatal death (James, *ibid* 1973, **246,** 235). The effect of smoking is thus to augment the < 2500-g group with some relatively healthy babies, who would otherwise be in a higher weight group.

In a study involving nearly 4000 births, Palmgren *et al.* (*Acta obstet. gynec. scand.* 1973, **52,** 183) have reported incidences of prematurity of 6·5% with a maternal smoking rate of less than ten cigarettes daily and 6·2% with more than ten cigarettes daily, compared with an incidence of 3·3% in non-smoking mothers. The mean birth weight was 220 g lower in the infants of smokers. Placental bleeding and infarction were more common among non-smokers (23·5 and 16·2% respectively) than among moderate smokers (14·4 and 11·3%) or heavy smokers (18·2 and 4·6%) and tended to occur more often in women with toxaemia, the incidence of which was 10·5, 8·3 and 5·9%, respectively in the three groups. The authors suggest that the reduction in maternal toxaemia associated with smoking may reflect the hypotensive and renal-stimulating effects of thiocyanate produced by the detoxication of cyanide present in smoke, or perhaps the abortifacient effect in smoking, which could reduce the number of potentially toxaemic mothers. The frequency of abortion was twice as high in smokers of more than ten cigarettes daily as in non-smokers. The fact that perinatal mortality in premature infants (defined as those weighing less than 2500 g at birth) was higher in the non-smoking group (29·5%) than in the smoking groups (16·5 and 18·2%) could thus be related in some degree to the relative occurrence of placental bleeding and infarction and maternal toxaemia and therefore provided some support for the foregoing comments of James (1973, *loc. cit.*).

The effect of smoking on the foetal heart rate was studied in 22 pregnant women aged 15–41 years by Cloeren *et al.* (*Arch. Gynaek.* 1974, **216,** 15). Tachycardia was detected ten times during 27 separate investigations, decreased fluctuations seven times, and a combination of the two eight times. Monitoring of the foetal heart was carried out for 30 minutes under rest conditions, and then for a further 30 minutes after the 4–12 minutes during which a cigarette was smoked. The effect of smoking on the uteroplacental and cardiac blood pools was studied in ten of these patients. In all cases there was an increase in the uteroplacental blood pool, although in half this was preceded by a transient decrease. Reciprocal changes were recorded in the cardiac blood pools in six of the patients. These effects may represent a direct action of nicotine on the sympathetic nervous system, or an indirect action resulting from a decrease either in placental circulation or in the concentration of oxygen in the maternal arterial blood.

Maternal cigarette-smoking has also been shown to result in a trend towards lower serum-bilirubin levels in the foetus and newborn (Nymand, *Lancet* 1974, **II,** 173). Thus in 1459 newborn offspring of non-smokers, the proportion with less than 100 μmols bilirubin/litre serum (the level of clinical observability) was 61·3%, a significantly lower incidence than the 69·0% in 1024 neonates born to smokers. At the other end of the scale, 12·6% of the neonates in the non-smoking group compared with 8·5% of those in the smoking group had a level above 200 μmols/litre. The cyanide in tobacco smoke has been implicated also in this effect, with the tentative suggestion that its detoxication in the foetal liver results in the induction of glucuronyl transferase and the consequent enhancement of the excretion of conjugated bilirubin from the liver.

Animal studies

The effect on pregnant mice of chronic exposure to cigarette smoke has been investigated by Wagner *et al.* (*Eur. J. clin. biol. Res.* 1972, **17,** 943). The effect of a fixed daily exposure at different phases of the gestation period was studied and, in addition, mice

were exposed to various concentrations of smoke over the entire gestation period. The different exposure groups showed no significant differences in the numbers of foetuses produced or in foetal weights. Even with exposure to the smoke from 30 cigarettes burned for 8 minutes on 2–5 occasions daily from 2 weeks before mating to day 14 of gestation, no foetal abnormalities appeared. There was, however, a marked reduction in maternal weight gain among the exposed animals and this was paralleled in non-pregnant mice exposed to the same concentrations of smoke.

Starting from the observation of a significant negative correlation between the birth weight of infants and the carboxyhaemoglobin (COHb) concentration of maternal blood in 176 smokers and 177 non-smokers in Denmark, Astrup *et al.* (*Lancet* 1972, **II,** 1220) studied the effects of carbon monoxide (CO) on pregnant rabbits. Exposure to 180 ppm CO, which produced concentrations of 16–18% COHb, or to 90 ppm CO, which produced 8–9% COHb, was maintained throughout gestation until day 30. A mean reduction in birth weight of about 20% and a neonatal mortality rate of 35% in the first 24 hours (compared with 1% in the controls) resulted from the higher level of exposure. The lower exposure level caused an 11% reduction in birth weight and a 24-hour neonatal mortality of 9·9%. In the group exposed to 180 ppm CO, three offspring from 18 rabbits had limb abnormalities.

The conclusions to be drawn from the evidence at present available seem to be that several different factors in the smoking mother's environment may determine deleterious effects on the infant and the growing child, and that any pronouncement on the relative importance of these factors must take into account selection at the perinatal stage.

[P. Cooper—BIBRA]

STARCH IN THE PERITONEUM, LUNG AND KNEE JOINT

Post-operative reactions to corn starch derived from the gloves of surgeons have been discussed in some detail in recent volumes (*Cited in F.C.T.* 1974, **11,** 261). It has been suggested, in view of the delay in the appearance of the reaction and its response to steroid and anti-inflammatory treatment, that corn-starch granuloma may constitute a hypersensitivity reaction.

Ehrlich *et al.* (*Sth. med. J., Nashville* 1974 **67,** 443) have reported two further cases of starch peritonitis. Two women, aged 29 and 35 years, were admitted for surgery on Stage II-B squamous cell carcinoma of the cervix. Exploratory laparotomy and selective lymphadenectomy were carried out uneventfully in both cases, and both subsequently received radiation therapy in the pelvic area. However, 33 days after her operation, the younger patient developed diffuse abdominal pain, which did not respond to antibiotics. When a second exploratory laparotomy was carried out, multiple dense adhesions and small white nodules were found on the visceral and parietal peritoneum. Considerable blood was lost in the course of surgery and the patient died during the operation. The second patient was more fortunate in that she remained asymptomatic for 3 months until a hysterectomy was carried out. At this operation multiple adhesions and numerous white nodules were found on the peritoneal tissue and uterus. This patient recovered uneventfully.

Histology on post-operative specimens from both women revealed small, well-organized granulomas containing epithelial cells and foreign-body giant cells. Entrapped in fibrin in the inflamed areas were numerous particles, which were identified as starch by staining with Lugols solution and by inspection under polarized light. Symptoms of a starch reaction generally appear 10–40 days after an operation, and can usually be diagnosed by the presence of starch granules in aspirated ascitic fluid. Ehrlich *et al.* (*loc. cit.*) report that during surgical procedures as many as 22·6% of surgical gloves show a perforation, through which starch can escape into the wound even when the outer surface of the gloves has been washed and wiped with a wet sponge. Absorption of the starch may be delayed by clumping and the resulting prolongation of exposure to the irritant may lead to the development of granulomas histologically indistinguishable (apart from the presence of starch) from tubercular lesions. These authors advocate treatment of the condition with steroids or anti-inflammatory agents.

Warshaw (*Lancet* 1972, **II,** 1054) reported his experience of reactions to starch in the peritoneal cavity and has followed this with a paper describing a case of pleuritis attributed to a starch reaction (Warshaw & Mills, *Surgery, St, Louis* 1974, **75,** 296). A 44-year-old man underwent a right lower lobectomy for carcinoma. A finding of pleural thickening on day 4 after the operation was followed by complaints of pain and wound discomfort accompanied by a raised temperature on day 8. Thoracentesis (removal of fluid through a puncture in the thoracic wall) produced a sterile serosanguinous fluid containing many mononuclear leucocytes and birefringent granules characteristic of starch. After drainage of 300 ml of fluid, the fever abated and the patient's recovery was uneventful. Pleural fluid from other thoracotomy patients, whose recovery was not complicated by this type of reaction, did not contain starch granules. Warshaw & Mills (*loc. cit.*) suggest that this syndrome is analogous to that of starch peritonitis and should subside spontaneously. These authors are not convinced of the efficacy of anti-inflammatory treatment.

In order to assess its effects on synovial tissue, various quantities of starch were implanted in the knee joint of adult mongrel dogs. Singh *et al.* (*Clin. Orthop.* 1974, **99,** 285) estimated that the amount of glove powder that can be removed from a surgical glove by wiping is 200 mg. Assuming that not all of this would necessarily be deposited under actual conditions of surgery, they implanted 25, 50 or 100 mg starch into the left suprapatellar pouch of three groups of eight dogs. In addition, the suprapatellar pouch of the right knee of each dog was opened and the synovium was rubbed with a clean gloved finger to simulate the surgical treatment of the test knee. Two animals from each group were killed after 3 days and 1, 2 and 4 weeks, and samples of fluid

and sections of representative areas were taken from the synovial capsules of each knee.

The control joints showed some inflammatory response and a polymorphonuclear infiltrate during week 1 after treatment, but by the end of week 2 this had largely subsided, leaving only some fibrosis at week 4. A very severe inflammatory response was observed in animals treated with 100 mg glove powder. The cell lining of the synovium was lost completely and infiltration with polymorphonuclear leucocytes and a marked increase in vascularity were noted in the first 2 weeks. Some lymphocyte infiltration, with haemorrhage and haemosiderin-laden macrophages and fibroblastic activity, was noted at this stage. Granules of starch were still identifiable at the end of 4 weeks, at which time round-cell infiltration of the synovial layers was noted. Moderate inflammation and the increase in vascularity still persisted. Similar but less severe responses were obtained following implantation of 50 or 25 mg starch powder. Smears of synovial fluid were sterile and showed dose-related counts of polymorphonuclear leucocytes during weeks 1 and 2 after treatment.

Singh *et al.* (*loc. cit.*) infer that inflammation is directly related to the quantity of starch present and that higher levels delay recovery. There was no evidence of a granulomatous response to the powder in the joints. They suggest that contamination of a human joint with glove powder during surgery would aggravate the synovial inflammatory response, and that surgeons should be meticulous about wiping all traces of powder from their gloves before operating. Such a recommendation, however, fails to solve the problem mentioned by Ehrlich *et al.* (*loc. cit.*) regarding the perforation of surgical gloves. The suggestion that in about 23% of cases, corn-starch contamination of a wound would be likely to occur whether or not the exterior of a glove had been wiped, again raises the question of whether the powdering of surgical gloves is really necessary at all (*Cited in F.C.T.* 1974, **12**, 264). It is still not clear whether human peritoneal reactions to corn starch are isolated responses in certain hypersensitive individuals. The finding of an asymptomatic granulomatous response by Ehrlich *et al.* (*loc. cit.*) indicates that these reactions may be symptom-free, and Warshaw & Mills (*loc. cit.*) suggest that some starch reactions will clear spontaneously, two factors that would complicate attempts to establish the actual incidence of such reactions.

[F. A. Charlesworth—BIBRA]

ACRYLIC CEMENT REACTIONS

Allergic reactions to acrylic dentures have been attributed to monomeric methyl methacrylate (MM), while acrylic dough containing this monomer has been shown to be a primary irritant in rabbits (*Cited in F.C.T.* 1971, **9**, 145; *ibid* 1972, **10**, 596). In a comparative study of the embryotoxic potential of various methacrylate esters (*ibid* 1973, **11**, 1151), some increase in resorptions and gross malformations was associated with administration of the methyl ester. However, the cardiovascular reactions to acrylic implants used in orthopaedic surgery of the hip are evoking particular interest at present, and have prompted many recent reports, as evidenced by the high proportion of 1972–1974 references in the list terminating a recent Special Article on this subject (*Lancet* 1974, **II**, 1002). These reports have put forward an extraordinary variety of suggestions regarding the possible factors involved in the development of the cardiovascular reactions described.

Clinical observations

From a study of 58 patients who underwent hip or femoral-head replacements, Cadle *et al.* (*Br. med. J.* 1972, **4**, 107) concluded that monomeric MM absorbed into the circulation from the resin surface might be responsible for the cardiovascular reactions sometimes observed after the packing of the bone cavity with the partly polymerized mixture of monomer, polymer powder and catalyst, but they threw little light on the mechanism involved. They pointed out that vascular collapse had been observed generally in the older and less fit patients undergoing femoral-head replacement and suggested that any potentially hypotensive anaesthetic agent, such as halothane, was likely to increase the hypotension induced by acrylic resin usage. Fowler (*ibid* 1972, **4**, 108), on the other hand, has stressed the importance of avoiding the complication of fat embolism in such patients by extracting all the fat from the reamed bone cavity before inserting the cement. Ellis (*ibid* 1973, **1**, 236) questioned an assertion (Brittain & Ryan, *ibid* 1972, **4**, 667) that careful preparation and an appraisal of the degree of plasticity of the cement before insertion into the femoral shaft were important factors in reducing hypotension and rejected suggestions that aeration of the mixture to disperse residual MM monomer was of prime importance.

Modig *et al.* (*Acta anaesth. scand.* 1973, **17**, 276) have reported the case of a 51-year-old woman with necrosis of the right femoral head, in whom acrylic cement was used to fix an acetabular prosthesis. Care was taken to allow partial setting of the cement before insertion, to diminish absorption of free monomer into the general circulation. The femoral prosthesis was inserted about 15 seconds after introduction of the cement into the cavity. The patient's mean pulmonary arterial pressure rose from 25 to 31 mm Hg during the 20–45 seconds following the prosthesis insertion, her blood pressure fell from 100 to 50 mm Hg in the period between 25 and 90 seconds after the insertion and her airway resistance rose. Within 3 minutes, arterial oxygen tension fell from 60 to 35 mm Hg and arterial saturation from 95 to 74%. Such changes were indicative of peripheral vasodilatation with increased pulmonary vascular resistance and bronchiolar constriction, but the authors did not commit themselves on the course of events beyond suggesting that these effects might be attributable to the escape of bone-marrow elements or monomeric MM into the circulation. Milne (*Anaesthesia* 1973, **28**, 538) reported cardiac arrest and

hypotension respectively in two patients in whom acrylic cement had been used for hip prosthesis. The temporary use of a tourniquet to reduce absorption of hypotensive materials was ineffective. Two deaths from cardiopulmonary collapse during hip operation and insertion of an acrylic prosthesis, reported by Kepes *et al.* (*J. Am. med. Ass.* 1972, **222,** 576), were followed up by an autopsy examination of one patient, which revealed widespread marrow emboli in the pulmonary arteries. However, Coventry (*ibid* 1973, **223,** 442), pointing out that such emboli are commonly found in patients with femoral-neck fracture, favoured absorption of MM as the cause of cardiac arrest rather than fat or air embolism.

A more detailed study of 45 patients (Fearn *et al. Acta orthop. scand.* 1972, **43,** 318) has shown a biphasic effect on blood pressure. When the acetabulum was packed with acrylic cement, 16 patients showed an initial rise in systolic blood pressure followed in eight of them by a fall, while an initial fall in 24 was followed in 12 by a rise; no change occurred in five. When the femur was packed with cement an initial rise in systolic pressure occurred in 24 patients, ten of whom showed a subsequent fall, in 20 there was an initial fall followed in 16 of them by a rise, and one patient showed no change. It was considered that the cardiovascular response to the thermal, mechanical or chemical stimulus derived from the use of methacrylate mixture was influenced by the patient's blood pressure at the start of treatment and by the concentration of halothane in the anaesthetic gas used. More recently, McMaster *et al.* (*Clin. Orthop. rel. Res.* 1974, no. 98, p. 254) have suggested a relationship between the development of a hypotensive response and a blood-volume deficit. They consider that adequate blood-volume replacement during the operation can minimize a loss of vascular tone due to circulating monomer.

Animal studies

Various authors, including McMaster *et al.* (*loc. cit.*), have sought the answer to this problem or support for their particular theories in animal studies.

Three pharmacological studies of 12 different methacrylate esters have been published by an American group. The first (Mir *et al. J. pharm. Sci.* 1973, **62,** 778) was an investigation into the effect of the esters on the isolated perfused rabbit heart in concentrations of 0·1, 0·01 and 0·001% in Locke's solution. Dimethylaminoethyl methacrylate was the most active pharmacologically and produced cardiac arrest at 0·01%. Lauryl methacrylate was the least active as a cardiac depressor. Most esters significantly reduced both cardiac rate and force of contraction, but the effect on coronary flow was less marked, and the magnitude of the changes produced by the esters in these three respects precluded any single criterion which might denote toxicity. MM had an irreversible effect on the isolated heart at all three concentrations tested. At the 0·01% level it reduced the heart rate, force of contraction and coronary flow by 56, 74 and 51%, respectively, while the higher concentration caused cardiac arrest.

The second of these papers (*idem, ibid* 1973, **62,** 1258) reports the effects of methacrylates on the contraction of isolated guinea-pig ileum, when applied in dilutions between 0·2 and 0·001%. Most of the esters inhibited spontaneous ileal contraction and antagonized the stimulant effect of acetylcholine and barium chloride, but the effects were readily reversible except in the case of 1,3-butylene dimethacrylate. MM acted in this way. Of the other methacrylates studied, the dimethylaminoethyl ester stimulated contraction of the preparation even in the presence of atropine. The authors of this report suggest that reduction in the tone of the smooth muscle of the arterioles and the resulting vasodilatation may be a factor in the hypotension associated clinically with the use of acrylic cements.

In their third paper (*idem, ibid* 1974, **63,** 376), these authors report the effects of iv infusions of methacrylates into anaesthetized dogs. All esters increased the respiration rate, decreased the heart rate and altered the electrocardiographic pattern. With the lower esters, including MM, there was a biphasic response comprising an abrupt fall in blood pressure followed by a rise. The longer-chain compounds elicited only a hypotensive response, while an anomaly was again presented by dimethylaminoethyl methacrylate, which caused hypertension.

McLaughlin *et al.* (*J. Bone Jt Surg.* 1973, **55A,** 1621) performed simulated hip arthroplasty in dogs, using a cement containing ^{14}C-labelled MM monomer to trace diffusion of the monomer into the circulation. Concentrations of [^{14}C]MM in the blood of the inferior vena cava were maximal 3 minutes after implantation of the cement in the acetabulum and femur and fell gradually over the next 16 minutes. Only about 0·5% of the total amount of implanted monomer was detected in the venous circulation and none appeared in the arterial blood. When labelled monomer was injected iv in doses of 25 or 50 mg/kg into dogs, maximal arterial-blood concentrations were reached in 30 seconds and no monomer was detectable after 3 minutes. After a dose of 75 mg/kg, monomer persisted in the arterial blood for 5 minutes. The lungs appeared to act as a major clearing organ for MM. A decrease in pulmonary function occurred only after injection of a dose equivalent to 35 times the dose of monomer calculated to be absorbed into the circulation during total hip replacement in men.

The reactions produced in dogs by plug implants of acrylic resin in the long bones have been described by Homsy *et al.* (*Clin. Orthop. rel. Res.* 1972, no. 83, p. 317). Two samples of resin mixes, with and without 15% powdered stainless steel or 15% titanium dioxide as an inert heat-absorbing filler, were tested. A leak of monomer into the central venous system, sufficient to produce a maximal concentration of 1·2 mg/100 ml, was detected after implantation within 3 minutes with an implant/body weight ratio of 1·9 g/kg. Similarly, studies in patients undergoing standard femoral-head arthroplasty revealed monomer levels in the central venous system as high as 1·3 mg/100 ml. When monomer was injected iv into dogs, low doses produced hypotension and large ones (125 mg/100 ml) caused respiratory arrest and pulmonary haemorrhage, but no other pathology was detected at autopsy. Transient blood levels below 50 mg MM/100 ml were not found to cause any canine morbidity, and although the actual blood level likely to evoke a toxic response in man cannot be defined, the evidence suggests that it must greatly exceed the level

(estimated at about 1 mg/100 ml) to which a patient undergoing acrylic arthroplasty would be exposed.

Support for this view of an effective dose some 50 times higher than the levels encountered clinically came from a histopathological and haemodynamic study of monomeric MM in dogs reported by Holland *et al.* (*ibid* 1973, no. 90, p. 262). Anaesthetized dogs were given three doses, each of 0·05 ml MM/kg, at 30-minute intervals, the injections being given via the portal vein to one group of three dogs, via the carotid artery to a second group and via the thoracic aorta to a third. The animals were killed for examination 30 minutes after the final injection, having been kept on a respirator in the interim. At autopsy the lungs showed small lobar haemorrhages, more marked after the carotid artery injections than after those by the other two routes. Microscopically, foci of congestion, oedema, intra-alveolar and intrabronchial haemorrhages, necrosis and disruption of the alveolar walls were seen. In the liver there were subcapsular haemorrhages in all three animals given the portal vein injection, with less severe haemorrhages in two of those injected in the thoracic aorta. Acute sinusoidal congestion with scattered haemorrhagic foci and hepatic cell necrosis appeared, especially in the subcapsular region. In the kidneys, lesions were apparent only after injections via the thoracic aorta and comprised widespread microscopically apparent damage to the glomeruli and to some extent to adjacent proximal tubules. The glomerular lesions ranged from acute congestion and focal necrosis to complete necrosis. No damage was observed in the brain, spleen or gastro-intestinal tract.

Hepatotoxic effects of MM have also been demonstrated in mice by Mallory *et al.* (*ibid* 1973, no. 93, p. 366), who administered solutions of 1–20% MM in olive oil by oesophageal instillation of a single dose of 0·1 ml and examined the liver histology of the mice 72 hours later. Dosage concentrations below 6% caused no liver changes, but above this level, the degree of liver damage and the numbers of mice affected were directly related to the dose, concentrations of 15–20% causing massive fatty infiltration with alteration and disruption of liver nuclei in 70–80% of the animals.

Apart from the pathological findings in the dog study reported by Holland *et al.* (*loc. cit.*), marked falls in blood pressure followed each of the injections lasting on average 3, 2 and 0·6 minutes after the first injection into the portal vein, carotid artery and thoracic aorta, respectively, and progressively longer after the second and third injections by each route. The rapid hypotensive response to thoracic aorta injection was followed by a strong rebound hypertensive reaction after the first injection and by a similar but less marked effect after the second.

Higher doses of MM monomer (0·5 ml/kg) were given to dogs in a series of iv injections alternating with controlled incremental haemorrhages (McMaster *et al. loc. cit.*) in connexion with these authors' already mentioned contention that the degree of hypotensive response in orthopaedic surgery could be related to the extent of the blood volume deficit. Selecting this dose as the smallest which gave consistently reproducible blood-pressure responses when administered via the external jugular vein to dogs with a normal blood volume, these authors demonstrated some correlation between the reduction in blood volume and the degree of hypotensive response to the MM injection. The heightened response did not persist beyond a 20% volume depletion however. A similar effect was reported by Berman *et al.* (*Clin. Orthop. rel. Res.* 1974, no. 100, p. 265), who showed that iv injection of MM monomer decreased peripheral resistance and blood pressure both in normal dogs and in those in which the blood volume had been reduced, but the final mean blood pressure was lower and the hypotension was more persistent in the latter group. Moreover, while cardiac output was increased by the MM injection in dogs with a normal blood volume, it was decreased in the hypovolemic dogs.

Other possible factors

The papers reviewed so far in this article have in general supported the contention that whatever conditions may influence the degree and incidence of the hypotensive response sometimes seen in surgery involving the use of acrylic cement, it is the MM monomer which is actually responsible for eliciting this reaction. Indeed, Ellis & Mulvein (*J. Bone Jt Surg.* 1974, **56B**, 59) have reported a study which they consider puts the responsibility for this effect clearly on the MM monomer rather than on any other constituent of the acrylic cement. Dogs were given three successive injections consisting, respectively, of the liquid component of a commercial acrylic resin, pure MM monomer and water (1 ml in each case), the order of the injections being different in each dog and time being allowed between each for the dog's cardiovascular state to return to normal. While the effects of the water injection were negligible, both the test materials reduced mean arterial pressure and increased heart rate and cardiac output to a closely comparable degree. There was therefore no evidence to suggest that minor constituents, accounting together for only about 0·1% of the liquid component of the cement, played any part in the effects produced.

Nevertheless, there are certain inconsistencies in the results obtained both with acrylic cement and with MM monomer injections, even in the same species of experimental animal, and the whole problem is still far from solved. The suggested involvement of air or fat embolism has already been mentioned and certain physical effects of the surgical procedure are also contenders for consideration. The heat generated by the polymerization process received some early attention (Frost, *Br. med. J.* 1970, **3**, 524) but Pelling & Butterworth (*ibid* 1973, **2**, 638) have shown that this is unlikely to play a significant part and that the implantation process itself may be harmful. They studied in rabbits and cats the cardiovascular response to forced insertion of acrylic bone cement, plasticine or soft paraffin wax into the medullary cavity of the femur, the materials, all of similar consistency, being tamped down with a stainless-steel rod. With each substance there was an acute fall in blood pressure within a few seconds of the insertion in both species. In 11% of all the insertions in rabbits, there was a second fall in blood pressure of variable duration usually accompanied by a rise in central venous pressure and a change in respiration rate. Thus, as appears to be the case with the clinical

use of acrylic cement, the cardiovascular response to insertion of material into the femoral cavity is a complex reaction involving various factors, but the hypotensive response in these animals was clearly unrelated to the entry of MM monomer into the circulation, since it was as readily induced by methacrylate-free materials. In this and a related paper (Butterworth & Pelling, *Br. J. Pharmac.* 1973, **48,** 330P), these authors comment on the presence of fat and marrow cells in the circulation and lungs of many of the treated animals and suggest that while the cardiovascular response appears to be produced primarily by a nervous mechanism, the occasional response of longer duration may be attributable to subsequent embolic effects.

A final factor, which should not be overlooked, has been put forward by Bloch *et al.* (*Med. J. Aust.* 1973, **1,** 1037), who have stressed that the greatest threat to the success of total hip replacement is infection. If this occurs it may necessitate very severe salvage operations. Their experiments demonstrated the survival of *Staphylococcus aureus* and *Bacillus subtilis* in samples of MM monomer, thus indicating that MM cannot be regarded as self-sterilizing and that the hazard of infection of the surgical wound must be minimized by carefully controlled asepsis in the operating environment. Another approach to this problem seems to be the incorporation of antibiotics into the acrylic cement. A recent report (Gardner & Medcraft, *Lancet* 1974, **II,** 891) indicates that fucidic acid has shown promise in this connexion, having a negligible effect on the mechanical properties of the cement and leaching out from the structure over a period of time. Further trials on antibiotic-bone cement mixtures are in hand.

The problem of infection was one of the aspects of acrylic-cement surgery examined by the Working Party on Acrylic Cement in Orthopaedic Surgery. Set up in 1971, this group has recently submitted its final report to the Department of Health and Social Security (*Lancet* 1974, **II,** 1002). The report concludes that use of acrylic bone cement in total hip replacements for arthritic cases is not associated with any greater risk of cardiac arrest than other major surgery in that age group, but examines in detail the possible aetiology of the cardiovascular reactions associated with femoral-head replacement. In addition to the factors discussed above, it considers the possible involvement of pharmacologically active substances released from tissues as a result of cytotoxicity or evolution of heat, raised intramedullary pressure and hypersensitivity. It also comments on the lack of reports associating cardiovascular reactions with the *in situ* polymerization of acrylic cement in faciomaxillary surgery. In the absence of any conclusion about the actual cause of the reactions that occur, the report's recommendations for the use of acrylate cements are aimed at eliminating or reducing those factors which may play some part in inducing or exacerbating adverse effects and at encouraging a state of readiness for combatting the effects when they do occur. One awaits with interest the results of further investigations in this field, said to be in progress in at least three centres in the UK.

[P. Cooper—BIBRA]

FLUOROCARBON PROPELLANTS IN PLAY AGAIN

We recently reviewed the fluorocarbon question and found that, in spite of much controversy, these propellants had aquitted themselves relatively well and were expected still to be around at the end of the day (*Cited in F.C.T.* 1974, **12,** 554). However, a new team has entered the field with a set of five papers presenting a somewhat different outlook.

The first of this set deals with cardiac arrhythmia in the mouse—an approach already given a fair share of attention (*ibid* 1974, **12,** 551). Aviado & Belej (*Toxicology* 1974, **2,** 31) have contrived, however, to tackle the problem in a slightly different way. Anaesthetized Swiss mice, weighing 25–35 g, were exposed for 6 minutes to a propellant gas through face masks. A second group was given a challenge dose of 6 μg adrenaline/kg iv 2 minutes after the start of the inhalation of the propellant. Continuous electrocardiograms (ECGs) were recorded. Atrioventricular block or ectopic ventricular beats occurred with six of the 15 gases tested (trichlorofluoromethane (F11) at an atmospheric concentration of 10%, dichloromonofluoromethane (F21) and trichlorotrifluoroethane (F113) each at 10%, vinyl chloride at 20% and methylene chloride and trichloroethane at 40%). The remaining nine gases failed to produce arrhythmias in concentrations up to 40%, or 60% in the case of monochlorodifluoroethane (F142b).

Additional treatment with adrenaline provoked arrhythmias during inhalation of 10% propane, 20% isobutane, dichlorotetrafluoroethane (F114), octafluorocyclobutane (FC-318) or chloropentafluoroethane (F115) or 40% chlorodifluoromethane (F22). F115 also produced ventricular fibrillation. The three remaining propellants (dichlorodifluoromethane (F12), difluoroethane (F152a) and F142b) did not sensitize the heart to adrenaline in concentrations of 40 or 60%. Pretreatment with cholinergic or β-adrenergic blocking agents had no effect on the incidence of arrhythmia, suggesting that the effects of the fluorocarbons were not mediated through these receptors. On the basis of these findings, Aviado & Belej (*loc. cit.*) reclassified these fluorocarbons into three groups according to their ability to cause cardiac arrhythmia in the mouse.

These opening moves were followed by an exercise on the acute effects of fluorocarbons on the respiration and bronchopulmonary system of the rat. Friedman *et al.* (*ibid* 1973, **1,** 345) placed tracheotomized and anaesthetized Mendel–Osborne rats, fitted with pleural space catheters, in whole-body plethysmographs. Pulmonary resistance and compliance, transpulmonary pressure, respiratory rate, air flow, tidal and minute volumes and electrocardiographic data were all recorded. The propellants (seven compounds

in all—F21, F11, F114, F12, F115, FC-318 and isobutane) included representatives of each of the three groups defined by Aviado & Belej (*loc. cit.*) and were administered in increasing concentrations until death occurred.

Lethal concentrations ranged between 19% (F21) and 48% (FC-318). Disappearance of electrical activity from the heart was related to the lethal concentration in most cases, as was bradycardia. F114 and F115 caused transient tachypnoea, while the remaining five propellants appeared to have a slightly depressing effect on respiratory rate. Reduced tidal volume, unrelated in onset to the lethal concentration, was observed with all seven fluorocarbons tested. F12 and F21 did not increase airway resistance, unlike the other five compounds. Effects on pulmonary compliance were related to the tidal volume and pulmonary resistance. Analysis of these results enabled Friedman *et al.* (*loc. cit.*) to regroup their seven fluorocarbons, and to deduce that the cardiac effects of these compounds were not necessarily associated with a lethal concentration. Nevertheless the authors consider the use of F11 and F21 as aerosol propellants inadvisable, both on account of their relatively low lethal concentrations in the rat and because they are more potent than other fluorocarbons in inducing cardiac arrhythmia in the mouse.

Having investigated bronchopulmonary effects in the rat, this group of workers next turned their attention to cardiopulmonary toxicity in the mouse (Brody *et al. ibid* 1974, **2**, 173). The three compounds investigated in this case were F11, which induced cardiac arrhthmia in the mouse and increased pulmonary resistance and decreased pulmonary compliance and tidal volume in the rat, F12, which did not induce arrhythmia in the mouse but decreased pulmonary compliance and tidal volume in the rat, and F152a, which had none of these effects at the concentrations tested. A cannula was inserted in the trachea of each of 96 anaesthetized mice, which were then placed in whole-body plethysmographs. The parameters measured were the velocity of tracheal air flow, transpulmonary pressure, tidal volume and pulmonary resistance and compliance. Metered volumes of propellant were administered directly into the trachea. A second series of respiratory measurements was carried out 24 hours after intratracheal injection of 0·2 mg papain to provoke lung lesions and the mice were subsequently killed and examined histologically. ECGs were examined in other papain-treated animals under fluorocarbon exposure.

Unexpectedly, it was found that far lower concentrations of F11 and F12 (1–2%) were required to increase pulmonary resistance and decrease compliance than were necessary to produce cardiac arrhythmia (10– > 40%). The responses of the mouse respiratory tree to agents (halothane and acetaldehyde) known to provoke a decrease in pulmonary resistance in other species were tested and it was found that while halothane increased pulmonary resistance and decreased pulmonary compliance in the mouse, acetaldehyde had the reverse effects, indicating that mouse lungs respond by increasing or decreasing resistance or compliance. Pretreatment with atropine reduced or blocked the changes in pulmonary resistance caused by F11 and F12, suggesting that the response was mediated by the vagus, but did not affect the decrease in pulmonary compliance. The overt effects of intratracheally administered papain were the functional symptoms of pulmonary congestion and oedema, which were subsequently demonstrated histologically, but the effects of a 4-minute inhalation of 2·5–5% fluorocarbon were not aggravated by the lesions.

Concentrations of 10% F11 were again found to produce spontaneous cardiac arrhythmia, while challenge with 6 μg adrenaline/kg shortened the time of onset and enhanced the reduction in heart rate and depression of the QRS complex. In papain-pretreated animals this enhanced effect appeared without adrenaline challenge, and injection of adrenaline had no additional effect. Exposure to 40% F12 for 4 minutes did not cause arrhythmia although ECG changes were observed, notably a reduction in heart rate and an increase in the height of the QRS complex. The intensity of the heart-rate change was reduced by adrenaline injection or papain pretreatment. Combination of all three treatments produced a conspicuous increase in QRS height. Exposure of papain-treated animals to 40% F152a for 4 minutes caused cardiac arrhythmia, although this was not induced by F152a alone or with an adrenaline challenge.

The relative sensitivity of the three types of response could be assessed from this single experiment. Cardiac arrhythmia appeared with concentrations of 10 or 40%, bronchopulmonary function was affected by about 1–2%, and respiratory tidal volume, rate and minute volume were affected by 2·5–5%. The authors suggest that "these experiments seem to confirm earlier claims that the use of certain propellants to administer bronchodilator drugs in aerosol form to patients with bronchopulmonary disease could conceivably lead to death by cardiac arrest".

Moving on from the mouse, the fourth paper in this series presents an investigation into the cardiotoxicity of 15 propellants in monkeys (Belej *et al. ibid* 1974, **2**, 381). The hearts of anaesthetized monkeys, cannulated for artificial respiration, were exposed and a strain gauge was sutured to the left ventricular surface for measurement of myocardial contraction. ECGs and aortic blood pressure were recorded, the latter by means of a cannula in the femoral artery. F11 was used as a reference compound, monkeys being exposed to 0·5, 1·25, 2·5 and 5% for 5-minute periods alternating with 10 minute exposures to uncontaminated room air. The effects of infusion with 0·5 or 1·0 μg adrenaline/kg/minute during fluorocarbon exposure and/or of myocardial ischaemia due to occlusion of the left anterior descending coronary artery for 20 minutes before propellant administration were studied in some animals.

Treatment with F11 provoked a dose-related fall in aortic blood pressure and depressed myocardial contractility, while the ECG showed a heart-rate acceleration, which was not dose-related. With 5% F11, ventricular premature beats and atrioventricular block appeared in two out of seven animals. Infusion of adrenaline reduced the threshold for the appearance of arrhythmia to 2·5%, and its sympathomimetic effect prevented the appearance of hypotension or reduced myocardial contractility. Occlusion of the coronary artery further reduced the proarrhythmic concentration of F11 to 1·25%. Combination of these

two treatments depressed the minimal proarrhythmic concentration further, to 0·5%, and also provoked a significant depression in myocardial contractility. The remaining 14 propellants were similarly tested, and then classified according to their effects on the primate heart.

F11, F21, F113, trichloroethane and methylene chloride were grouped in Class 1, consisting of propellants causing arrhythmia and myocardial depression with inhaled concentrations of 2·5–5%. Class 2 comprised compounds causing arrhythmia and myocardial depression with inhaled concentrations of 5–10%, namely F114, F12 and isobutane. No arrhythmia but myocardial depression with inhalation of 2·5–10% were characteristics of Class 3, to which vinyl chloride, F22 and F142b belonged. Finally FC-318, F115 and F152a (Class 4) caused no arrhythmia and no myocardial depression in concentrations of 10–20%. When arranged from F11 through to F152a in order of their ability to produce arrhythmia and to depress myocardial contractility, the listing of the compounds is not essentially different from that found in similar studies in the dog (Reinhardt *et al. Archs envir. Hlth* 1971, **22,** 265) and mouse (Aviado & Belej, *loc. cit.*), although the sensitivity of the different species to the various groups differs in degree. While the mechanism of these cardiovascular effects is still uncertain, Belej *et al.* (*loc. cit.*) state that depression of myocardial contractility is a direct effect of fluorocarbon on the heart, as was previously indicated by the action of F12 on rat ventricular muscle *in vitro* (*Cited in F.C.T.* 1974, **12,** 554), while arrhythmia appears to be a combination of a direct effect on the heart and an indirect effect involving the adrenals.

In view of the results of the study in monkeys, it was decided to use canine heart-lung preparations to study six propellants, FC-318, F115, F152a, F11, F113 and trichloroethane (Aviado & Belej, *Toxicology* 1975, **3,** 79). The heart-lung preparations were produced by the classical method of Knowlton & Starling (*J. Physiol.* 1912, **44,** 206) and, in addition, coronary sinus outflow and aortic blood flow (jointly a measure of cardiac output), heart rate and myocardial contractile force (determined with a Walton strain gauge arch) and left atrial pressure were measured during exposure to 2·5, 5·0, 10 or 20% propellant/air mixtures. Cardiac output was controlled by varying the height of the venous reservoir, as there is no functional innervation in this type of preparation, and a ventricular-function curve was derived by raising the reservoir to a series of known heights and plotting against each other the resulting cardiac output and left atrial pressure (filling pressure). A shift to the right indicated depression—a reduction in output for the same filling pressure.

Three Class 1 compounds, F11, F113 and trichloroethane all affected ventricular function in a dose-related manner, although only F11 caused a reduction in myocardial contraction. The remaining three propellants, FC-318, F115 and F152a, were tested at higher concentrations of 10 and 20% instead of 2·5 and 5%. Again all three caused a shift in the ventricular-function curve to the right compared with controls. In addition, 20% FC-318 depressed the force of myocardial contraction, as did 10 and 20% F152a, but F115 had no effect on this parameter at either concentration. It is of note that while not every compound depressed myocardial contractility, all produced a change in the ventricular-function curve. In effect, measurement of function of the whole heart was more informative than determination of the activity of a segment of muscle on the heart surface. The authors sum up their study in the following statement: "At the present time, there is no known propellant for aerosols which does not depress ventricular function in the canine heart". Unfortunately, they add no corollary as to the significance of these results with regard to the human situation, and omit to draw attention to the concentrations to which this comment is relevant.

Other workers (Thompson & Harris, *Toxic. appl. Pharmac.* 1974, **29,** 242), having already observed cardiac arrhythmias, arterial hypotension and direct depression of myocardial contractility due to fluorocarbons, have investigated the time course of the disappearance of some of the cellular effects in the heart after the termination of exposure of cats to the propellant. They felt that sensitization to injected adrenaline, as reported by Reinhardt *et al.* (*loc. cit.*), would be an effective measure of such propellant effects. The experiments were confined to a single fluorocarbon, F12, to which 13 anaesthetized cats were exposed via tracheal cannulae. The femoral vein was catheterized for adrenaline administration and the femoral artery for arterial pressure measurements, and ECGs were recorded. In preliminary work, 0·25–2·0 μg adrenaline/kg injected immediately after the end of a 4-minute inhalation of a mixture of F12, oxygen and nitrogen (25:25:50, by vol.) provoked fatal ventricular fibrillation or severe multifocal tachycardia, so in subsequent experiments, 4 or 8 μg adrenaline/kg was injected 3, 5, 8 or 10 minutes after the end of a 4-minute inhalation of 37% F12. Three or more ventricular premature contractions (VPCs) in 10 seconds were used as the index of myocardial sensitization. Adrenaline alone caused two isolated VPCs or less in unexposed animals. The incidence of ventricular tachyarrhythmias induced by adrenaline varied inversely with the time after the end of F12 inhalation. Measurement of blood concentrations of F12 showed a peak 2 minutes after the beginning of the inhalation and the maintenance of a constant level for the remaining 2 minutes at 11·5 ± 1·75 mg/100 ml. The levels fell to 0·08 ± 0·016 mg/100 ml 10 minutes after cessation of exposure. There were no significant differences between treated and untreated animals in arterial pressure or sino-atrial heart-rate responses to adrenaline.

Thompson & Harris (*loc. cit.*) concede that this method may be an artificial way of detecting cellular effects induced by F12 in the heart. However. it confirmed that sensitization of the heart to the effects of adrenaline continues to a gradually decreasing extent for up to 10 minutes after exposure to 37% F12. While adrenaline contributes to the severity of these arrhythmias, this does not appear to provide any explanation for the falling incidence during the post-exposure period or for the variation in individual susceptibility. It would seem to be of value to determine whether cumulative tissue effects during repeated dosing might contribute to some of the sudden deaths occurring just after cessation of propellant inhalation (*Cited in F.C.T.* 1971, **9,** 730).

It has been claimed (Chiou, *J. Am. med. Ass.* 1974,

227, 658) that the biological half-life of fluorocarbon in man is longer than has usually been thought. The sharp decline in the blood concentration of a drug in the early phase is primarily due to its distribution to other tissues, and biphasic changes in F11 blood levels have been apparent in man after inhalation of the fluorocarbon (Dollery *et al. Lancet* 1970, **II,** 1164). Chiou (*loc. cit.*) suggested that the biological half-life of F11 may be 90 minutes, and he also pointed out that the massive differences in the absorption and elimination of propellants in different patients can complicate the interpretation of available data.

Dollery *et al.* (*Clin. Pharmac. Ther.* 1974, **15,** 59) have since reported further work on blood levels of fluorocarbon after the use of pressurized aerosols for drug administration by asthmatic subjects. Blood samples were taken from eight subjects after one or two puffs of a proprietary inhaler containing salbutamol or isoproterenol, each inhalation coinciding with the early part of a deep inspiration. The samples were capped, kept on ice and then prepared for analysis by gas chromatography using halothane as an internal standard. Blood/gas and blood/muscle partition coefficients were estimated *in vitro*. A computer programme had been written to derive the arterial and myocardial concentrations of a fluorocarbon following any cycle of puffs or breath-holding pattern. Other variables were lung volume, tidal volume, myocardial blood flow and blood/gas and blood/heart muscle partition coefficients. Making certain assumptions regarding the amount of an expelled dose actually inhaled, it was found that the computer model described quite well the clinically observed elimination of F11 and F12, the fluorocarbons tested. Peak arterial levels of F11 determined in the asthmatic patients ranged from 0·53 to 4·5 μg/ml (two puffs) and from 0·26 to 1·9 μg/ml (one puff), while those of F12 ranged from 0·2 to 4·7 μg/ml (two puffs) and from 0 to 2·3 μg/ml (one puff), the peaks occurring in all cases about 10–20 seconds after the last puff. These levels were substantially less than those found to produce adverse effects in dogs (Clarke & Tinston, *Ann. Allergy* 1972. **30,** 536). From the computer model it would appear that to reach the levels of F11 and F12 found to be effective in the dog (20 and 35 μg/ml, respectively), a puff would have to be taken on every breath for 12–24 breaths—a massive overdosage, which may possibly have occurred in some asthmatic subjects desperate for relief. During this study no patient showed any untoward effect.

While the risk of cardiotoxicity is still present for those who use drug aerosols to excess, the outlook for users of domestic aerosols is more cheerful. Marier *et al.* (*Can. med. Ass. J.* 1974, **111,** 39) set out to establish whether normal household use of aerosol products would lead to the production of hazardous levels of fluorocarbon. The use of 13 household aerosols with a known content of propellant (fluorocarbon or hydrocarbon) was monitored for a 4-week period. The aerosols included were a deodorant, hairspray, frying-pan spray, room freshener, insect repellent, insecticide, furniture polish, spot remover, wall cleaner, window cleaner, soil-proofing agent, depilatory and bathroom cleaner. Twenty normal healthy housewives taking part in the study were requested to refrain from using aerosols as far as possible during the fortnight before and the fortnight after the 4-week period, during which a complete diary of use of the sprays was kept and the quantities used were assessed by weighing the containers before and after the test. Each subject was given a full medical examination after week 1 of the pre-exposure abstinence period, at the end of the exposure period and at the end of the second abstinence period. Blood levels of fluorocarbon were assessed at the end of each of the first 6 weeks and at the end of the study.

No significant changes in the general health of these subjects were found during the 4-week period, although the total fluorocarbon exposure during this time was estimated to be as high as 815 g in one case. Studies of heart and lung function, haematology and biochemical parameters were included. The average exposure/subject was 21·6 g fluorocarbon/day. The subjects had been given a prescribed regimen of aerosol use to follow and they emphatically declared that this level was far higher than their normal level of use. Nevertheless, no fluorocarbon was detected in the blood of any user during the study, even though the analytical methods were sensitive down to 0·004 ppm F11, 0·1 ppm F12 and 4 ppm F114. The only effects noted were sneezing and coughing and some skin and eye irritation due to the actual components of the aerosols. Marier *et al.* (*loc. cit.*) feel that very high concentrations of fluorocarbons can be reached only under conditions of abuse, and are satisfied that household use of fluorocarbon-propelled aerosols is unlikely to be hazardous.

While the authors of the first group of papers considered in this review obviously feel that no fluorocarbon has been shown to be without hazard at the levels of exposure used in their animal studies, it would seem important to correlate some of this work with human exposure. Dollery *et al.* (1974, *loc. cit.*) have indicated that very excessive use of drug aerosols could induce cardiac arrhythmia and death and have suggested that the design of aerosol packs could be modified so that an overdose could not be administered. However, with normal use, the benefit of the sprays to patients with bronchial and asthmatic conditions may be inestimable. In the household situation it again appears to be the abuse of fluorocarbon propellants in aerosols that may present some toxic hazard rather than any normal levels of use.

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TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS

PRESERVATIVES

2835. Nitrates fail to depress thyroid function in dogs

Kelley, S. T., Oehme, F. W. & Hoffman, S. B. (1974). Effect of chronic dietary nitrates on canine thyroid function. *Toxic. appl. Pharmac.* **27**, 200.

It is well known that high concentrations of nitrate in drinking-water or in vegetables such as spinach will produce methaemoglobinaemia in infants following its reduction to nitrite (*Cited in F.C.T.* 1965, **3**, 357), and the presence of nitrate or nitrite together with secondary amines in food or drink may sometimes lead to the formation of nitrosamines (*ibid* 1970, **8**, 76). In rats, nitrate may interfere with the iodine-concentrating mechanism of the thyroid and so exert an antithyroid effect (Lee *et al. J. Nutr.* 1970, **100**, 1121).

Kelley *et al.* (cited above) have studied the correlation between a prolonged intake of dietary nitrate and thyroid malfunction in beagle dogs, which were given drinking-water containing 300, 600 or 1000 ppm sodium nitrate. After 1 yr on this regimen, monthly blood samples were taken for 4 months from the adults in each group. The animals were bred within their respective groups and blood samples were also taken from the puppies at the age of 9 wk. No significant differences were demonstrated between the groups either in total serum thyroxine or in the resin sponge uptake of labelled triiodothyronine (T-3 test), and there were no signs of hypothyroidism either in the adults or puppies. Thus, there was no evidence that nitrate in concentrations up to 1000 ppm in the drinking-water consumed for at least 1 yr had any effect on canine thyroid function.

AGRICULTURAL CHEMICALS

2836. Aldrin, dieldrin and endrin as teratogens

Ottolenghi, Anna D., Haseman, J. K. & Suggs, F. (1974). Teratogenic effects of aldrin, dieldrin, and endrin in hamsters and mice. *Teratology* **9**, 11.

To study their effects on pregnancy and embryonic development, single oral doses equivalent to 50% of the LD_{50}s of aldrin, dieldrin and endrin were administered by oral intubation to pregnant hamsters and mice. The doses given to hamsters (on day 7, 8 or 9 of gestation) were 50, 30 and 5 mg/kg and to mice (on day 9) 25, 15 and 2·5 mg/kg for aldrin, dieldrin and endrin, respectively. Such doses did not provoke overt toxic reactions, such as convulsions, respiratory embarrassment, nosebleeds or diarrhoea. The doses were administered in corn oil, and two control groups, one intubated with corn oil and one untreated, were maintained. Laparotomy was performed on day 14 of gestation for hamsters and on day 18 for mice for the counting of dead, live and resorbed foetuses. Surviving foetuses were weighed and then examined and processed for the detection of malformations.

All three compounds markedly increased the incidence of foetal deaths in hamsters treated on day 7 or 8, but in animals treated on day 9 the increase was significant only with dieldrin, which exerted its maximal foetotoxic effect when administered on day 7. The pesticide treatments also caused a significant reduction in foetal weight compared with corn oil-treated controls. Congenital abnormalities among hamster foetuses included cleft palate, open eye and webbed foot. These were associated with all three compounds and often occurred in combination. Of 216 abnormal hamster foetuses, 133 had only one abnormality, 51 had two and 32 had three or more. A further abnormality, fused ribs, appeared as the sole defect in 11 foetuses and was almost exclusively associated with endrin administration. Aldrin, on the other hand, was responsible for most of the cleft lips found, while dieldrin was alone in producing a limited number of foetuses with combinations of exencephaly, platycrania, micrognathia and/or ectrodactyly accompanied by open eye and webbed foot.

In mice, foetal survival and foetal weight were not significantly affected by any of the treatments. Open eye and webbed foot were the most common abnormalities associated with aldrin, and cleft palate and webbed foot with dieldrin. Of 66 abnormal mouse foetuses, 53 had only one anomaly. Mouse foetuses with one or more malformations in the aldrin-, dieldrin- and endrin-treated groups, respectively, comprised 33, 17 and 5% of total live foetuses, compared with 22, 33 and 28% in hamsters given the same treatments on day 8.

The results indicate that, in hamsters, day 8 is the period of greatest sensitivity. The frequent association of webbed foot and open eye with low foetal weight suggests that these structural effects may merely be a reflection of growth retardation.

2837. Chlorinated pesticides through the generations

Adams, Mildred, Coon, F. B. & Poling, C. E. (1974). Insecticides in the tissues of four generations of rats fed different dietary fats containing a mixture of chlorinated hydrocarbon insecticides. *J. agric. Fd Chem.* **22**, 69.

Carnio, J. S. & McQueen, D. J. (1973). Adverse effects of 15 ppm of *p,p'*-DDT on three generations of Japanese quail. *Can. J. Zool.* **51**, 1307.

Most long-term feeding studies of DDT and other

chlorinated hydrocarbon insecticides have tended to concentrate on the possible carcinogenicity of such compounds (*Cited in F.C.T.* 1974, **12,** 764) rather than on their possible effects on reproductive performance and the health of successive generations.

The first paper cited above describes a four-generation experiment. Rats were fed from weaning on a nutritionally adequate diet, 20% of which (38% on a calorie basis) consisted of cottonseed oil, lard, soyabean oil or hydrogenated vegetable-oil shortening, or heated samples of one of the first two, and which contained in each case a mixture of DDT, DDE, DDD, BHC, lindane, dieldrin, heptachlor epoxide, methoxychlor and Perthane. Control groups were given the various dietary fats without added insecticide. Adults of the F_0, F_1 and F_2 generations were killed after the weaning of the second litter and F_3 rats 12 wk after weaning, and tissues were taken for analysis. Representative weanlings from each group were also killed for tissue analysis.

In the control groups, the highest concentrations of DDT, DDE and DDD were found in the body fat of adult rats (at levels of 0·37, 0·48 and 0·30 ppm, respectively), and other tissues generally contained less than 0·10 ppm of residues. Rats fed the insecticide mixture in unheated or heated fats showed higher body-fat concentrations of DDT, DDE and DDD in the F_1 than in the F_0 generation, but in general generation differences were small and showed no continuing increase with succeeding generations. Increased levels of DDT, DDE, DDD and dieldrin (but not BHC and lindane) were stored in the livers and carcasses of weanlings in the insecticide-fed groups and there was some increase in the levels of the DDT-type compounds and dieldrin in the adult livers, but again there was no evidence of persistent enhancement through successive generations. Detectable levels of methoxychlor and Perthane were not found in these tissues, but measurable levels of lindane, BHC and heptachlor epoxides were found in the body fat of some treated rats. The tissue concentrations of the compounds appeared to be little affected by the type of fat used as the vehicle, except in the case of dieldrin, higher tissue concentrations of which occurred in the F_1 generation with the cottonseed-oil diet than with the other fats.

The second paper cited concerns the administration of 15 ppm *p,p'*-DDT in the diet to Japanese quail (*Coturnix coturnix*) over three generations. The first, F_0, generation was fed the test or control diet from the age of 60 days, from which time they were allowed to lay eggs for a 50-day period, those laid between days 37 and 50 being incubated and hatched. The resulting F_1 generation was fed the parental diet from birth, mated at day 60 and allowed to produce eggs for 110 days, the F_2 generation being hatched from eggs laid near the end of this period. Between the first and third generations, egg production diminished slightly, fertility and percentage fertility declined substantially, and the production of abnormal (shell-less and thin-shelled) eggs and eggs with an enhanced DDT content increased. By the F_2 generation, abnormal eggs constituted 23·3% of the total egg production. There was a build-up of both *p,p'*-DDT and DDD over successive quail-chick generations, the residue levels in the F_2-generation eggs being significantly higher than those from the F_0 generation.

These two papers suggest the possibility of a successive build-up of insecticide residues in some animal species though not in others, differences probably being determined by metabolic considerations.

2838. Mutagenic action of ethylene thiourea in bacteria

Seiler, J. P. (1974). Ethylenethiourea (ETU), a carcinogenic and mutagenic metabolite of ethylenebis-dithiocarbamate. *Mutation Res.* **26,** 189.

Ethylenebis(dithiocarbamate) fungicides give rise to ethylene thiourea (ETU) as a metabolite in mammals (Lyman, in *Pesticide Terminal Residues*, edited by A. S. Tahori, Butterworths, London, 1971) and as a decomposition product of certain cooking procedures (*Cited in F.C.T.* 1974, **12,** 779). ETU has been shown to produce hepatomas in mice and thyroid hyperplasia and thyroid cancer in rats, and to be teratogenic in rats (*ibid* 1973, **11,** 702; *ibid* 1974, **12,** 282). Evidence of its mutagenicity is therefore not particularly surprising.

Seiler (cited above) has compared the mutagenicity of ETU with that of related urea and thiourea derivatives in the 'his G-46' strain of *Salmonella typhimurium* grown in agar-tryptone culture. Aminopurine, used as a positive control, had a relative mutagenic activity of >30 (the incidence of spontaneous reversions being represented as 1·0). Compared with this, the indices for comparable concentrations of ETU (2·5), *N*-ethylthiourea (2·0), thiourea (1·0), propyleneurea (1·0), *N*-ethylurea (1·0) and ethyleneurea (0·9) show ETU, with a fairly weak—but significant—level of mutagenic activity, clearly in the lead in relation to the other compounds tested.

Investigation of the dose-response relationship for ETU indicated that whereas ETU in concentrations of 100–1000 ppm exhibited significant mutagenic activity in *S. typhimurium*, higher concentrations (such as 25,000 ppm) killed otherwise viable bateria and so masked any mutagenic effect. The overt mutagenic effect of 10,000 ppm ETU was still significant but was weaker than that of 1000 ppm, the relative mutagenic activity being only 1·6 instead of 2·5.

PROCESSING AND PACKAGING CONTAMINANTS

2839. The biochemical effects of phthalate esters

Daniel, J. W. & Bratt, H. (1974). The absorption, metabolism and tissue distribution of di(2-ethylhexyl) phthalate in rats. *Toxicology* **2,** 51.

Stein, M. S., Caasi, Priscilla I. & Nair, P. P. (1974). Influence of dietary fat and di-2-ethylhexyl phthalate on tissue lipids in rats. *J. Nutr.* **104,** 187.

Much interest has centred recently on phthalate esters, particularly those used widely as plasticizers, and this has been reflected in the number of papers concerned with their toxicity and environmental

occurrence. Attention has also been paid to various aspects of their metabolism and biochemical reactivity (*Cited in F.C.T.* 1975, **13**, 146; Rowland, *Fd Cosmet. Toxicol.* 1974, **12**, 293).

The first paper cited above describes the fate of ^{14}C-labelled di-(2-ethylhexyl) phthalate ([^{14}C]DEHP) given to rats in single oral doses and by repeated daily feeding. After a single dose of 2·9 mg [^{14}C]DEHP/kg, 42% of the radioactivity was excreted in the urine and 57% in the faeces within 7 days, some 14% being excreted by way of the bile. When the animals were fed 1000 ppm unlabelled DEHP daily for 7 days prior to receiving the dose of [^{14}C]DEHP, the 4-day totals for urinary, faecal and biliary excretion of radioactivity accounted, respectively, for 57, 38 and 9% of the administered dose. In rats fed a diet containing 1000 ppm [^{14}C]DEHP continuously for up to 35 days, ^{14}C activity in the liver attained within about 1 wk the equivalent of 40–50 ppm DEHP, while the level in the abdominal fat reached 7–9 ppm within 2 wk. After daily feeding of 5000 ppm [^{14}C]DEHP, equilibrium levels in liver and fat were reached by days 9–14 and averaged about 120 and 80 ppm respectively. Brain contained 2–3 ppm and heart 15–20 ppm DEHP. In rats maintained on this diet for 49 days and then given normal diet, liver radioactivity declined with a half-life of 1–2 days while that in fat declined rather more slowly. During wk 1 of feeding with 5000 ppm [^{14}C]DEHP, the relative liver weight rose to some 50% above normal and thereafter remained constant throughout treatment, reverting to normal 1 wk after the end of the feeding period. Electron microscopy of the liver revealed only a slight increase in smooth endoplasmic reticulum. After five daily doses of 500 mg DEHP/kg, hexobarbitone sleeping time was reduced in male and female rats by 39 and 34% respectively. Intravenous infusion of [^{14}C]DEHP was followed by its rapid disappearance from the blood and retention of 60–70% of the dose of radioactivity in the liver and lung during the first 2 hr; a subsequent fall of some 50% occurred in the concentration in these organs during the next 22 hr. During this time, 32–58% of the ^{14}C activity was recovered from the urine and 20–38% from the faeces. Studies on the metabolic products of orally administered DEHP present in the fat, liver and urine identified the main metabolites as the acid, alcohol and ketone resulting from ω- and (ω-1)-oxidation of mono-(2-ethylhexyl) phthalate, itself the product of DEHP hydrolysis by pancreatic lipase, and thus supported and extended other recent studies (*Cited in F.C.T.* 1975, **13**, 146).

The second paper cited describes the feeding to rats of a fat-free diet supplemented with either 0·1% DEHP or 4% stripped lard, or both, for 44 days. DEHP feeding increased liver weight whether or not fat was given at the same time. In the absence of fat, DEHP caused a slight but not statistically significant reduction in body-weight gain, whereas when fat and DEHP were fed simultaneously, the growth-promoting effect of the fat appeared to be potentiated. A similar interaction between dietary fat and DEHP was indicated in the recorded weights of the epididymal fat pad. Accumulation of DEHP was evident in the heart and epididymal fat pad, but not in the liver (presumably because of metabolism) but when fat was given together with DEHP the total lipid content of the liver was increased.

This study suggests that while the independent effects of DEHP and dietary fat tend to be antagonistic, the two materials are capable of interacting when consumed simultaneously, so that dietary DEHP should be considered as having a potential influence on lipid metabolism.

THE CHEMICAL ENVIRONMENT

2840. Life with nickel

Schroeder, H. A., Mitchener, Marian & Nason, A. P. (1974). Life-term effects of nickel in rats: Survival, tumors, interactions with trace elements and tissue levels. *J. Nutr.* **104**, 239.

It has been suggested that nickel (Ni) may be an essential element for young animals (*Cited in F.C.T.* 1972, **10**, 595). The paper cited above describes the results of the life-time administration of 5 ppm Ni in the drinking-water of 104 rats from weaning until their natural death 2–4 yr later. At the same time, possible interactions between Ni and other essential metals were studied. The drinking-water of both the Ni-treated rats and the controls also contained 50 ppm zinc (Zn), 10 ppm manganese (Mn), 5 ppm copper (Cu), 5 ppm chromium (Cr), 1 ppm cobalt and 1 ppm molybdenum.

Consumption of Ni increased growth rates in the early part of the study but did not affect survival, longevity, the incidence of tumours, or the frequency of occurrence of specific pathological lesions. It increased the concentrations of Cr in the heart and spleen and of Mn in the kidneys, and decreased the Zn concentration in the lung, the Mn concentration in the spleen and the Cu concentrations in both of these organs. There was no evidence of any tissue accumulation of Ni. No effect was observed on the uric acid concentration in serum. Three findings in this study consistent with the concept of Ni as an essential trace metal in rats were its enhancement of growth, its lack of toxicity and its failure to accumulate in tissues, but these indirect indications cannot be said to establish the essentiality of the metal.

2841. Dealing with inorganic tin

Hiles, R. A. (1974). Absorption, distribution and excretion of inorganic tin in rats. *Toxic. appl. Pharmac.* **27**, 366.

The biological fate of tin (Sn) after single oral, multiple oral and single iv doses of stannous (Sn^{II}) and stannic (Sn^{IV}) fluoride, Sn^{II} and Sn^{IV} citrate and Sn^{II} pyrophosphate ($Sn_2P_2O_7$) was studied in rats by administration of compounds labelled with ^{113}Sn. After single oral doses, equivalent to 20 mg Sn/kg, 98·6–99·7% of the label was recovered in the faeces, with 1% or less appearing in the urine. The small

amounts of ^{113}Sn retained in the body 48 hr after dosing were mainly in the liver, kidneys and skeleton. With Sn^{II} compounds significantly more ^{113}Sn appeared in the urine and tissues than with Sn^{IV}. Distribution did not differ significantly between fluoride and citrate compounds, but with pyrophosphate there were lower concentrations of ^{113}Sn in the urine and most tissues than with the corresponding fluoride or citrate. After injection of 2 mg Sn/kg iv as Sn^{II} or Sn^{IV} citrate, some 30% of the dose of radioactivity was excreted in the urine. Biliary excretion accounted for 11% of Sn^{II} but for none of the Sn^{IV}. After daily oral dosage for 28 days, the average percentage of the dose absorbed was less than that from a single oral dose, but bone showed an increased ^{113}Sn accumulation roughly proportional to the increased level of exposure. The calculated half-life of ^{113}Sn in bone was 20–40 days. Foetuses from rats given Sn orally throughout gestation showed no significant concentration of the metal.

Thus the low toxicological hazard of tin in inorganic forms can be attributed to its low absorption, low tissue accumulation and rapid turnover in the tissues.

2842. Complicating the retention picture of inspired vapours

Egle, J. L., Jr. (1973). Retention of inhaled acetone and ammonia in the dog. *Am. ind. Hyg. Ass. J.* **34,** 533.

The absorption and excretion of acetone in man and dogs exposed to considerable atmospheric concentrations (100–1000 ppm) of the vapour have been described (*Cited in F.C.T.* 1975, **13,** 151). Since in smaller concentrations both acetone and ammonia feature with aldehydes among the constituents of the gaseous phase of tobacco smoke, a study of the possible interaction between these compounds is of interest. It has been estimated that the vapour phase of cigarette smoke contains some 42 μg acetone and 12 μg ammonia in each 40-ml puff.

Dogs under pentobarbitone anaesthesia were allowed spontaneously to inhale air containing acetone (0·36–0·80 μg/ml), ammonia (0·15–0·50 μg/ml), acetone plus acetaldehyde (0·50 and 0·40–0·70 μg/ml, respectively) or ammonia plus acetaldehyde (0·40 and 0·40–0·70 μg/ml, respectively). The uptake of acetone by the whole respiratory tract was around 52%, while that of ammonia was some 25% higher. In both lower and upper respiratory tracts the retention of ammonia was greater than that of acetone when each was inhaled alone. At all the ventilatory rates studied (5–40/min), the retention of acetaldehyde was increased by addition of acetone; but with the simultaneous inhalation of ammonia, acetaldehyde retention decreased at lower and increased at higher respiratory rates.

The moral of these findings is that when several different compounds are inhaled at the same time, retention of one of them may be altered by the presence of another.

2843. Acrylamide neuropathy revealed

Thomann, P., Koella, W. P., Krinke, G., Petermann, H., Zak, F. & Hess, R. (1974). The assessment of peripheral neurotoxicity in dogs: Comparative studies with acrylamide and clioquinol. *Agents & Actions* **4,** 47.

Monomeric acrylamide has been shown to cause peripheral neuropathy in man (*Cited in F.C.T.* 1971, **9,** 912) and various animal species, including the rat (*ibid* 1974, **12,** 579). The paper cited above provides details of its effect in beagles and the results of a comparative study on clioquinol (iodochloro-8-hydroxyquinoline) in the same species are presented as evidence that clioquinol offers no significant hazard of peripheral neuropathy.

Two groups of dogs were fed acrylamide in gelatin capsules on 7 days/wk in daily doses of 5 mg/kg for 60 days or 15 mg/kg for 22 days. Animals on the lower dose showed signs of sedation, jaw-muscle weakness and ataxia after the first 3 wk. Those on the higher dose showed in addition mydriasis (pupil dilation), conjunctivitis, salivation, laboured breathing, spasticity and stiffness of the hind limbs, muscle twitching and convulsions. Towards the end of the treatment period their appetite and general condition were poor. Hyperreflexia of stretch reflexes persisted for 10 days after withdrawal of the higher dose, but were normal after a 30-day recovery period. Impairment of nerve function was demonstrated in the saphenous nerve after the high-level dosing, conduction velocity being most reduced with less marked changes in chronaxy and the absolute refractory period. Only the latter parameter was affected in dogs given 5 mg/kg/day. At autopsy, the dogs given 15 mg/kg/day showed a suppurative lobular pneumonia, which resolved to some extent in those kept alive for 30 days after cessation of treatment. Fragmentation and swelling of the myelin sheaths and axons of peripheral nerves were associated with both levels of treatment. These lesions were most marked in those dogs on the higher dose level allowed the longest recovery period but were not evident in those killed immediately on termination of the high-level dosing. Distal branches of the sciatic nerve were generally more severely affected than were proximal branches.

In contrast, clioquinol administered similarly in capsules in doses of 30, 100 or 200 mg/kg/day for 12 months produced no significant neurological abnormalities of function or structure.

2844. Support for bithionol

Powell, H. C. & Lampert. P. W. (1974). Bithionol: A possible substitute for hexachlorophene. *Pediatrics, Springfield* **52,** 859.

The possibility that the topical application of strong preparations of hexachlorophene (HCP) to young infants soon after birth may lead to damage to the central nervous system has been widely discussed (*Cited in F.C.T.* 1974, **12,** 563). The present report considers an alternative antibacterial believed not to have the same adverse effects.

The central nervous lesion produced by HCP is a type of cerebral oedema characterized by separation of myelin lamellae and resulting vacuolation of the myelin sheath (*ibid* 1974, **12,** 564). Bithionol (BI), another antibacterial agent, was investigated to determine whether it was capable of producing these lesions in adult mice. This study was undertaken initially because animals poisoned with cuprizone and

isonicotinic acid have been found to have nervous-tissue lesions similar to those caused by HCP, and the common factor between all these three chemicals and BI is their ability to chelate copper. Three groups of ten adult female Swiss-Webster mice were given 1000 ppm HCP or 1000 or 2000 ppm BI in the diet for 2 wk, and were then decapitated. Brains were fixed in glutaraldehyde and examined microscopically.

A reduction in body weight, lethargy and hind-limb paresis were noted in the group receiving HCP, and the characteristic spongiform changes in the brain tissue occurred. No such effects were observed in the BI-treated groups, so that no support was obtained for an association between this type of cerebral lesion and a compound's copper-chelating properties. On the basis of the negative findings, however, the authors recommend that BI should be considered for use as a substitute for HCP under certain clinical conditions. Brief mention is given to the photosensitizing properties of BI [the severity of which resulted in an FDA ban on its use in cosmetics (Code of Federal Regulations, Title 21, Chapter I, Sec. 3.60)] and it is suggested that: "Future topical use would require preparations incorporating photoprotective agents, and shielding of patients from sensitizing light sources".

[Are we now to believe that infants should spend their first days of life hidden from the light? This aspect apart, the study reported here does not seem in itself to be comprehensive enough or to augment previous knowledge sufficiently to justify a recommendation for the possible use of bithionol as a replacement for hexachlorophene.]

2845. Ethylene oxide cyclic tetramer and spermatogenesis

Leong, B. K. J., Ts'o, T. O. T. & Chenoweth, M. B. (1974). Testicular atrophy from inhalation of ethylene oxide cyclic tetramer. *Toxic. appl. Pharmac.* **27,** 342.

The cyclic tetramer of ethylene oxide (1,4,7,10-tetraoxacyclododecane; EOCT) has metal-chelating properties, which are reflected in its industrial use. Preliminary observations in rats exposed to EOCT levels in air between 1·2 and 63·8 ppm for 6 hr/day for up to 8 days showed that inhalation led to extreme anorexia, loss of weight, debility, hindquarter incoordination, testicular atrophy, auditory hypersensitivity, tremors and convulsions and sometimes death. Survivors recovered slowly from all the effects except the testicular atrophy, which persisted for 4 months and prompted the further investigations described in this paper.

Male Sprague–Dawley rats were exposed to 0·5 or 1·0 ppm EOCT vapour for 7 hr daily on 5 days/wk for 3 wk. In both groups, marked testicular atrophy and degeneration of the germinal epithelium occurred and the atrophy persisted for 4 months after cessation of exposure. During this time the rats showed normal sexual activity but remained sterile. Examination immediately after EOCT exposure revealed atrophy of the prostate gland and seminal vesicles, but the appearance and size of these organs reverted towards normal during the following 2–3 wk. Injection of testosterone in daily ip doses of 10 mg/kg for 1 wk before and throughout the EOCT exposure effectively prevented the atrophy of the prostate and seminal vesicles but not that of the testes. In addition to these effects, 1 ppm EOCT depressed conditioned behavioural responses and food and water consumption, retarded growth and induced tremors. With the 0·5 ppm exposure such effects were milder, and in both cases they were reversible.

It seems likely that the effect of EOCT on the testes involves the chelation of trace metals, such as zinc and manganese, which are essential for spermatogenesis, but this is at present an unproven hypothesis.

2846. Haloether formation from formaldehyde not a working problem

Kallos, G. J. & Solomon, R. A. (1973). Investigations of the formation of bis-chloromethyl ether in simulated hydrogen chloride-formaldehyde atmospheric environments. *Am. ind. Hyg. Ass. J.* **34,** 469.

The reported carcinogenicity of bis-chloromethyl ether (BCME) in rats and mice has raised the urgent question of whether hazardous concentrations of BCME can be formed by interaction between formaldehyde (HCHO) and hydrogen chloride (HCl) in moist air in working environments (*Cited in F.C.T.* 1974, **12,** 552). Using a method of BCME determination which was both specific for this ether and sensitive to a limit of 0·1 ppb ($b = 10^9$) for a sample of 15 litres of air, the authors of the paper cited above studied the vapour-phase reaction between HCHO and HCl at different concentrations in glass or plastics (Saran) containers at room temperature.

The combination of HCHO and HCl in concentrations above their threshold limit values (e.g. of 100 or 200 ppm of each) in a 22-litre glass flask or a 10-litre plastics bag at a relative humidity of 40–60% did not produce detectable levels of BCME during periods exceeding 14 hr. With concentrations of 500–3000 ppm of each compound, BCME concentrations of only a few ppb resulted from a similarly prolonged reaction. These findings indicate that the reaction between HCHO and HCl does not present health problems to workers in chemical plants, since even at concentrations beyond those which can be tolerated by man, these compounds do not produce significant quantities of BCME.

2847. A look back at chloromethyl methyl ether

Beavers, E. M. (1974). Lung cancer in chloromethyl methyl ether workers. *New Engl. J. Med.* **290,** 971.

In an article published during last year (*Cited in F.C.T.* 1974, **12,** 551), brief reference was made to a paper by Figueroa *et al.* (*New Engl. J. Med.* 1973, **288,** 1096), who reported a roughly eightfold enhancement of lung-cancer incidence among men exposed to chloromethyl methyl ether (CMME) at a chemical plant.

This assessment has since been disputed in the letter cited above, in which a representative of the company concerned claims that the data used were inadequate and in many cases inaccurate. It is stated that many more workers were exposed to CMME in the plant than was originally indicated and that statistical analysis of the data up to the end of 1970 shows no significant difference in lung-cancer incidence

between employees at the plant and Philadelphia residents as a whole in any age group except in that covering 45–54 yr. In this group the incidence was higher among the plant workers, but the increase over the general population was said to have been roughly constant throughout the period 1954–1970, whereas no employees in this group who died of lung cancer could be shown to have been at risk of exposure to CMME before 1960. All the deaths from lung cancer in that group were stated to have occurred in heavy smokers living in heavily industrialized areas of the city.

These arguments are, in turn, largely rejected by the original group (Figueroa & Weiss, *New Engl. J. Med.* 1974, **290**, 972), so that the question cannot be considered settled. It seems, however, that the statement reported in our previous article should not be accepted without reservation.

2848. Teratogenic studies on chloroform

Thompson, D. J., Warner, S. D. & Robinson, V. B. (1974). Teratology studies on orally administered chloroform in the rat and rabbit. *Toxic. appl. Pharmac.* **29**, 348.

Schwetz, B. A., Leong, B. K. J. & Gehring, P. J. (1974). Embryo- and fetotoxicity of inhaled chloroform in rats. *Toxic. appl. Pharmac.* **28**, 442.

Because chloroform is widely used as an industrial solvent and chemical intermediate and was at one time an important inhalation anaesthetic, studies of its toxicity have been predominantly concerned with the inhalation and parenteral routes of administration. Nevertheless, chloroform is used in various oral medications, often as a preservative, and some studies on the effects of oral ingestion have been published. Acute oral LD_{50} values in the region of 1 g/kg have been reported (Kimura *et al. Toxic. appl. Pharmac.* 1971, **19**, 699), for example, and the relationship between diet and the oral toxicity of chloroform has been studied (McLean, *Br. J. exp. Path.* 1970, **51**, 317; McLean & McLean, *Br. med. Bull.* 1969, **25**, 278).

The effect of orally administered chloroform on embryonic and foetal development in the rat and rabbit has now been reported (first paper cited above). Rats were given total doses of 0, 20, 50 or 126 mg/kg/day in corn oil on days 6–15 of gestation and rabbits received 0, 20, 35 or 50 mg/kg/day in corn oil on days 6–18. Doses were given by gavage, the total daily dose for the rats being administered in two equal amounts given about 7 hr apart. In both species, the higher dosage levels caused overt signs of toxicity in the adult, including a reduction in food consumption and body weight and some evidence of hepatotoxicity. Moreover, a reduction in foetal weights in litters from rats given 126 mg/kg/day and in those from rabbits given 20 or 50 mg/kg/day was indicative of some degree of foetotoxicity. However, none of the dose levels administered was embryocidal or teratogenic in either species.

This finding contrasted with the results of a study (second paper cited above) in which rats were exposed to an atmosphere containing 30, 100 or 300 ppm chloroform for 7 hr/day on days 6–15 of gestation. Exposure to chloroform in this way showed some maternal toxicity, particularly at the highest level, but the evidence of embryo- and foetotoxicity was much more striking. Exposure to 300 ppm caused a significant increase in the incidence of foetal resorptions, a decrease in foetal weight and length and a reduction in conception rate (15% compared with 88% in controls). The incidence of resorptions was dose-related, as was an increase in the incidence of anomalies of the vertebrae and sternebrae and in the occurrence of subcutaneous oedema, all of which may be considered as indicators of retarded foetal development. In addition, actual malformations (acaudia and imperforate anus) were found in three of the 23 litters produced by rats exposed to 100 ppm chloroform but did not occur in other groups.

Inhaled chloroform, while showing only slight evidence of teratogenicity was thus shown to be markedly embryotoxic, whereas the effect of chloroform administered orally was limited to a mild foetotoxicity. This suggests that the maternal blood levels of chloroform in the inhalation study were considerably higher than in the oral study, as might be expected, but no data are available on this point.

2849. The fate of inhaled methylchloroform

Eben, Anneliese & Kimmerle, G. (1974). Metabolism, excretion and toxicology of methylchloroform in acute and subacute exposed rats. *Arch. Tox.* **31**, 233.

Methylchloroform (1,1,1-trichloroethane; MC) is known to have some neurological effects in man (*Cited in F.C.T.* 1972, **10**, 273) although it is widely used as a relatively safe alternative to other trichlorinated solvents, so further information on its fate in the body does not come amiss. The paper cited above describes effects of exposing rats to an atmosphere containing 220 or 440 ppm MC for 4 hr. Urine samples were collected over the following 3–4 days for determination of trichloroethanol (TCE) and trichloroacetic acid (TCA). A further group of rats was exposed to 204 ppm MC for 8 hr daily for 5 days/wk for 3 months, and similar urine analyses and certain other determinations were carried out.

In most animals exposed to 220 or 440 ppm MC, most of the TCE produced appeared in the urine within 24 hr. Excretion of both TCE and TCA was approximately dose-dependent. In the subacute trial, urinary levels of TCE increased continuously to a maximum between days 55 and 65 and decreased thereafter. TCA concentration in the urine rose to about 20 μg/24 hr within the first few days and then remained fairly constant. Mean TCE excretion rose from about 93 μg/24 hr in wk 1 to 435 μg/24 hr in wk 10, with the daily TCA excretion varying from 12–20 μg. With increasing TCE concentrations in the urine, the TCE:TCA ratio rose from 4·6 early in the trial to 15–20 later. In the blood, levels of MC and TCE remained almost constant throughout the study and chloral hydrate could not be detected. No MC was detected in the fat, brain, liver, kidneys, heart or spleen. During the exposure period no behavioural differences and no differences from controls in respect of appearance and body-weight gain were observed. Haematology, tests for hepatic and renal function and blood-glucose levels remained normal. At autopsy no histological lesions were found.

This study thus lends support to previous indications of the relatively innocuous nature of MC under

the types of condition in which it is most likely to be used as an industrial solvent.

2850. MBK-induced polyneuropathy

Mendell, J. R., Saida, K., Ganansia, M. F., Jackson, D. B., Weiss, H., Gardier, R. W., Chrisman, C., Allen, N., Couri, D., O'Neill, J., Marks, B. & Hetland, L. (1974). Toxic polyneuropathy produced by methyl N-butyl ketone. *Science, N.Y.* **185,** 787.

Further information is now available on the suggested association between industrial exposure to methyl *n*-butyl ketone (MBK) and the development of peripheral neuropathy, a comment on which appeared in a recent issue (*Cited in F.C.T.* 1975, **13,** 290).

Following the diagnosis (in June 1973) of a polyneuropathy in a worker from a plant manufacturing plastics-coated and colour-printed fabrics, an extensive screening programme covering 1161 workers at the plant revealed clinical evidence of polyneuropathy in 79 and abnormal electromyograms in 182. Severely affected individuals showed distal muscle weakness and loss of deep tendon reflexes in both upper and lower extremities, while sensory impairment involved loss of sensitivity to superficial pain, temperature change, light touch and vibrations in the feet and fingers. There were strong indications that these conditions were associated with exposure to MBK, which had been used as a dye solvent and cleaning agent in the plant since the latter half of 1972. No cases of polyneuropathy occurred before December 1972, and attack rates seemed to correlate with factory areas of high MBK usage and increasing hours of exposure.

Small groups of chickens, rats and cats were therefore exposed for 24 hr/day on 7 days/wk to an MBK-contaminated atmosphere. Chicks were exposed initially to 200 ppm and rats and cats to 600 ppm, but these levels were subsequently reduced to 100 and 400 ppm respectively, to minimize complications from inanition and weight loss. All the exposed animals developed peripheral neuropathy, overt signs of muscular weakness appearing at wk 4–5 of treatment in chicks, at wk 5–8 in cats and at wk 11–12 in rats. Abnormal electromyograms were obtained in cats from wk 4–6, the main findings being those seen also in the workers studied, namely positive waves, slowing of nerve conduction velocity and fibrillation potentials. The histological changes were similar in all three species and consisted of focal, often abrupt, axonal swelling surrounded by an abnormally thin myelin sheath, located either along the nerve fibre or close to the nodes of Ranvier. Near the latter points were demyelinated areas of varying length, and changes in the axons (accumulation of neurofilaments and loss of neurotubules) occurred at the sites of axonal swelling. Some areas of myelin denudation were found without any associated axonal swelling.

So far it has not been possible to compare these histological findings with those in nerve biopsy material from the affected workers, but they resemble the changes associated with certain other forms of polyneuropathy. Moreover, it has been calculated that the number of hours of exposure preceding the development of clinical neuropathy was closely comparable in the workers and experimental animals, and the clinical signs of neuropathy and the major electromyographic abnormalities were similar in the men and animals.

The authors of this paper question whether the recommended threshold limit value of 100 ppm for MBK provides an adequate margin of safety.

2851. More favourable data on hydrogenated terphenyls

Adamson, I. Y. R. & Furlong, Judith M. (1974). The fate of inhaled and ingested tritiated terphenyls in mice. *Archs envir. Hlth* **28,** 155.

Previous experiments have shown that HB-40, a hydrogenated terphenyl mixture used as a nuclear-reactor coolant, is toxic after ingestion only in very heavy doses unlikely to be encountered in practice (*Cited in F.C.T.* 1974, **12,** 794). Inhalation of concentrations well above those encountered in industrial atmospheres has been shown in mice to be offset by cell renewal in the lung and adaptation in the liver (*ibid* 1974, **12,** 284).

The work cited above was designed to study the tissue deposition and clearance of tritiated HB-40 following its inhalation or ingestion by mice. No accumulation of radioactivity from an HB-40 aerosol containing particle sizes ranging from 1 to 20 μ occurred after five successive daily 4- or 7-hr exposures of the animals to an aerosol concentration of 2×10^{-3} g/litre in air. Immediately after the inhalation periods, radioactivity in the gut was significantly increased, but it returned to the control value by the next day. No significant changes could be detected in liver or kidney scintillation counts, and lung counts were only slightly and transitorily increased. Peak incorporation of labelled material into gut, liver and kidney occurred 4–5 hr after ingestion of HB-40 and had fallen considerably within 24 hr. After 1 wk, no significant radioactivity could be detected in these organs. Autoradiographic studies 4–6 hr after ingestion indicated a low tissue uptake of HB-40, with random distribution in the gut epithelium, a diffuse scatter throughout the liver cells and a distribution in the kidney directed towards the tubules rather than the glomeruli. The latter finding ties in with the occurrence of tubular necrosis following prolonged experimental ingestion of terphenyl coolants (*ibid* 1974, **12,** 794). No excess of activity over background levels was detectable by autoradiography after 24 hr.

Thus the picture of HB-40 remains one of a compound of low toxicity which does not remain long in the body after inhalation or ingestion.

2852. Dermatitis from stearyl alcohol and propylene glycol

Shore, R. N. & Shelley, W. B. (1974). Contact dermatitis from stearyl alcohol and propylene glycol in fluocinonide cream. *Archs Derm.* **109,** 397.

Stearyl alcohol has been implicated in the occasional case of contact dermatitis (*Cited in F.C.T.* 1970, **8,** 239), and propylene glycol, widely used as a solvent of very low toxicity, has been shown to produce a minimal degree of direct irritation when applied to the skin of rabbits, piglets and man (*ibid* 1973, **11,** 346).

In a case now reported, these two compounds were ingredients of a cream preparation of the anti-inflammatory corticosteroid, fluocinonide. Despite the presence of this corticosteroid, a severe contact dermatitis developed in a young woman who was being treated for linear scleroderma by daily topical application of the fluocinonide cream in conjunction, after a time, with Saran-film occlusion of the treated areas at night. She developed reactions only at the sites that were occluded, no response being seen when treatment was restricted to open application. Closed-patch tests with the cream formulation were strongly positive, but tests with a fluocinonide ointment were negative. Treatment was continued with this ointment without ill effect. Patch testing with ingredients of the cream showed that the patient was sensitive to both commercial stearyl alcohol and propylene glycol. Since the latter was present in both the cream and ointment, further tests were carried out and these indicated that the effect of propylene glycol was one of primary irritancy, which occurred with the 60% level in the cream or with a 10% concentration in water but not with the 5·7% present in the ointment. High-grade stearyl alcohol did not produce a positive patch-test reaction but the component of commerical stearyl alcohol responsible for the dermatitis was not isolated. Among the 3–7% impurities in the commercial material were oleyl, palmityl and other alcohols, together with bound solvent, such as acetone, petroleum ether or thiophene. Tests with the cream base alone produced a stronger positive reaction than those with the formulated product, thus confirming the ability of fluocinonide to moderate the inflammation, although it could not mask the marked allergic reaction to the stearyl alcohol impurities when this was enhanced by the propylene glycol irritation and the increased skin absorption promoted by occlusion.

2853. Toxicology of triethylene glycol dinitrate

Andersen, M. E. & Mehl, R. G. (1973). A comparison of the toxicology of triethylene glycol dinitrate and propylene glycol dinitrate. *Am. ind. Hyg. Ass. J.* **34,** 526.

Ethylene glycol dinitrate, used in antifreeze dynamite, has been reported to cause sudden fatal circulatory collapse of workers in manufacturing plants. Propylene glycol dinitrate (PGDN) has been suggested as a safer replacement, although it is liable to produce severe debilitating headache, vasodilatation, hypotension and methaemoglobinaemia (*Cited in F.C.T.* 1970, **8,** 111; *ibid* 1973, **11,** 1152). The toxicity of another dinitrate, triethylene glycol dinitrate (TEGDN) has now been studied and compared with that of PGDN. Both compounds were stabilized with an inert diluent of low volatility and tested by various routes in rats, guinea-pigs, rabbits and mice as well as *in vitro* on isolated rat phrenic nerve–diaphragm preparations.

The ip 24-hr LD_{50}s of TEGDN and PGDN, respectively, in males were 945 and 1047 mg/kg for mice, 700 and 402 mg/kg for guinea-pigs and 796 and 479 mg/kg for rats, while the oral and sc LD_{50}s of the two compounds in rats were 1000 and 250 mg/kg and 2520 and 530 mg/kg respectively. Both dinitrates caused methaemoglobinaemia, but TEGDN acted less rapidly than PGDN. Lethal doses of PGDN produced ataxia, lethargy, respiratory depression and severe methaemoglobinaemia. Lethal doses of TEGDN caused, in addition, tremor and excessive sensitivity to auditory and tactile stimuli. Terminal methaemoglobin levels in these rats were 30–40% after TEGDN treatment and 70–85% after PGDN. Rats surviving an ip LD_{50} of either compound were examined for changes in plasma-enzyme activities. TEGDN produced sustained increases in all the enzymes studied (aspartate aminotransferase, lactic dehydrogenase, creatine kinase and alkaline phosphatase), whereas PGDN produced moderate increases only in the last two of these.

In the nerve-muscle preparation, concentrations above 1·5 mM-TEGDN blocked nerve-stimulated contraction but did not affect the response to direct stimulation of the muscle. Daily dermal applications of either compound in a dose of 21 mmols/kg for 2–3 wk were largely fatal to rabbits, the mean time to death being 16–17 days in both cases. However, the PGDN-treated rabbits gained weight during treatment and appeared normal until just before death, whereas those given TEGDN lost about 20% of their starting weight during treatment. A study of food consumption in guinea-pigs given TEGDN in ip doses of 100–400 mg/kg/day for 15 days indicated that even the lowest dose reduced food intake and retarded weight gain to some extent, while the higher doses had a more marked effect.

2854. Another hazard of glue sniffing

Taher, S. M., Anderson, R. J., McCartney, R., Popovtzer, M. M. & Schrier, R. W. (1974). Renal tubular acidosis associated with toluene "sniffing". *New Engl. J. Med.* **290,** 765.

We recently reported two cases of polyneuropathy in young men who had taken to sniffing hexane–toluene vapours from a plastics-bonding glue (*Cited in F.C.T.* 1975, **13,** 157). The present paper describes two further cases involving toluene sniffing and showing a previously unrecorded complication.

One subject, a 23-yr-old Mexican, was admitted to hospital with generalized paralysis following toluene sniffing. Standard biochemical studies showed a reduced level of serum potassium, and treatment with 400 mequiv potassium chloride over 36 hr restored normal muscle strength. Hypokalaemia and hyperchloraemic metabolic acidosis were found on the occasion of subsequent admissions for muscle weakness after toluene sniffing. On each occasion, potassium replacement produced a dramatic restoration of muscle strength and correction of the serum-electrolyte balance. A similar hypokalaemic acidosis was found in a 20-yr-old American Indian girl who initially reported because of nausea after sniffing aerosol paint containing 60·4% toluene for 3–5 days. Treatment with potassium chloride again led to recovery. Re-evaluation of both subjects after a symptom-free, non-sniffing period showed normal clinical values.

Kidney involvement has not previously been associated to any significant extent with toluene inhalation. Microscopic pyuria, haematuria and proteinuria have been noted in some toluene sniffers, but others engaged in this pursuit have been found to have normal urinary sediments. However, it is possible that some cases may have been complicated by the abuse

of other solvents. In the two cases now reported, high toluene levels in the blood were consistently associated with metabolic acidosis, resulting primarily from impairment of renal tubular acidification. The biochemical investigations carried out by these authors suggested that toluene affects the generation of a hydrogen-ion gradient by the distal tubule leading to classic (type I) renal tubular acidosis. This appears to be completely reversible when toluene sniffing terminates, and no after-effects have been observed.

Unfortunately, since previous reports of toluene intoxication have not included measurement of serum electrolytes and urinary acidification in animals or man, it is not possible to determine the extent to which this complication occurs. The increasing incidence of toluene abuse may lead to the more frequent observation of this syndrome in the future.

2855. No chromosome hazard from spray adhesives

Cervenka, J. & Thorn, Hattie L. (1974). Chromosomes and spray adhesives. *New Engl. J. Med.* **290**, 543.

Although the ban on sales of spray-adhesive products imposed by the US Federal Consumer Product Safety Commission in August 1973 (*Federal Register* 1973, **38**, 22569, 23355 & 25216) was subsequently withdrawn (*ibid* 1974, **39**, 3582), the concern the ban provoked was reflected in the number of studies that were undertaken to test the validity of the original asumptions. The results obtained have prompted much criticism of the basis for the ban and have raised doubts as to what evidence of chromosome breakage ought in future to be taken as an indication of possible genetic hazard (*Cited in F.C.T.* 1975, **13**, 156).

The authors cited above selected 14 subjects who had been exposed to spray adhesives almost daily, generally in the course of their work, for periods ranging from 6 months to 4 yr and who had been in contact with the products to within 3 wk of the cytogenetic analysis. Chromosomes from peripheral blood samples from these subjects were compared with those from ten matched controls without any history of exposure to spray adhesives. In the test subjects a total of 3421 mitoses showed 50 chromatid and isochromatid breaks, three acentric fragments and two dicentric chromosomes, representing a 1·6% incidence of abnormalities. The highest incidence was 3·2% in one subject, but those individuals with a history of heaviest exposure showed an incidence of no more than 1·3% breaks. In the non-exposed controls, a total of 1594 metaphases showed 21 breaks and one acentric fragment, representing a 1·38% incidence of abnormalities. There was therefore no significant difference between exposed and control subjects in the incidence of chromosomal abnormalities.

NATURAL PRODUCTS

2856. Identification of a bracken carcinogen

Evans, I. A. & Osman, M. A. (1974). Carcinogenicity of bracken and shikimic acid. *Nature, Lond.* **250**, 348.

Shikimic acid (3,4,5-trihydroxy-1-cyclohexene-1-carboxylic acid) was first reported as a naturally occurring compound nearly 90 yr ago, but it was not until the early 1950s that it was recognized as an important plant metabolite, playing a major role in the biosynthesis of essential aromatic amino acids from glucose. This acid has been identified in a large number of plant tissues including apples and wheat (Bohm, *Chem. Rev.* 1965, **65**, 435) and this widespread compound has now been implicated as one of the carcinogenic factors in bracken. We have previously reported attempts to isolate the tumour-inducing fraction of bracken, including work by the authors named above and their colleagues (*Cited in F.C.T.* 1972, **10**, 603; *ibid* 1974, **12**, 285).

The paper cited describes the effects of administering shikimic acid to mice. The main constituent of a bracken fraction which gave positive results in assays for acute toxicity, mutagenicity and carcinogenicity in mice was shown by low-temperature mass spectrometry to be shikimic acid, a compound unlike previously identified chemical carcinogens in both its structure and its widespread distribution. Because shikimic acid seemed an improbable carcinogen, the minor constituents of the fraction also received a considerable amount of attention. However, administration of a single dose of shikimic acid to 10-wk-old mice, orally or by ip injection, produced cancerous or precancerous lesions. Eight out of 13 animals given a single dose of 1, 5, 10, 15 or 20 mg ip and one animal given 100 mg by gavage developed Grade I or II neoplasms of the gastric mucosa or reticulum-cell A or B leukaemias. These animals died 24–60 wk after treatment. No tumours were found in five other mice that died 52–68 wk after ip treatment or in 57 control mice killed after comparable periods of time.

In view of these results a study of dominant lethal mutations was carried out. Groups of five 10-wk-old male mice (weighing 25–30 g) were given 25 mg shikimic acid in 0·5 ml H_2O (approximately equal to the LD_5) ip, or 80 mg in 0·2 ml H_2O by gavage. Ten controls were given 0·5 ml normal saline ip. In each of the 8 wk of the spermatogenic cycle, the males were mated with four virgin females. Twelve days after mating, the females were killed and scored for total implantations, deciduomas and late deaths. The numbers of dead implants expressed as a percentage of total implants (the Mutagenic Index) was plotted against time after injection to indicate the incidence of dominant lethal mutations. Over the whole 8 wk, the average control figure was 4·4%, while the group given shikimic acid orally showed a peak figure of 13·6% at wk 7 and the figures for the ip-treated group were consistently high from wk 2 onwards, with a maximum of 22·1%. The results obtained for shikimic acid in this experiment were of the order of those obtained elsewhere for aflatoxin and benzo[*a*]pyrene. Further work is being carried out on the effects of small daily doses and on the response of other experimental animals.

A second fraction from bracken has been found to contain another tumour inducer, more powerful than shikimic acid and capable of producing squamous carcinomas of the forestomach as well as tumours of the glandular stomach after single doses, but details of this work have not yet been published. This fraction has also been shown to produce the syndrome of acute bovine bracken poisoning.

2857. Abnormal metabolites of the sweet potato

Boyd, M. R., Burka, L. T., Harris, T. M. & Wilson, B. J. (1974). Lung-toxic furanoterpenoids produced by sweet potatoes (*Ipomoea batatas*) following microbial infection. *Biochim. biophys. Acta* **337**, 184.

In 1972, we reported the unhappy tale of an unusual lung disorder, which caused the death of 69 of a herd of 275 Hereford cattle and followed the consumption of mouldy sweet potatoes (*Cited in F.C.T.* 1972, **10**, 884). The group of workers who at that time confirmed the chemical structure of ipomeanol—a lung-oedema factor produced by infection of sweet potato with *Fusarium javanicum*—have since extended their work.

Exogenous stimuli such as chemical irritation, freezing and microbial disease will cause sweet-potato roots to produce and accumulate "abnormal" metabolites, some of which are toxic to animals. It is now suggested that four closely related 1,4-dioxygenated 1-(3-furyl)pentanes comprise the lung-oedema factor produced by mould-damaged sweet potatoes. The compounds are 4-ipomeanol (1-(3-furyl)-4-hydroxy-1-pentanone; 4-IPO), 1-ipomeanol (1-(3-furyl)-1-hydroxy-4-pentanone; 1-IPO), ipomeanine (1-(3-furyl)-1,4-pentanedione; IPI) and 1,4-ipomeadiol (1-(3-furyl)-1,4-pentanediol; IPD).

4-IPO, 1-IPO, IPI and IPD have been synthesized and spectral and physical data have been obtained. The lung-oedema factor was produced by incubation of sweet-potato slices (previously decontaminated with sodium hypochlorite) with a suspension of *F. solani* for 6 days at 22°C. The slices were then frozen and ground and the powder was extracted with acetone and then ethyl ether to give, on evaporation, 6·6 g oily residue/kg potatoes. Thin-layer and column chromatography were carried out on the crude extract. Silyl derivatives were prepared for gas chromatography and used for purification by means of high-pressure liquid chromatography. Comparison of spectral and physical data with those of synthesized samples confirmed the presence of the four compounds, ipomeanine being present only in traces.

Oral or ip administration of a few milligrams of the crude extract in aqueous propylene glycol produced marked pulmonary toxicity in the mouse, rat, guinea-pig and rabbit. In mice, significant pathology was generally limited to the lungs following a lethal dose, although sublethal doses sometimes caused hepatic and renal necrosis. Pulmonary responses indistinguishable from those evoked by the crude extract were observed in mice given 4-IPO, 1-IPO, IPI and IPD. The LD_{50} values of the four compounds in mice were, respectively, 38, 79, 26 and 104 mg/kg orally, 36, 49, 25 and 67 mg/kg ip and 21, 34, 14 and 68 mg/kg iv. The animals suffered increasing dyspnoea before death, which occurred within 6–24 hr, and their lungs showed widespread intra-alveolar oedema and vascular congestion. Occasional animals still surviving after 3–5 days had some renal damage.

Little is known about the long-term effects of this group of compounds. It is possible that the effects of the individual compounds are not merely additive but synergistic, or that the compounds are to some extent interconvertible by the sweet potato, the fungal contaminant or the animal ingesting them. The authors point out that these toxic furanoterpenoid metabolites often occur in slightly blemished potatoes sold for human consumption, and are not destroyed by normal cooking procedures. Therefore, quantitative analytical methods for sweet-potato toxins should be developed, and further investigations are needed to assess the potential effects in man of repeated ingestion of low levels of the toxins in the diet.

2858. Tannic acid and fertility of mice

Peaslee, Margaret H. & Einhellig, F. A. (1973). Reduced fecundity in mice on tannic acid diet. *Comp. gen. Pharmac.* **4**, 393.

In view of demonstrations of the depressant effect of tannic acid on the growth of various species of experimental animal and its postulated interference with the neurosecretory activity of the hypothalamus (*Cited in F.C.T.* 1971, **9**, 923; Peaslee & Einhellig, *Toxic. appl. Pharmac.* 1973, **25**, 507), it is hardly surprising that some attention has now been devoted to the possible effects of high dietary levels of this acid on the reproductive cycle in females.

The study cited above was designed to investigate fertility in white mice fed a diet containing 8% tannic acid either for 9–11 wk prior to breeding or continuously from weaning and throughout the breeding period. In the first case, only the females were given the test diet and they were mated with control males known to be fertile, while in the second experiment both males and females were fed the test diet. Treatment of females prior to breeding had no significant effects on subsequent litter weights or size, but there appeared to be some lengthening of the breeding cycle in these mice. Animals fed the diet continuously produced litters containing fewer and smaller pups than were found in control litters. Both first and second litters from treated pairs showed a slower rate of weight gain than controls and the first litters had a significantly poorer survival rate. The extension of the breeding cycle in these treated mice was much more marked than that recorded in the shorter experiment and together with the reduction in litter size was attributed to some disturbance in hormonal control caused by the tannic acid treatment.

2859. Toxins from mouldy rice

Glinsukon, T., Yuan, S. S., Wightman, R., Kitaura, Y., Büchi, G., Shank, R. C., Wogan, G. N. & Christensen, C. M. (1974). Isolation and purification of cytochalasin E and two tremorgens from Aspergillus clavatus. *Pl. Fds for Man* **1**, 113.

Some time ago (*Cited in F.C.T.* 1972, **10**, 606) we referred to a report that *Aspergillus clavatus*, isolated from mouldy barley, produced a neurotoxin which caused transient tremors and more persistent muscular incoordination in mice. The fungus was also asso-

ciated with the development of hepatic and pancreatic granuloma, with cloudy swelling, focal necrosis and regenerative metaplasia in the liver and with tubular degeneration and cortical metaplasia in the kidney.

A. clavatus has since been isolated from mouldy glutinous rice, consumption of which had been involved in cases of fatal human illness in Thailand. The paper cited above reports the identification of three toxins from this isolate. Two of the compounds, injected ip into weanling rats in a dose of 500 mg/kg body weight, rapidly induced fine tremors and hypersensitivity (which persisted for 5 days), inhibited growth and caused death in about 8 days, but autopsy revealed no specific histopathology in any of the major organs. The third toxin, known as cytochalasin E, was partially inactivated by heat and unstable at acid pH. It induced ataxia and coma in rats and led to death, apparently due to circulatory collapse, within 2–18 hr according to the dose and route of administration. An ip dose of 2·6 mg/kg caused severe congestion of the liver, kidney, lung, spleen and intestine, lung haemorrhages, cerebral oedema and excessive accumulation of peritoneal fluid.

Various toxins produced by food-borne fungi are being studied in relation to their possible significance in human disease in Thailand, and the *A. clavatus* toxins have figured in these studies. However, although the fungus was apparently associated with a case of Reye's syndrome in a young Thai boy, none of the three toxins it produces induced in rats effects resembling Reye's syndrome, which occurs mainly in children aged 3–8 yr and is characterized by severe cerebral oedema, fatty degeneration of the liver, kidney and heart, hypoglycaemia, convulsions, coma and generally death. Incidence of the syndrome follows distinct geographical and seasonal patterns in Thailand, and some association with aflatoxin B_1 has been reported (Shank *et al. Fd Cosmet. Toxicol.* 1971, **9**, 501).

COSMETICS, TOILETRIES AND HOUSEHOLD PRODUCTS

2860. Ammoniated mercury hits the kidney

Kibukamusoke, J. W., Davies, D. R. & Hutt, M. S. R. (1974). Membranous nephropathy due to skin-lightening cream. *Br. med. J.* **2**, 646.

Aminomercuric chloride (ammoniated mercury; AMM) has been shown to undergo percutaneous absorption, and the cases of six women who developed signs of mercurialism after applying bleaching creams containing 1–3% AMM for 2 yr or more have been reviewed (*Cited in F.C.T.* 1973, **11**, 1158).

The paper cited above describes the development of the nephrotic syndrome (involving facial and peripheral oedema, proteinuria, hypoalbuminaemia and raised levels of plasma cholesterol) in a 21-yr-old African woman who had applied a skin-lightening cream containing 10–15% AMM nightly for 1–2 yr. Use of the cream was discontinued and a diuretic was given to counteract the oedema, and as a result the nephrosis remitted within 6–9 months. A kidney-biopsy specimen taken during the illness showed minimal glomerulonephritis and tubular dilatation with flattening of the epithelial lining of some tubules. Electron microscopy confirmed the tubular damage, and together with the immunofluorescence findings indicated that either an immune complex of mercury with a blood protein or an antigen released by direct tubular-cell damage was probably responsible for the syndrome.

[This case reinforces the long-contended argument for the removal of ammoniated mercury from the therapeutic armamentarium, whether it is used for treatment or cosmetic purposes.]

2861. More on the hairspray problem

Blessing, M. H. (1974). Storage of vinylpyrrolidone-vinylacetate (VP-VA) in rats following endotracheal and subcutaneous injection. *Virchows Arch. Abt. A path. Anat.* **362**, 115.

Polyvinylpyrrolidone has produced a macrophage reaction in the lungs of rats, and possibly allergic reactions in a few individuals (*Cited in F.C.T.* 1967, **5**, 590; *ibid* 1974, **12**, 434). Vinylpyrrolidone–vinyl acetate (VP–VA), a high-molecular-weight copolymer used in cosmetic hairsprays, is a closely related compound which presents similar difficulties in toxicity evaluation owing to the differences in particle size respirable by different species. It is possibly for this reason that results of various inhalation studies on VP–VA in guinea-pigs, rabbits and rats have proved difficult to reproduce.

The paper cited above describes experiments which were designed to obviate differences in respirable particle size and which involved single or repeated endotracheal or sc injection of a saline solution of VP–VA into rats. The use of pure copolymer avoided the possibility of side effects attributable to propellants, solvents, perfumes or other additives. Animals were killed and examined between 1 and 365 days after treatment. Within 1 or 2 days of endotracheal injection, the alveolar spaces of all the lung lobes were packed with macrophages, and 3–4 days later numerous large macrophages were present in the lung interstitium, particularly in the peribronchial and perivascular lymphatics. After 4–6 months, some lung deposits were still present, particularly in the alveoli close to bronchi and vessels as well as near fibrous septae. No VP–VA deposits were observed in other organs except for small scattered deposits in the spleen of animals that had received repeated endotracheal injections.

After sc injections most of the dose of VP–VA was stored in the spleen, in the subcapsular region of which large reticular cells, transforming into vacuolated cells, appeared 2–3 days after injection. After about 6 days, the red pulp was completely filled with storage cells. Residual storage cells were still present in the spleen after 1 yr. After repeated sc injections of VP–VA, a few groups of large vacuolated cells were seen in the portal region of the liver lobules and in the podocytes of the kidney glomeruli and the epithelium of the tubules. Very infrequently, storage was observed in the reticular cells of the bone

marrow and lymph nodes and sometimes large solitary macrophages were seen in the lung interstitium.

There was no evidence that VP–VA produced any inflammatory changes in the pulmonary or other tissues. The polymer appears to be eliminated from the lung partly by way of interstitial macrophages and the lymphatic system, but mainly by transfer to the gut via the mucociliary route to the oesophagus. After a single sc dose, VP–VA was demonstrated in the urine within 30 min, the maximum rate of excretion being reached after about 1·5 hr.

2862. A hairdresser's dilemma resolved

Reiss, F. & Fisher, A. A. (1974). Is hair dyed with para-phenylenediamine allergenic? *Archs Derm.* **109,** 221.

The commonest ingredient of permanent hair colouring formulations is *p*-phenylenediamine (PPD) which has been associated with an incidence of sensitization of some 10% in patients with contact dermatitis (*Cited in F.C.T.* 1973, **11,** 147). As far as the general population is concerned, however, the authors cited above quote a figure of 1 in 50,000 for cases of allergic dermatitis arising from the application of PPD as a hair dye. They point out that the allergenic or sensitizing potential of hair dyes based on PPD apparently resides in the diamine or its oxidation products alone, and not in additives which are always present.

The question they set out to answer is whether individuals who have already developed allergy to PPD (hairdressers for the most part) can safely handle hair which has been dyed with it, without suffering a recurrence of their reaction. Twenty individuals, 19 women and one man, who had given strongly positive patch-test reactions to PPD were patch-tested with samples of hair which had been dyed 24 hr before with one of two commercial PPD preparations (Miss Clairol Creme Formulae, shade nos 52 and 30). The dyed hair was moistened with water and applied under a closed patch on the back of the subject, where it was left for 48 hr. The test procedure was repeated 3 wk later. No person showed any positive reaction, either after the first 48-hr contact or 3 wk later.

Evidently, hairdressers who become sensitized to PPD preparations need not worry about coming into contact with hair which has already been dyed with such preparations, because the allergen becomes completely oxidized or fixed during the dyeing process.

METHODS FOR ASSESSING TOXICITY

2863. Ornithine carbamoyltransferase as a test for liver damage

Divincenzo, G. D. & Krasavage, W. J. (1974). Serum ornithine carbamyl transferase as a liver response test for exposure to organic solvents. *Am. ind. Hyg. Ass. J.* **35,** 21.

Many suggestions have been made regarding the value and simplicity of tests for the levels of circulating liver enzymes in serum as indicators of liver damage and impaired liver function (*Cited in F.C.T.* 1973, **11,** 930). Investigation of serum urocanase showed that determinations of this enzyme were more time-consuming and provided no better index of liver damage than the routine serum glutamic–oxalacetic and glutamic–pyruvic transaminase tests (*ibid* 1974, **13,** 588), but more promising results were obtained with sorbitol dehydrogenase (*ibid* 1975, **13,** 294).

Now it is the turn of another enzyme, ornithine carbamoyltransferase (OCT), a mitochondrial enzyme promoting condensation of L-ornithine and carbamoyl phosphate to L-citrulline and orthophosphate in the urea cycle of the liver, to receive close scrutiny in this connexion. According to the authors cited above, OCT offers a sensitive and specific indication of the acute hepatotoxicity induced by certain organic solvents. Blood samples were taken from guinea-pigs 24 hr after they had been given an ip injection of a corn-oil solution of carbon tetrachloride, bromotrichloromethane, trichloroethylene, methylene chloride, chloroform, tetrachloroethylene, 1,1,2,2-tetrachloroethane, 1,1- or 1,2-dichloroethane, 1,1,1- or 1,1,2-trichloroethane, Freon 112 or 113, carbon disulphide, benzene or toluene, or of undiluted ethanol, methanol, isopropanol, *n*-butanol, diethyl ether, acetone, dioxane, *n*-amyl acetate, methyl isobutyl ketone, tetrahydrofuran, methyl ethyl ketone, *n*-butyl acetate, xylene, *n*-hexane, dimethylsulphoxide, *N*,*N*-dimethylformamide or vinyl acetate.

Preliminary observations had indicated that serum OCT activities were closely comparable in the dog, cat and rat, the levels being about 1 IU, but were slightly higher (about 2 IU) in the rabbit and guinea-pig. In this guinea-pig study, increases in serum OCT to more than 5·0 IU were considered significant.

Each solvent was tested in a range of doses, and routine liver-histology studies carried out on the same animals showed a close correlation between tissue damage and a rise in serum OCT. A low order of hepatotoxicity was ascribed to any solvent that increased OCT activity significantly only at a dose above 500 mg/kg, those causing a significant increase at a dose between 50 and 500 mg/kg were described as moderately hepatotoxic and those that were effective in this respect at a dose below 50 mg/kg were designated highly hepatotoxic. On this scale, bromotrichloromethane and carbon tetrachloride were identified as highly toxic, chloroform, hexane, tetrachloroethylene, 1,1,2-trichloroethane and vinyl acetate as moderately toxic, and methyl and butyl alcohols, amyl and butyl acetates, methyl ethyl and methyl isobutyl ketones, 1,2-dichloroethane, diethyl ether and xylene as only slightly toxic. The other solvents tested had no detectable effect on OCT levels or liver histology at levels well above 500 ppm.

2864. Assessing nephrotoxicity

Wright, P. J. & Plummer, D. T. (1974). The use of urinary enzyme measurements to detect renal damage caused by nephrotoxic compounds. *Biochem. Pharmac.* **23,** 65.

Enzyme assays are playing an increasingly important part in the assessment of the toxicity of compounds. The preceding item is concerned with the more 'popular' question of serum-enzyme studies, but the paper considered here suggests that the measurement of urinary enzyme concentrations might be used to simplify studies of nephrotoxicity. Several compounds either known to be nephrotoxic or suspected of such activity were administered to rats by ip injection and the activities of four enzymes were determined in urine samples collected for up to 7 days after the injection.

It was found that rats injected with uranyl nitrate, a known nephrotoxin, in a dose of 25 mg/kg showed an 11-fold increase in alkaline phosphatase (AlkP) excretion during the first 12 hr, and a 90-fold increase in lactate dehydrogenase (LDH) excretion during the 24–36 hr post-injection period. Urinary excretion of AlkP returned to normal limits within 48–60 hr, whereas LDH excretion remained elevated for 72–84 hr. Acid phosphatase (AcP) excretion was not significantly affected by the treatment and glutamic dehydrogenase (GDH) rose slightly above the upper limit of normal only at 48–60 hr. Urine volumes were in the normal range throughout except in the 12 hr immediately after injection, when the volume was increased, but both proteinuria and glucosuria were demonstrated and all but one of the 11 rats died between days 4 and 7.

Injection of 4-nitrophenylarsonic acid (25 mg/kg) in neutral solution increased LDH excretion 200-fold at 24–36 hr, the level remaining elevated up to 84 hr but returning to normal limits within 7 days. AlkP excretion was mildly affected only up to 36 hr having returned to normal by 60 hr. AcP and GDH were slightly increased at 24–36 hr, the period during which both proteinuria and glucosuria were most severe and there was a slight polyuria.

In contrast to these findings, 4-aminocatechol (10 mg/kg) had no effect on urine volumes or enzyme activities and the only change with 4-nitrocatechol (5 mg/kg) was a slight rise in AcP activity at 0–12 hr. With both compounds, the urines showed no more than a trace of proteinuria, no glucose and no blood. Although 4-aminocatechol has been identified as an abnormal metabolite of phenacetin in man and has been suspected of being responsible for kidney damage, there was no biochemical evidence of its nephrotoxicity in rats.

The authors conclude from this study that LDH determinations offer the most sensitive indication of acute renal damage and recommend the use of urinary assays of this enzyme in studies on possible nephrotoxic agents.

BIOCHEMICAL PHARMACOLOGY

2865. Arachidonic acid and the eye

Dawson, G. & Newell, F. W. (1974). Arachidonic acid and retinal pigmentary degeneration. *Lancet* **I**, 1119.

An investigation of the plasma-fatty acid levels of a woman with pigmentary degeneration of the retina and progressive spino-cerebellar degeneration revealed an arachidonic acid ($C_{20:4n-6}$; AA) level of 18%, whereas the normal female range is 5–9%. Both neutral lipid and phospholipid fractions had a considerably raised proportion of AA, but triglycerides and esterified cholesterol were primarily affected. Alteration of the patient's diet failed to modify the AA levels. When fibroblasts from the patient were incubated with [^{14}C]AA for 24 hr in a serum-free medium, they incorporated much greater amounts of ^{14}C into triglyceride, esterified cholesterol and phospholipid fractions than did normal human fibroblasts. At the same time, [^{14}C]linoleate incorporation into triglycerides, esterified cholesterol and phospholipids, but not that of [^{14}C]palmitate or [^{14}C]stearate, was increased compared with controls. AA is derived from linoleic acid ($C_{18:2n-6}$), and lipids of the outer segment of the retina are normally rich in unsaturated fatty acids, especially $C_{22:6n-6}$.

Plasma-AA levels in patients with pigmentary degeneration of the retina and in their immediate relatives were compared with levels in groups of patients with a variety of other retinal diseases or with unrelated disorders. It was found that in six of nine women with retinitis pigmentosa and in seven of 15 closely related women, plasma-AA levels exceeded 9%, the upper limit of the normal range. No similar abnormality was associated with optic atrophy, choroiderma, night blindness or retinitis pigmentosa associated with diabetes. In men, plasma AA appears to show a wider normal range and further studies are in hand to clarify the significance of the levels found in male patients with retinitis pigmentosa. The overall evidence suggests that an abnormality in the composition of retinal phospholipids resulting from a defect in arachidonate metabolism may lead to degenerative changes in the membrane.

CANCER RESEARCH

2866. NTA and nitrite in carcinogenicity studies

Greenblatt, M. & Lijinsky, W. (1974). Carcinogenesis and chronic toxicity of nitrilotriacetic acid in Swiss mice. *J. natn. Cancer Inst.* **52**, 1123.

The tertiary amine, nitrilotriacetic acid (NTA), is a chelating agent which has been considered as a partial replacement for sodium triphosphate in household detergents. In the most recent of our series of reviews on the possible biological effects of NTA (*Cited in F.C.T.* 1974, **12**, 421), we mentioned a carcinogenicity study carried out in rats and designed

to examine, in particular, the possible *in vivo* formation of a nitroso compound in rats treated simultaneously with NTA and sodium nitrite ($NaNO_2$). Unfortunately a very high tumour incidence in all the animals, including the controls, reduced the value of this study (*ibid* 1974, **12,** 423), but the same group has now reported a similar study in mice.

The test compounds were administered to groups of random-bred Swiss mice in the drinking-water. The solutions were given at night and each mouse consumed 5 ml of the given solution on each of 5 nights/wk for 26 wk. The mice were weighed every 4 wk and survivors were killed at wk 37–38 for autopsy.

When NTA was given to 40 male and 40 female mice in a concentration of 5 g/litre, no male mice developed lung tumours and the incidence in females was 12%. The incidence of lung tumours in untreated male and female controls was 19 and 11% respectively. Addition of $NaNO_2$ (1 g/litre) to the same level of NTA in the drinking-water produced a tumour incidence of 33% in males and 16% in females, but a product of the chemical reaction between nitrous acid and NTA, *N*-nitrosoiminodiacetic acid, given in the drinking-water at a concentration of 2 g/litre caused no increase in the incidence of tumours, only 4–5% of these animals being affected. The increased incidence in animals treated with the NTA/$NaNO_2$ mixture was not significant when the sexes were considered separately, but overall this treatment did result in a significant increase. *N*-Nitrosopiperidine (0·1 g/litre), included as a positive control because of its potent carcinogenic activity, induced tumours in 50 and 40% of males and females, respectively. Mice given 1 g $NaNO_2$/litre without NTA had a lung tumour incidence comparable with that in the untreated controls (12% in males and 16% in females).

[The results of this experiment are rather odd. NTA itself showed no evidence of carcinogenicity, but when it was combined with $NaNO_2$ a small but significant increase in tumours occurred. The putative product of the interaction of these two compounds, however, was associated with a tumour incidence somewhat below that in controls, and no increase occurred with $NaNO_2$ alone. One is left to speculate whether these results may not be entirely fortuitous. Perhaps the study would have been more conclusive if it had been extended to the full 80 wk usually considered appropriate for carcinogenicity studies in the mouse.]

2867. Nitrosamines in and out of the bladder

Hawksworth, G. & Hill, M. J. (1974). The *in vivo* formation of N-nitrosamines in the rat bladder and their subsequent absorption. *Br. J. Cancer* **29,** 353.

Several studies have been concerned with the possible formation of *N*-nitrosamines in the mammalian gut. These centred originally mainly on the stomach (*Cited in F.C.T.* 1970, **8,** 77) where the low pH favours the necessary nitrosation of secondary amines, but the ability of thiocyanate present in human saliva to catalyse this reaction has also been considered (Boyland *et al. Fd Cosmet. Toxicol.* 1971, **9,** 639), as has the production of nitrosamines in the small or large intestine as a result of bacterial intervention (*Cited in F.C.T.* 1974, **12,** 156). The authors named above consider that the latter is an unlikely *in vivo* source of nitrosamines, but describe experiments demonstrating that a similar reaction can occur in the infected urinary bladder of the rat if the nitrate intake is sufficiently high.

An *Escherichia coli* culture was introduced into the bladders of Sprague–Dawley rats, which were subsequently kept in metabolism cages and given drinking-water containing 5 mg sodium nitrate/ml. Approximately 90% of the nitrate consumed was recovered in the urine. After nitrate had been administered for 4 days, 500 μg piperidine hydrochloride was given by gastric intubation on two successive days to six rats with infected bladders, to two uninfected nitrate-treated control rats and to two infected rats given distilled water to drink instead of nitrate solution. Urine from all these rats was collected for 24 hr after each dose. The urine from half of the test animals but from none of the controls was found to contain *N*-nitrosopiperidine (NNP) in an amount equivalent to the nitrosation of 0·04% of the amine dose. This compared with a 0·4% conversion *in vitro* when the same dose of amine was incubated for 18 hr in a broth culture with a nitrate concentration similar to that found in urine. Similar results were obtained using pyrrolidine instead of piperidine.

Subsequently it was demonstrated that the radioactivity from [^{14}C]dimethylnitrosamine or [^{3}H]nitrosopiperidine ([^{3}H]NNP) introduced into the rat bladder was rapidly absorbed into the circulating blood, the rate of absorption being unaffected by the presence of infection. With labelled dimethylnitrosamine, peak blood levels of radioactivity were reached in 30–60 min, and after 4 hr 8% of the total dose was present in the blood and a further 8% in the major organs (liver, stomach and kidney), while 33% remained in the urine and most of the balance was presumably excreted as $^{14}CO_2$. After administration of [^{3}H]NNP, absorption was somewhat slower and the organ distribution of radioactivity was limited almost exclusively to the liver and kidney. When [^{3}H]NNP was introduced in a similar way into the hamster bladder, however, significant amounts were found also in the stomach, small intestine and lungs.

Both piperidine and pyrrolidine are present in normal human urine and nitrosamines have been reported in the urine of patients with urinary tract infections, but the significance of these facts, and of the possibility of rapid absorption of the nitrosamine from the bladder, in terms of human health has yet to be established.

Fd Cosmet. Toxicol. Vol. 13. p. 411. Pergamon Press 1975. Printed in Great Britain

LETTERS TO THE EDITOR

SACRIFICE AND MORTALITY

Sir,—The sheer economy and clarity of the request 'Drop dead!' is quite unmatched by the pretentious obscurity of other adaptations of the English language by North Americans. For years most Englishmen have twitched on hearing or reading, in accounts of laboratory experiments, that animals were 'sacrificed', knowing full well that the slaughters referred to were not carried out for religious reasons as offerings to deities. The shorter word 'killed' is always more appropriate. Recently, an even worse distortion of the English language has crept into reports on studies on animals: 'deaths' are being referred to as 'mortalities'. According to the Shorter Oxford Dictionary, 'mortality' is "the condition of being mortal or subject to death", "loss of life on a large scale, as by war or pestilence", "mortals collectively", or "the number of deaths in a given area or period from a particular disease". None of these definitions encourages the use of 'mortalities' to mean 'deaths'.

F. J. C. Roe,
4 Kings Road,
Wimbledon,
London, SW19 8QN,
England

[We can enlarge on Dr. Roe's experience with monstrosities such as 'euthanize' and 'euthanatize'. After waging a losing battle over 'sacrificed', 'medicated' and 'mortality', one attains a state of resignation to the inevitable. There are more important issues to be concerned about in Toxicology—Ed.]

ENVIRONMENTAL FACTORS IN THE ORIGIN OF CANCER

Sir,—We regret that the section on nitrosamine carcinogenesis in our paper "Environmental factors in the origin of cancer and estimation of the possible hazard to man" (*Fd Cosmet. Toxicol.* **13**, 251) was prepared without adequate care and therefore is erroneous. We ask that readers accept our apology for this mistake and disregard that section of the paper.

H. B. Jones and A. Grendon,
Donner Laboratory,
Lawrence Berkeley Laboratory,
University of California,
Berkeley, Cal., USA

Fd Cosmet. Toxicol. Vol. 13, p. 413. Pergamon Press 1975. Printed in Great Britain

FORTHCOMING PAPERS

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

In vivo chromosome-damaging effect of cyclohexylamine in the Chinese hamster. By G. F. van Went-de Vries, J. Freudenthal, A. M. Hogendoorn, M. C. T. Kragten and L. G. Gramberg.

Lack of effect of blood sampling-induced haematopoiesis on *in vivo* chromosome damage by cyclohexylamine in Chinese hamsters. By G. F. van Went-de Vries, M. C. T. Kragten and R. A. van den Bosch.

Failure to induce tumours in guinea-pigs after concurrent administration of nitrite and diethylamine. By N. P. Sen, Dorothy C. Smith, C. A. Moodie and H. C. Grice.

Zur Frage einer kombinierten Wirkung eines chemischen Mutagens und strahlensterilisierter Nahrung im Mutagenitäts- und Reproduktionstest bei der Maus. By H. W. Renner.

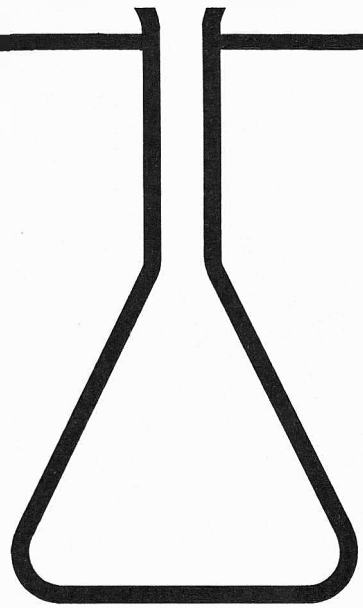
Dominant lethal mutations in male mice fed γ-irradiated diet. By P. S. Chauhan, M. Aravindakshan, A. S. Aiyar and K. Sundaram.

Quelques effets à moyen terme du lindane sur les enzymes microsomales du foie chez le rat. By M. A. Pelissier, Ph. Manchon, S. Atteba et R. Albrecht.

Tissue distribution, excretion and biological effects of [^{14}C]tetrachlorodibenzo-*p*-dioxin in rats. By J. R. Allen, J. P. Van Miller and D. H. Norback.

The carcinogenicity of aflatoxin M_1 in rainbow trout. By J. H. Canton, R. Kroes, M. J. van Logten, M. van Schothorst, J. F. C. Stavenuiter and C. A. H. Verhülsdonk. Toxicological tests on flavouring matters. II. Pyrazines and other compounds. By J. M. Posternak, J. J. Dufour, C. Rogg and C. A. Vodoz. (Summary of Toxicological Data)

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



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Published monthly to aid the study of interactions between organisms and the environment

Edited by PAUL JARVIS, Edinburgh
HARRY SMITH, Nottingham

Aims and Scope

Current Advances In Ecological Sciences provides a monthly current awareness service for biologists, ecologists and environmental scientists trying to keep abreast of the ever-increasing literature, both journals and books, in their areas of interest.

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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.

Instructions to Authors

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

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

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

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

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

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