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RESEARCH SECTION

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INFORMATION SECTION

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Research Section

ANALYSIS OF VOLATILE *N*-NITROSAMINES IN ALCOHOLIC BEVERAGES

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(*Received 4 June 1979*)

Abstract—A rapid extraction procedure for the analysis of volatile *N*-nitrosamines in alcoholic beverages is described. The technique involves liquid–liquid extraction, using dichloromethane, on a Preptube followed by concentration of the eluate and analysis on a gas chromatograph interfaced to a Thermal Energy Analyser. Detection limits, based on a 15-ml sample, vary from 0·2 μg/litre for *N*-nitrosodimethylamine (NDMA) to 0·5 μg/litre for *N*-nitrosopyrrolidine. Volatile nitrosamines were not found in any of the wines, sherries, liqueurs, brandies, gins, vodkas and rums that were analysed. All 18 brands of domestic and imported beer contained NDMA, at levels ranging from 0·4 to 7·0 μg/litre and six out of seven brands of Scotch whisky were also shown to contain NDMA, at levels between 0·3 and 2·0 μg/litre.

INTRODUCTION

There have been numerous reports suggesting an association between the consumption of alcoholic beverages and cancer in man (McGlashan, 1969; Rothman, 1975; Williams & Horm, 1977). The strongest associations are for cancer of the mouth, pharynx and larynx (Pell & D'Alonzo, 1973; Schmidt & deLint, 1972), oesophagus (Gsell & Loffler, 1962; Wildner & Gibel, 1966; Wynder & Bross, 1961), and liver (Leevy, Gellene & Ning, 1964; Lee, 1966). The possibility that the causative agent is a trace contaminant has been considered by many workers (Bogovski, Walker, Castegnaro & Pignatelli, 1974; Rothman, 1975). *N*-Nitrosamines are of special interest in this respect since a number of them are known to induce cancer of the oesophagus in animals.

In 1968, using polarography and thin-layer chromatography, McGlashan, Walters & McLean claimed to have found *N*-nitrosodimethylamine (NDMA) at levels up to 2000 μg/litre in home-brewed Zambian spirits. Subsequently McGlashan, Patterson & Williams (1970) showed that furfural would have interfered in the method and the findings were shown to be false by gas chromatography–mass spectrometry (GC–MS). Using an improved GC–MS method, sensitive at the 100 μg/litre level, Collis, Cook, Foreman & Palframan (1971) found no evidence of the presence of volatile nitrosamines in similar spirits from East Africa. Using thin-layer chromatography and a GC method capable of detecting 25 μg/litre, Sen & Dalpe (1972) could find no evidence of *N*-nitrosamines in a number of beers, whiskies, sherries or rums. By oxidizing the *N*-nitrosamine to the *N*-nitramine, and then using GC–electron capture, Castegnaro, Pignatelli & Walker (1974) developed a method sensitive to 1 μg nitrosamine/litre and using this NDMA was detected in 14 out of 30 farm-produced apple cider distillates at levels between 1 and 10 μg/litre (Bogovski *et al.* 1974). Using the clean-up procedure of Sen & Dalpe (1972), followed by GC–MS Bassir & Maduagwu (1978) found 0·5 μg NDMA/litre in a sample of Nigerian palm-wine. They also confirmed the finding by GC–MS.

Spiegelhalder, Eisenbrand & Preussmann (1979) distilled beer samples from mineral oil (Fine, Rounbehler & Oettinger, 1975) and then, using GC interfaced to a Thermal Energy Analyser (TEA), showed that 111 out of 158 German beer samples were contaminated with NDMA at mean levels of between 0·2 and 11·2 μg/litre. Their results were confirmed by GC–MS.

We report here on a much simplified analytical technique involving a single step liquid–liquid extraction on a Preptube, followed by concentration and analysis by GC–TEA. The method, sensitive at the 0·2 μg/litre level for NDMA, was used to screen a wide variety of popular US and imported alcoholic drinks.

EXPERIMENTAL

Materials. Liqueurs, wines, whiskies, brandies, beers, etc., were purchased from liquor stores in Massachusetts and at state liquor stores in New Hampshire. The seals on all bottles were broken just before analysis. Standard nitrosamine solutions and Preptubes were obtained from the Analytical Services Laboratory of Thermo Electron (Waltham, MA). The organic solvents were glass-distilled and were obtained from Burdick and Jackson (Muskegon, MI).

Apparatus. Gas chromatographic analyses were carried out using an isothermal GC (Model 661, Thermo Electron Corp., Waltham, MA), interfaced to a TEA analyser (Model 502LC, Thermo Electron Corp.). The GC column was a 14-ft stainless steel tube (outer diameter 1/8 in.), packed with 10% Carbowax 20 M containing 0·5% KOH on Chromosorb WHP 80/100 (Analabs, New Haven, CT). The GC was operated at 125 and 150°C, with a helium carrier-gas flow of 50 ml/min. The TEA analyser oven temperature was 450°C. The high-pressure liquid chromatographic (HPLC) analyses were carried out using a Varian 8500 LC pump (Varian Instrument Division, Palo Alto, CA) interfaced to a TEA-502. The LC column (3·2 × 250 mm) was 10 μ Lichrosorb Si60 (Altex Corp., Berkeley, CA). The solvent system was acetone–isooctane (7:93, v/v).

Table 1. *Detection limits (signal to noise ratio = 3:1) of seven volatile N-nitrosamines recovered from alcoholic beverages*

N-Nitrosamine	Detection limit (μg/litre)	Recovery efficiency %
N-Nitrosodimethylamine	0·2	45–62
N-Nitrosodiethylamine	0·2	47–68
N-Nitrosodipropylamine	0·4	57–77
N-Nitrosodibutylamine	0·5	61–81
N-Nitrosopiperidine	0·5	67–82
N-Nitrosopyrrolidine	0·5	62–83
N-Nitrosomorpholine	0·5	69–97

Procedure. Samples containing more than 20% (v/v) alcohol, were diluted with distilled water before analysis. Dilution was necessary to retain the alcohol on the Preptube. Samples with less than 20% (v/v) alcohol were analysed without prior dilution. The Preptube was pre-wetted with 10 ml dichloromethane and 15-ml aliquots of the samples were loaded onto it. The samples were eluted with 5 × 10 ml dichloromethane and the eluates were collected in a Kuderna-Danish evaporator, to which 0·3 ml isooctane was added as a keeper, and the volume was reduced to approximately 1 ml in a water bath at 55°C. Aliquots (20 μl) of each sample were then analysed by GC–TEA. NDMA-positive samples were confirmed using two methods. Firstly, the retention time for NDMA was confirmed using HPLC. Secondly, GC–high resolution MS was used with the magnetic analyser set to resolve the exact mass of NDMA ($C_2H_6N_2O$–74·0480; resolution 10,000, peak matched against a perfluoroalkane standard), from other background materials. Detection of an exact mass fitting the composition for the molecular-ion of NDMA, combined with the coincident retention time, confirmed the presence of NDMA (Spiegelhalder *et al.* 1979). Recovery studies were conducted by spiking the liquor samples with 5 μg/litre of each of the following *N*-nitrosamines: NDMA; *N*-nitrosodiethylamine (NDEA); *N*-nitrosodipropylamine (NDPA); *N*-nitrosodibutylamine (NDBA); *N*-nitrosopiperidine (NPIP); *N*-nitrosopyrrolidine (NPYR); *N*-nitrosomorpholine (NMOR).

RESULTS AND DISCUSSION

The range of recoveries and the detection limits (at a 3:1 signal to noise ratio) for the seven nitrosamines in the spiked liquor samples are listed in Table 1. The GC–TEA chromatogram of a wine sample to which a mixture of these nitrosamines (5 μg/litre of each) had been added is shown in Fig. 1. The 71 different brands of alcoholic beverages which were analysed are listed in Table 2. NDMA was present in all the beers at levels from 0·4 to 7·0 μg/litre, and in six out of the seven Scottish whiskies at levels from 0·3 to 2·0 μg/litre. Typical GC–TEA chromatograms of a Scotch whisky and a beer sample are shown in Figs 2 and 3, respectively. Volatile nitrosamines were not found in any of the wines, sherries, liqueurs, brandies, gins, vodkas and rums that were analysed.

To ensure the NDMA was not being formed as an artefact during extraction and/or analysis, three experiments were carried out. Firstly, when excess sulphamic acid and α-tochopherol were added to the beer and whisky samples, no decrease in the measured

Table 2. *N-Nitrosodimethylamine content of alcoholic beverages*

Type	Country of origin	Alcohol content (%)	NDMA μg/litre GC–TEA	NDMA μg/litre HPLC–TEA
	Wine			
Pink	NY, USA	13·7	ND	
White Sauterne	CA, USA	12·5	ND	
Dry white table	CA, USA	11·5	ND	
Red Chianti	CA, USA	12·5	ND	
Rose	NY, USA	12	ND	
Fruity	CA, USA	11	ND	
Rose	CA, USA	11·5	ND	
White	CA, USA	12	ND	
Rose	NY, USA	20	ND	
Dessert	CA, USA	19	ND	
White dinner	Ontario, Canada	13	ND	
White	W. Germany	10	ND	
White	W. Germany	10	ND	
Rose	Italy	9	ND	
Red	Italy	9	ND	
Rose	Portugal	12	ND	
White sparkling	Portugal	12	ND	
Red Bordeaux	France	11·5	ND	
White Burgundy	France	12	ND	

(*Continued on next page*)

Table 2. (*Continued*)

Type	Country of origin	Alcohol content (%)	NDMA μg/litre GC–TEA	NDMA μg/litre HPLC–TEA
	Sherry			
Dry	Spain	20	ND	
Pale dry	NY, USA	18	ND	
Orange (dessert wine)	CA, USA	19	ND	
	Liqueur			
Coffee	Jamaica	32·5	ND	
Orange	USA	30	ND	
Orange peel	MA, USA	26	ND	
Orange	France	40	ND	
	Gin			
Dry	MA, USA	40	ND	
Dry	London, England	47	ND	
Dry	London, England	47·3	ND	
	Brandy			
Grape	CA, USA	40	ND	
Cognac	France	40	ND	
Grape	Greece	42	ND	
Blackberry	Holland	35	ND	
	Vodka			
Grain	PA, USA	40	ND	
Grain	CT, USA	50	ND	
Grain	CT, USA	40	ND	
	Rum			
Light, dry	Puerto Rico	40	ND	
Dark, heavy bodied	Virgin Islands	40	ND	
Light	Virgin Islands	40	ND	
	Whisky			
Blended	IL, USA	45	ND	
Blended	IN, USA	40	ND	
Sour mash	TN, USA	45	ND	
Blended	CA, USA	43	ND	
Sour mash	TN, USA	45	ND	
Blended	Canada	43.4	ND	
Blended	Ireland	43	ND	
Blended	Scotland	43·5	0·4	ND
Blended	Scotland	43	0·3	ND
Blended	Scotland	43	2·0	2·8
Blended	Scotland	43	1·2	1·0
Blended	Scotland	43	1·2	1·3
Blended	Scotland	43	1·7	1·7
Blended	Scotland	43·4	ND	ND
	Beer			
Light	France	NC	0·6	0·6
Light	Philippines	NC	3·4	3·6
Light	Japan	NC	3·7	3·7
Light	Greece	NC	0·5	ND
Light	Holland	NC	5·2	4·7
Light	Holland	NC	3·1	3·2
Light	Holland	NC	0·7	0·8
Dark	W. Germany	NC	5·3	4·8
Dark	Mexico	NC	0·4	ND
Dark	Ireland	NC	0·6	1·0
Lager	Australia	NC	1·8	1·8
Dark ale	United Kingdom	NC	6·4	7·0
Light	USA	NC	7·0	6·7
Light	USA	NC	1·8	1·7
Light	USA	NC	0·9	0·8
Light	USA	NC	4·4	4·0
Dark	USA	NC	1·4	0·9
Dark	USA	NC	3·1	3·0

NDMA = *N*-Nitrosodimethylamine ND = Not detected (less than 0·2 μg/litre)
NC = Not calculated GC–TEA = Gas chromatography–Thermal Energy Analysis
HPLC–TEA = High-pressure liquid chromatography–Thermal Energy Analysis

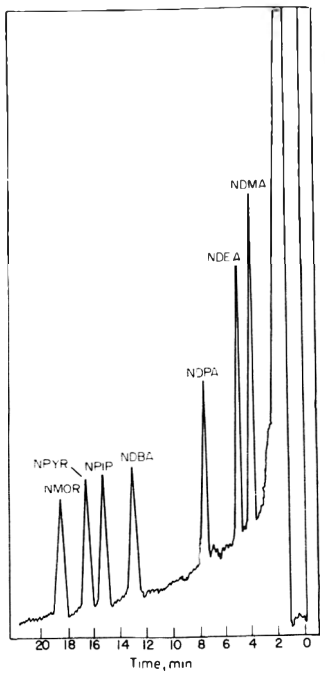

Fig. 1. GC–TEA chromatogram of a sample of wine to which 5 μg/litre each of NDMA, NDEA, NDPA, NDBA, NPIP, NPYR and NMOR had been added.

NDMA levels was observed, indicating that nitrosation was not occurring during analysis. Secondly, the addition of excess piperidine to the beer and whisky samples did not lead to the production of NPIP. Thirdly, synthetic beverage solutions (3% and 20% ethanol in distilled water) did not contain NDMA (at a detection limit of 0·2 μg/litre).

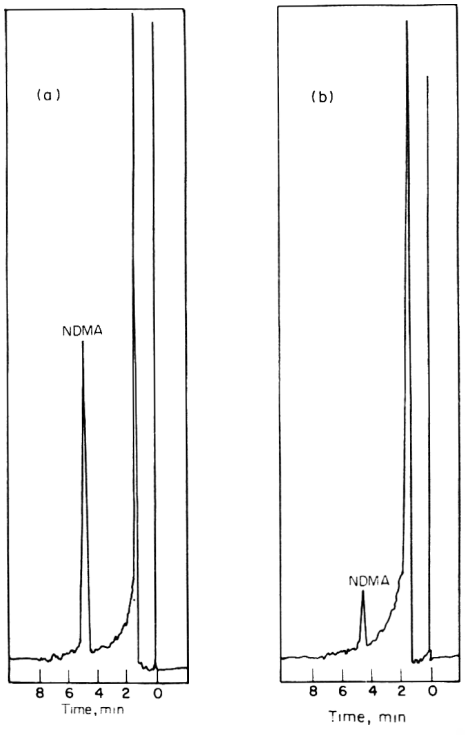

Fig. 2. (a) GC–TEA chromatogram of a 1·5 ng NDMA standard. (b) Typical GC–TEA chromatogram of a Scotch whisky sample, showing the presence of 1·7 μg NDMA/litre.

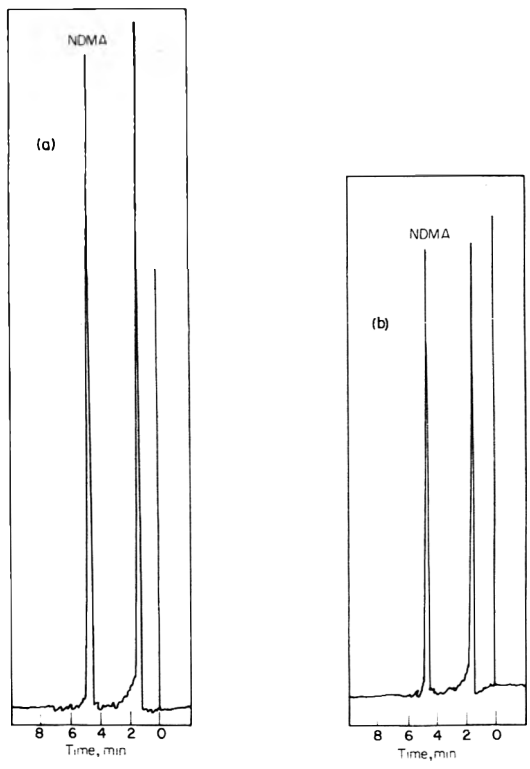

Fig. 3. (a) GC–TEA chromatogram of a 2·3 ng NDMA standard. (b) Typical GC–TEA chromatogram of a beer sample, showing the presence of 7 μg NDMA/litre.

The levels of NDMA in beer detected in this study are similar to those reported by Spiegelhalder *et al.* (1979) in German beers. Similar results were also obtained by E. A. Walker (personal communication 1979) in samples of US and European beers. The presence of NDMA in Scotch whisky has not previously been reported, but since both beer and Scotch whisky are made from barley, this finding is not surprising.

False-negative results using the GC–TEA have not been reported (Fine, 1979; Fine & Rounbehler, 1975; Gough, Webb, Pringeur & Wood, 1977; Havery, Fazio & Howard, 1978; Krull & Fine, 1979). It is therefore concluded that in wines, sherries, liqueurs, brandies, gins, vodkas and rums, volatile *N*-nitrosamines cannot be considered as possible causative agents in alcohol-related cancers.

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MUTAGENICITY OF CHINESE ALCOHOLIC SPIRITS

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(*Received 17 April 1979*)

Abstract—Using chromatography on XAD-2 columns, concentrates were made from 27 samples of commonly consumed Chinese alcoholic spirits and from five samples of selected imported alcoholic beverages. These concentrates were tested for mutagenic properties using the *Salmonella typhimurium*/microsomal test system. Mutagenic activity was detected in 17 of the 27 Chinese spirits but not in any of the imported drinks. The concentrates were shown to contain frameshift and/or base-pair substitution mutagens; some required metabolic activation and others did not. The data appeared to support various epidemiological studies suggesting that alcohol consumption might be an aetiological agent in cancer of the oesophagus.

INTRODUCTION

Various studies have suggested that alcohol taken in the form of spirits, beer and wine can be an important aetiological factor in cancer of the oesophagus (Warwick & Harington, 1973). Several mechanisms of action could be involved. It is possible that alcohol itself may be a tumour promoter or carcinogen for the oesophagus, or that the beverage may act as a carrier for carcinogenic chemicals derived from the grain used in the preparation of the brew, from materials added intentionally to impart a special flavour or from material dissolved from the surface of the containers used for brewing or distillation. A third possibility is that alcoholic drinks may cause malnutrition, which can subsequently affect the integrity of the oesophagus.

A search for carcinogens in alcoholic spirits consumed in the Transkei and other parts of Africa where there is a high incidence of oesophageal cancer was initiated by McGlashan and his colleagues (McGlashan, 1969; McGlashan, Patterson & Williams, 1970; McGlashan, Walters & McLean, 1968). These authors reported the presence of nitrosamine-like substances in some samples of home-made African spirits analysed by polarography and thin-layer chromatography. Such techniques were later proved to be unspecific for *N*-nitrosamines (Collis, Cook, Foreman & Palframan, 1971 & 1972).

Recently, several rapid *in vitro* systems for detecting chemical carcinogens have been developed (Poirier, 1976). Purchase, Longstaff, Ashby, Styles, Anderson, Lefevre & Westwood (1978) evaluated six short-term tests for the prediction of carcinogenicity and concluded that the Salmonella/microsome mutagenicity assay described in detail by Ames, McCann & Yamasaki (1975) had the most extensive application. In view of the relatively high incidence of oesophageal cancer in Hong Kong (Doll, 1969), we considered it important to find out whether commonly consumed local spirits contained any carcinogens. The present communication reports our findings using the Ames mutagenicity test.

EXPERIMENTAL

Chemicals. Aroclor 1254 was a gift from Monsanto Chemical Co., St. Louis, MO. Dimethylsulphoxide (DMSO), spectrophotometric grade, was purchased from Riedel de Haen, Germany, aflatoxin B_1 from Calbiochem, San Diego, CA, nitrosodimethylamine (NDMA) from Eastman Kodak Co., Rochester, NY, 2-aminofluorene from Tokyo Chemicals Inc., Japan, methyl-*N*-nitrosoguanidine (MNNG), nicotinamide adenine dinucleotide phosphate (NADP) and glucose-6-phosphate (G-6-P) from Sigma Chemical Co., St. Louis, MO, and XAD-2 resins from Applied Science Laboratories, State College, PA.

Samples of alcoholic spirits. Commonly consumed Chinese spirits of different varieties, mostly fermented from rice, glutinous rice and barley and in some cases flavoured with herbs, were purchased from local stores. These spirits are distilled 1–3 times and stored in large clay pots until they are exported or sold. In the latter case they are bottled on the premises when purchased. For comparison, five samples of imported beverages were also tested; these were two samples of beer, one of table wine, one of whisky and one of brandy.

Preparation of spirit concentrate. The alcoholic-spirit samples (about 600 ml; pH 5·5–6·0) were concentrated by passage through columns of the non-polar resin XAD-2, on which mutagenic substances were adsorbed. The columns were subsequently eluted with acetone according to the method described by Yamasaki & Ames (1977). Each of the eluted fractions was thoroughly mixed and then heated at 60°C under an atmosphere of nitrogen to evaporate the solvent. DMSO was then added to each of the dried residues (0·8 ml/100 ml of original sample) and these concentrates were subjected to the Ames mutagenicity test. For each experiment, a dose range of 10, 50, 100, 200 and 300 μl of concentrate was used. A 30% ethanol control was also included, since the alcohol content of many of the Chinese spirits was around this level.

Bacterial strains. Tester strains of *Salmonella typhimurium*, TA98 for the detection of frameshift

Table 1. In vitro *mutagenicity of Chinese alcoholic spirits*

Sample* no.	Induced revertants†/100μl DMSO concentrate/plate						
	TA98		TA1535		TA100		
	−S-9	+S-9	−S-9	+S-9	−S-9	+S-9	+S-9 + P‡
1	NS	NS	NS	596 (200)	3112	791	2475
2	1596 (200)	NS	NS	NS	0	905 (200)	1025
3	2515 (200)	NS	0	NS	475 (300)	0	438
4	0	0	225	625	0	0	2410 (300)
5	0	2760 (300)	NS	832	0	3465	4254 (200)
6	2380	0	0	0	0	NS	3580 (300)
7	0	NS	0	0	0	0	3420 (300)
8	0	NS	0	0	620	0	1236
9	NS	NS	NS	112	NS	5225 (300)	3837 (300)
10	1512	1482 (200)	NS	NS	0	1387	0
11	0	NS	0	453	0	NS	NS
12	310	4320 (300)	0	0	0	0	2200
13	0	3100 (200)	0	0	0	2630	0
14	0	0	0	1022 (300)	0	3050	3300 (200)
15	112	0	0	0	1775 (300)	0	4050 (300)
16	1374	0	0	1184 (300)	0	4260 (200)	0
17	0	0	0	0	0	2670 (300)	3265 (300)

DMSO = Dimethylsulphoxide NS = Not significant

*In total, 27 samples were studied, 17 of which gave positive results and were recorded. Five imported spirits investigated gave negative results.

†Spontaneous revertant colonies on control plates without mutagen and S-9 mix were subtracted (these were about 20 for TA1535, 40 for TA98 and 180 for TA100; slightly larger numbers arose on plates containing S-9 mix). Values greater than twice the number of spontaneous colonies were interpreted as significant and recorded. Numbers in brackets indicate volume (μl) of DMSO concentrate that gave the maximum number of induced revertants. Where no such number is given, the volume inducing the greatest response was generally 100 μl.

‡Modified procedure of Yahagi *et al.* (1977) for nitrosamine detection.

mutations and TA100 and TA1535 for base-pair substitution mutations, were generously supplied by Prof. Bruce N. Ames, University of California, Berkeley, CA. Overnight cultures of the bacteria in nutrient broth were used for the mutagenesis assay. A fresh cell suspension was used for each experiment.

Preparation of the S-9 mix. A 9000-**g** supernatant (S-9) of liver homogenate was prepared by the method of Ames *et al.* (1975) from a male Sprague-Dawley rat (body weight 200 g) induced with a single ip injection of a polychlorinated biphenyl mixture (500 mg Aroclor 1254/kg) and killed 5 days later. The S-9 mix used contained (per ml): S-9 (0·3 ml), $MgCl_2$ (8 μmol), KCl (33 μmol). G-6-P (5 μmol), NADP (4 μmol) and sodium phosphate buffer, pH 7·4 (100 μmol). S-9 mix was prepared freshly each time and bacterial contaminants were removed by passage through a sterile Swinex Filter unit (Millipore Corp., Bedford, MA) equipped with a Millipore filter (pore size 0·45 μm).

Mutagenesis assay. The assays were carried out as described by Ames *et al.* (1975). 0·1 ml of the bacterial tester strain, the sample to be tested and, where appropriate, 0·5 ml S-9 mix being added to 2 ml molten top agar. The contents were mixed and poured on to minimal-glucose agar plates containing a limited amount of L-histidine. After incubation for 2 days at 37°C, the colonies that had reverted to histidine prototrophy were counted. The sterility of the microsomal preparations and test samples was routinely checked to ensure that there was no bacterial contamination. The validity of the bacterial strains was also checked with known mutagens, such as aflatoxin B_1, MNNG, 2-aminofluorene and NDMA. For the detection of mutagenicity due to *N*-nitrosamines, a procedure (S-9 + P) described by Yahagi, Nagao, Seino, Matsushima, Sugimura & Okada (1977) was

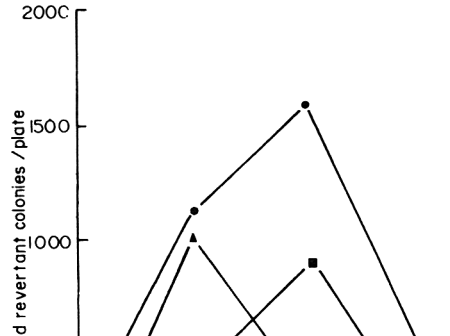

Fig. 1. Mutagenic dose-response curves of a DMSO concentrate of a sample (sample 2) of Chinese spirit, tested by the Ames Salmonella test using *S. typhimurium* strain TA98 without S-9 (●——●) and strain TA100 with the S-9 mix (■——■) and with 'S-9 + P', the modified procedure of Yahagi *et al.* (1977) designed for nitrosamine detection (▲——▲).

used: the test substance, S-9 mix and bacteria (TA100 strain only) were pre-incubated at 25°C for 20 min, then mixed with 2 ml molten top agar and finally poured on to a minimal-glucose agar plate.

RESULTS

Using the Ames/microsome test on the DMSO extracts of Chinese alcoholic spirits, we found that 17 out of 27 samples exhibited mutagenic activity (Table 1). Seven samples responded positively towards both TA98 and TA100, indicating the presence of both frameshift and base-pair mutagens, while the remaining ten samples contained base-pair mutagens only. In all positive samples, a dose-response relationship was observed, as exemplified by sample 2 (Fig. 1). The five imported alcoholic drinks, which included table wine, whisky and brandy, gave negative results. DMSO and 30% pure ethanol showed no evidence of mutagenicity at the concentrations used in our experiment.

DISCUSSION

The Ames Salmonella test is based on the empirical relation between mutagenesis and carcinogenesis: a molecular event (somatic mutation) may be shared by the two phenomena. This test has been validated in several laboratories, and Ames' own data (McCann & Ames, 1976; McCann, Choi, Yamasaki & Ames, 1975) showed that 90% of known carcinogens were mutagenic in the *Salmonella* test. The accuracy and sensitivity of the assay makes it a useful tool for obtaining information rapidly about the mutagenicity and potential carcinogenicity of chemicals, particularly of complex mixtures. Recently one of us was involved in work showing that several items of preserved Chinese food contained mutagenic substances and that WA rats fed Chinese salted fish had mutagenic urine (Fong, Ho & Huang, 1979; Ho, Huang & Fong, 1978). This was in agreement with earlier findings by Fong & Chan (1973a,b & 1977) and by Huang, Gough & Ho (1978), who showed by gas chromatography and mass spectrometry that low levels of *N*-nitrosamines were present in some preserved Chinese foods.

Using three tester strains of *S. typhimurium* to detect frame-shift and base-pair substitution mutations under different assay conditions, we have demonstrated that both direct-acting mutagens/carcinogens and carcinogens that require microsomal activation can occur in certain Chinese spirits (Table 1). Although the molecular structures of these mutagens have not been determined, it is likely that *N*-nitrosamines are present, as the Yahagi *et al.* (1977) procedure of pre-incubating the bacteria with S-9 mix and the test substance showed selective enhancement in the case of such substances. A linear dose-response relationship was evident in the spirit extracts at low concentrations (Fig. 1), but at high concentrations the number of induced revertant colonies decreased. Since DMSO, in the amounts used in our experiment had no toxic or mutagenic effects on the three tester strains, this lack of a dose-response relationship at high concentrations could have been due to some toxic effect that was inherent in the extracts and became manifest at such concentrations.

Ten samples of Chinese spirits gave negative results. Only one of these contained additives such as herbs, meat or other extraneous substances added in the fermentation/brewing process, and all of them were of the purer, triple-distilled, grade. The 17 mutagenic samples were either single-distilled or contained additives as described above. It appeared, therefore, that mutagenicity in Chinese spirits might be related to the type of ingredients and the method of brewing, distillation and/or storage. We did not find any mutagenic activity in the five samples of imported wine and liquors, but more samples must be screened before the possibility of mutagenicity can be entirely ruled out.

In summary, our results on the occurrence of mutagens in Chinese spirits suggest a risk of unknown magnitude to people who are exposed to them. Our data provide support for the hypothesis that alcoholic drinks could be a possible co-carcinogenic factor in the development of oesophageal cancer. On the other hand, our results raise a number of questions that require further investigation. To answer these questions, it will be necessary to determine the chemical structure of the mutagens present, to examine the conditions and reactions leading to their formation, and to conduct further epidemiological studies on the association between intake of such spirits and cancer of the oesophagus, both in Hong Kong and in other high risk areas in China.

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FORMATION D'UN CANCERIGENE, LE *N*-NITROSOCARBARYL, PAR INTERACTIONS ENTRE UN INSECTICIDE DE LA SERIE DES CARBAMATES, LE CARBARYL, ET LE NITRITE DE SODIUM DANS LE SUC GASTRIQUE DE RAT*

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Résumé—Nous avons étudié la formation d'un cancérigène, le *N*-nitrosocarbaryl, *in vitro* à 37°C dans du suc gastrique de rat par interaction entre un insecticide, le carbaryl (*N*-methyl carbamate de naphtyl-1), et le nitrite de sodium. Le *N*-nitrosocarbaryl se forme très rapidement et nous avons mis en évidence une relation linéaire entre le temps d'incubation et la quantité de *N*-nitrosocarbaryl apparue jusqu'à 45 min (rendement théorique d'environ 0,45% à 45 min). Il y a apparition de dérivé *N*-nitrosé même avec de faibles concentrations de carbaryl et de nitrite de sodium et la quantité formée est proportionnelle aux concentrations des deux précurseurs. Après 45 min la quantité de *N*-nitrosocarbaryl présente dans le suc gastrique diminue rapidement; différents facteurs doivent interférer en rendant difficile l'évaluation exacte du *N*-nitrosocarbaryl. Ces résultats montrent que le *N*-nitrosocarbaryl peut se former dans du suc gastrique de rat et ils posent un problème de risque potentiel de cancérogénèse.

Abstract—The formation of the carcinogen *N*-nitrosocarbaryl from the insecticide carbaryl (*N*-methyl-1-naphthyl carbamate) in the presence of sodium nitrite has been studied *in vitro* in rat gastric juice at 37°C. *N*-Nitrosocarbaryl was formed very rapidly and a linear relationship between the time of incubation and the amount of *N*-nitrosocarbaryl formed was apparent up to 45 min (when the yield was about 0·45% of the theoretical). The yields of the *N*-nitroso derivative were significant even at low concentrations of carbaryl and sodium nitrite and the amounts formed were related to the quantity of the two precursors. The amount of *N*-nitrosocarbaryl in the gastric juice decreased rapidly after the maximum concentration had been reached. A number of different factors may interfere with the accurate estimation of the *N*-nitrosocarbaryl present. However, these results show that *N*-nitrosocarbaryl can be produced in rat gastric juice and this possible formation constitutes a potential carcinogenic risk.

INTRODUCTION

Des pesticides de la série des carbamates pouvant être présents dans l'environnement et plus particulièrement sous forme de résidus dans les aliments, peuvent être à l'origine de la formation de *N*-nitrosocarbamates par réaction avec des nitrites. Ainsi, le carbaryl, puissant inhibiteur de la cholinestérase largement utilisé en agriculture dans de nombreux pays, peut être *N*-nitrosé *in vitro* à pH acide en solution aqueuse chlorhydrique ou acétique pour former du *N*-nitrosocarbaryl (Eisenbrand, Ungerer et Preussmann, 1975a; Elesperu et Lijinsky, 1973).

Par ailleurs, il est connu que des amines secondaires ou tertiaires peuvent être *N*-nitrosées également *in vitro* dans du suc gastrique d'homme et de différents animaux (Lane et Bailey, 1973; Sen, Smith et Schwinghamer, 1969) et *in vivo* dans l'estomac de rat et de lapin (Eisenbrand, Ungerer et Preussmann, 1974; Greenblatt, Kommineni, Conrad, Wallcave et Lijinsky, 1972; Lijinsky et Greenblatt, 1972).

A la suite de ces données, nous nous sommes demandés si le carbaryl ne pouvait pas être *N*-nitrosé directement et à des concentrations relativement faibles dans du suc gastrique de rat par interaction avec du nitrite de sodium, ces deux substances pouvant se trouver fréquemment et même conjointement dans les aliments de l'homme et de l'animal. La possibilité d'une telle formation dans le suc gastrique n'est pas à négliger lorsqu'on sait que le *N*-nitrosocarbaryl est un cancérigène de la partie antérieure de l'estomac quand il est administré par voie orale (Eisenbrand, Schmähl et Preussmann, 1976; Lijinsky et Taylor, 1976).

METHODES EXPERIMENTALES

Provenance des produits. Le carbaryl (*N*-methylcarbamate de naphtyl-1; Sevin®) a été fourni gracieusement par la Société PEPRO, Lyon; après recristallisation dans l'éthanol, son point de fusion est de 142°C et il est chromatographiquement pur (détermination par chromatographie sur couche mince, gel de silice). Le nitrite de sodium (Prolabo, Paris) est de pureté analytique. Nous avons synthétisé le [^{14}C-*Me*]carbaryl par action de la [^{14}C]methylamine (CEA, Saclay) sur du chloroformiate de naphtyl-1 selon la méthode de Knaak, Talland, Bartley et Sullivan (1965). Le [^{14}C]carbaryl conservé en solution benzénique à +4°C et contrôlé par radiochromatographie,

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a une pureté radiochimique supérieure à 99%. L'activité spécifique est de 32,2 mCi/mmol de carbaryl; le rendement de synthèse est de 75%.

Nous avons synthétisé du *N*-nitrosocarbaryl (*N*-méthyl *N*-nitrosocarbamate de naphtyl-1) selon la méthode de Elesperu, Lijinsky et Setlow (1974). Après plusieurs recristallisations dans l'éther, le *N*-nitrosocarbaryl répond aux caractéristiques physicochimiques données par ces auteurs; en outre, sa pureté a également été déterminée par la présence d'une seule tache lors de chromatographie en couche mince. Le produit est stable lorsqu'il est conservé à l'abri de la lumière et à −15°C.

Suc gastrique de rat. Le suc gastrique de rat est obtenu par la méthode de Shay en utilisant des rats OFA (Sprague-Dawley) de 190–200 g mis au jeûne pendant 24 hr avant de faire la ligature du pylore sous anesthésie légère à l'éther. Les rats sont sacrifiés 5 hr après la ligature, le suc gastrique est recueilli et le pH est évalué. Les sucs gastriques sont conservés à −15°C pendant une période n'excédant pas 7 jours; après décongélation le pH est vérifié, il est compris entre 1,35–1,40.

N-Nitrosation in vitro *dans le suc gastrique de rat.* Nous avons étudié la formation dans le suc gastrique de *N*-nitrosocarbaryl à partir de carbaryl et de nitrite de sodium d'une part en fonction du temps et d'autre part en fonction des concentrations des deux précurseurs.

Dans la première série d'expériences, 50 μmols de nitrite de sodium (PM = 69) et 10 μmols de [^{14}C]carbaryl (PM = 201) sont mis dans 5 ml de suc gastrique; l'activité spécifique est de 0,712 μCi/μmol de carbaryl, elle est obtenue par mélange avec du carbaryl non radioactif. Le suc gastrique contenant les deux précurseurs est placé dans un bain Gallenkamp thermostaté à 37°C, sous agitation (150 oscillations/min) et à l'abri de la lumière, le *N*-nitrosocarbaryl tendant à se détruire à la lumière. Après 5, 10, 15, 30, 45, 60, 75 et 90 min, la réaction est arrêtée par addition de 2,5 g de NaCl suivie immédiatement d'extractions par le dichlorométhane afin d'effectuer un dosage radiochimique du *N*-nitrosocarbaryl formé. Parallèlement, 5 ml de suc gastrique contenant soit le [^{14}C]carbaryl seul soit le nitrite de sodium seul sont portés à 37°C et traités dans les mêmes conditions expérimentales afin d'éliminer toute possibilité d'interférences notamment celle d'une radioactivité résiduelle du [^{14}C]carbaryl. Nous avons vérifié le pH du mélange réactionnel qui n'est pas modifié au cours de l'incubation.

Lors de la deuxième série d'expériences, du nitrite de sodium et du [^{14}C]carbaryl sont mis dans 5 ml de suc gastrique à des concentrations variant dans chaque cas en progression géométrique les unes par rapport aux autres de raison 2. Ainsi, en présence de 10 μmols de carbaryl la concentration la plus faible en nitrite de sodium est de 3,125 μmols et, en présence de 25 μmols de nitrite de sodium, elle est de 1,25 μmols de carbaryl. La *N*-nitrosation est arrêtée, comme indiquée ci dessus, après 30 min d'incubation à 37°C.

Extraction et dosage radiochimique du N-*nitrosocarbaryl.* Après avoir ajouté 2,5 g de NaCl dans le milieu réactionnel, on fait deux extractions avec chacune 15 ml de dichlorométhane. Les phases organiques sont rassemblées, séchées sur Na_2SO_4 et une prise aliquote est évaporée à sec au moyen d'un évaporateur rotatif. Afin d'évaluer le *N*-nitroso[^{14}C]carbaryl apparu, le résidu sec est repris dans de l'éther et chromatographié en couche mince sur gel de silice en utilisant comme solvant éther–hexane (1:1). La révélation est faite au moyen d'une lampe aux ultra-violets (λ = 254 nm) et les taches correspondantes au carbaryl (R_F = 0,23) et au *N*-nitrosocarbaryl (R_F = 0,78) sont 'grattées' au moyen d'un scalpel. Les particules de silice sont mises en suspension dans un mélange scintillant contenant 10 ml de Scintix®, à base de toluène et de Triton X100 (Isotec, Versailles), et de 10 ml d'eau. Il se forme un gel dont on mesure la radioactivité grâce à un spectromètre à scintillation liquide Intertechnique, type SL 30 (Intertechnique Ltd., Sheffield). L'affaiblissement lumineux est déterminé par standardisation externe automatique; le rendement de détection est de 77%. Les désintégrations par min sont transformées en nmols de carbaryl ayant été *N*-nitrosées afin d'évaluer les quantités de *N*-nitrosocarbaryl formé.

RESULTATS

Cinétique de formation du N-*nitrosocarbaryl dans le suc gastrique*

Dans nos conditions expérimentales, il se forme très rapidement du *N*-nitrosocarbaryl en présence de 10 μmols de carbaryl et de 50 μmols de nitrite de sodium, les quantités de *N*-nitrosocarbaryl formées augmentant en fonction du temps d'incubation, de 5 à 45 min. La figure 1 représente, pour chaque temps étudié, les moyennes des six valeurs expérimentales de *N*-nitrosocarbaryl formé ainsi que la droite de régression vraie calculée à partir de ces données (Schwartz, 1969). Cette droite a pour équation $Q = 0{,}766t + 10{,}95$ où sont représentées les quantités de *N*-nitrosocarbaryl formé Q, exprimées en nmols ou μg, en fonction du temps d'incubation t, exprimé en min; la pente de la droite de régression 0,766 ± 0,049 correspond à la cinétique de *N*-nitrosation. L'étude statistique de la pente de cette droite montre que la variation de la quantité de *N*-nitrosocarbaryl formé est fonction du temps d'incubation ($F = 243$; $P < 0{,}001$). La comparaison des variances entre le temps et la variance totale des données montre que la droite de régression est bien linéaire ($F = 2{,}55 < F^2_{20}$ à 5%).

Cinétique de 'disparition' du N-*nitrosocarbaryl dans le suc gastrique*

A partir de 45 min les quantités de *N*-nitrosocarbaryl présentes dans le suc gastrique diminuent en fonction du temps (Fig. 1). Comme précédemment, nous avons tracé la droite de régression calculée à partir des valeurs expérimentales obtenues aux quatre temps étudiés; elle a pour équation $Q = -0{,}509t + 66{,}53$, la pente de la droite −0,509 ± 0,039 correspondant à la cinétique de 'disparition' du *N*-nitrosocarbaryl. La quantité de *N*-nitrosocarbaryl disparu est fonction du temps ($F = 162$; $P < 0{,}001$) et la droite de régression est bien linéaire ($F = 0{,}84 < F^2_{20}$ à 5%).

Influence des concentrations en carbaryl et en nitrite de sodium sur la formation de N-*nitrosocarbaryl*

La figure 2 montre que la *N*-nitrosation augmente avec des concentrations croissantes de nitrite de

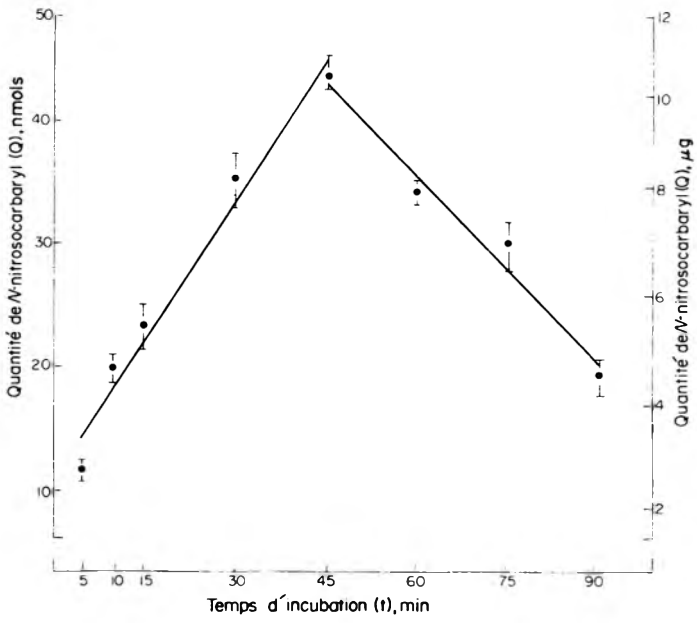

Fig. 1. Formation et 'disparition' de *N*-nitrosocarbaryl *in vitro* dans du suc gastrique de rat en présence de 10 µmols de carbaryl et de 50 µmols de nitrite de sodium. Chaque point représente la moyenne de six valeurs expérimentales ± l'erreur standard sur la moyenne.

sodium (en présence de 10 µmols de carbaryl) et de carbaryl (en présence de 25 µmols de nitrite de sodium). Cependant, ce phénomène ne peut pas être mis en évidence par une droite de régression. Par contre, nous avons pu calculer une parabole de régression par la méthode des moindres carrés (Lellouch et Lazar, 1974). Pour des concentrations variables en carbaryl, la parabole a pour équation $Q = 7{,}454c - 0{,}456c^2 - 0{,}342$ où sont représentées les quantités de *N*-nitrosocarbaryl formé Q, exprimées en nmols, en fonction des concentrations en carbaryl c, exprimées en µmols; la pente de la droite de régression est 7,454 ± 0,899 ($F = 68$; $P < 0{,}001$) et la courbure de régression est $-0{,}455 \pm 0{,}084$ ($F = 29$; $P < 0{,}001$). Il en est de même pour des concentrations variables c en nitrite de sodium où la parabole a pour équation $Q = 1{,}499c - 0{,}015c^2 + 0{,}734$; la pente de la droite de régression est 1,499 ± 0,208 ($F = 51$; $P < 0{,}001$) et la courbure de la régression est $-0{,}015 \pm 0{,}003$ ($F = 17$; $P < 0{,}001$). Dans le deux cas, le modèle mathématique choisi est significatif ($F = 0.94 < F^2_5$ à 5%). Il apparait donc que, dans le

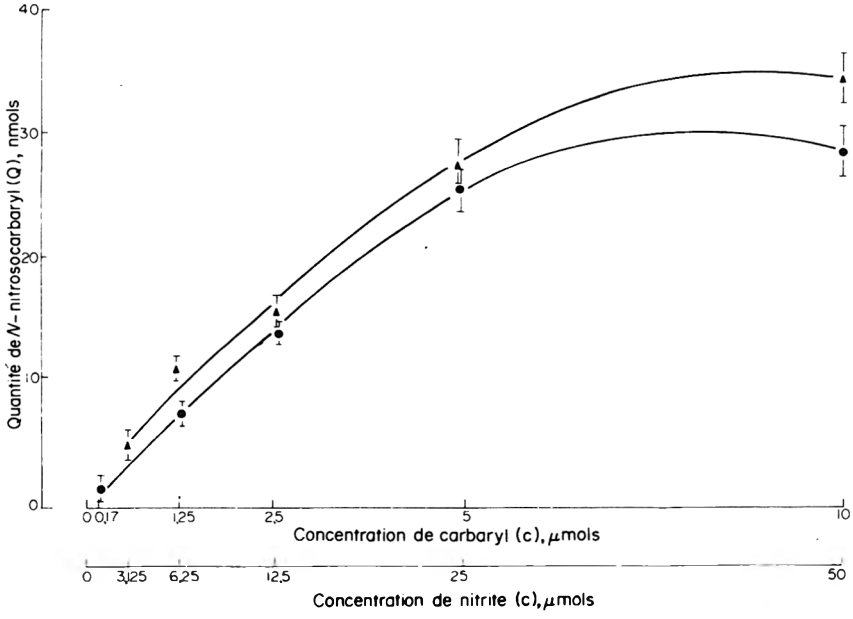

Fig. 2. Influence des concentrations en carbaryl (●) et en nitrite de sodium (▲) sur la formation de *N*-nitrosocarbaryl, pendant 30 min *in vitro* dans du suc gastrique de rat en présence soit de 25 µmols de nitrite de sodium soit de 10 µmols de carbaryl. Chaque point représente la moyenne de six valeurs expérimentales ± l'erreur standard sur la moyenne.

domaine des variations de concentrations étudiées, la quantité de *N*-nitrosocarbaryl formé obéit bien à une fonction quadratique.

DISCUSSION

Nos résultats montrent que dans nos conditions expérimentales le carbaryl réagit très rapidement avec du nitrite de sodium dans du suc gastrique de rat pour former, en quantités non négligeables, le dérivé *N*-nitrosé correspondant. Le suc gastrique est donc un milieu favorable à la formation de *N*-nitrosocarbaryl et nous avons mis en évidence une relation linéaire, jusqu'à 45 min, entre la *N*-nitrosation du carbaryl et le temps d'incubation à 37°C, en présence de 50 μmols de nitrite de sodium et de 10 μmols de carbaryl. Une telle possibilité devrait retenir l'attention du fait que le carbaryl et des nitrites peuvent se trouver dans les aliments en créant ainsi un risque potentiel. Bien que le rendement de synthèse soit relativement faible, de l'ordre de 0,45% à 45 min, il a une signification biologique étant donné que ce dérivé est un cancérigène (Eisenbrand *et al.* 1975a) notamment de l'estomac (Eisenbrand *et al.* 1976; Lijinsky et Taylor, 1976) et un mutagène (Blevins, Lee et Regan, 1977; Egert et Greim, 1976; Greim, Bimboes, Egert, Göggelmann et Krämer, 1977; Lee, Gold et Mirvish, 1977); en outre, il modifie les propriétés morphologiques et biologiques de cellules en culture (Quarles et Tennant, 1975).

La quantité de *N*-nitrosocarbaryl formé est fonction des concentrations des deux précurseurs présents dans le suc gastrique; ces résultats recoupent ceux trouvés dans la littérature concernant la *N*-nitrosation, en milieu aqueux acide, d'autres pesticides (Eisenbrand, Ungerer et Preussmann, 1975b). Cependant les courbes représentatives de ce phénomène sont des paraboles dans le domaine de variation des concentrations étudiées; il y a donc un seuil au dessus duquel les augmentations des concentrations en nitrite de sodium ou en carbaryl n'intervienment pratiquement plus dans la synthèse de *N*-nitrosocarbaryl. Il faut signaler, par ailleurs, que le nitrite de sodium se décompose en milieu acide (Turney et Wright, 1959) et aussi dans le suc gastrique de rat (G. Saint Blanquat, communication personnelle 1978); de ce fait, une diminution des concentrations en nitrite de sodium pourrait être un facteur limitant lors de la formation de *N*-nitrosocarbaryl. Mais cependant nous avons montré qu'en très petites quantités, de l'ordre de quelques μg, les deux précurseurs mis en présence dans le suc gastrique réagissent pour former du *N*-nitrosocarbaryl. Ce résultat n'est pas sans importance lorsqu'on sait d'une part, que les résidus dans les aliments sont de cet ordre de grandeur et, d'autre part, que des quantités minimes de nitrosamines cancérigènes administrées pendant une longue période peuvent conduire à une cancérisation.

Après 45 min, nous avons observé une diminution progressive de la quantité de *N*-nitrosocarbaryl présent dans le suc gastrique. Ce dérivé, instable en solution à la température ordinaire, l'est vraisemblablement encore beaucoup plus à 37°C; par ailleurs, selon Eisenbrand *et al.* (1975a), il est instable à pH 1, la stabilité maximale étant située entre les pH 3 et 5. D'autre part, il est probable que le *N*-nitrosocarbaryl ait des propriétés alkylantes comme de nombreuses nitrosamines cancérigènes et mutagènes (Magee, Nicoll, Pegg et Swann, 1975). De ce fait, il pourrait se fixer, dès sa formation, sur des protéines du suc gastrique ce qui expliquerait aussi sa 'disparition' du milieu, l'extraction par le dichlorométhane devenant alors impossible. Il est donc évident que plusieurs phénomènes se superposent et que la quantité de *N*-nitrosocarbaryl évaluée n'est peut être qu'une partie de la quantité effectivement synthétisée. De plus, l'apparition et la 'disparition' du *N*-nitrosocarbaryl pouvant intervenir simultanément, la détermination du temps nécessaire au rendement optimal est difficile à préciser. De telles difficultés ont déjà été signalées lors de la formation, *in vivo*, d'autres nitrosamines où le problème est encore beaucoup plus complexe (Sander, Labar, Ladenstein et Schweinsberg, 1975).

En conclusion, la possibilité de formation de *N*-nitrosocarbaryl dans le suc gastrique de rat à partir de carbaryl et de nitrite de sodium devrait retenir l'attention. En effet, ce phénomène de *N*-nitrosation du carbaryl n'est pas à négliger car il se produit avec des quantités minimes des deux précurseurs. De plus, le *N*-nitrosocarbaryl est cancérigène et mutagène et l'on sait que ces propriétés se manifestent quand les dérivés *N*-nitrosés sont administrés à trés petites doses. De ce fait, un problème de risque potentiel, difficile à évaluer, est posé.

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LYSINOALANINE FORMATION IN ALKALI-TREATED PROTEINS AND MODEL PEPTIDES*

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Abstract—Lysinoalanine formation in lactalbumin, casein and soya protein was studied as a function of pH, temperature and time. Formation was very rapid at elevated temperature and pH. At 90°C and pH 12 or 10, lysinoalanine levels in excess of those previously reported to cause nephrocytomegaly in rats were observed after only a few minutes of treatment. No detectable lysinoalanine was formed at slightly acidic pH values. Under very severe treatment conditions (pH 12, 90°C), lysinoalanine was formed very rapidly but was apparently destroyed upon further exposure. Lysinoalanine formation in a solution of polylysine with polyserine demonstrated both that unsubstituted serine served as a precursor to lysinoalanine formation and that significant interpeptide crosslink formation occurred under the conditions studied. Studies with model proteins and peptides supported the idea that cystine residues were the primary precursors of the lysinoalanine crosslink under moderately alkaline conditions, but that serine became a more important contributor as the severity of the alkaline treatment increased.

INTRODUCTION

Alkali treatment of proteins is known to result in the decomposition of cystine, serine, phosphoserine and threonine residues (Mecham & Olcott, 1949; Nicolet, 1931, 1932; Nicolet, Shinn & Saidel, 1942; Patchornik & Sokolovsky, 1964a), with concomitant formation of a number of amino acids not commonly found in nature (Bohak, 1964; Patchornik & Sokolovsky, 1964b; Horn, Jones & Ringel, 1941; Ziegler, Melchert & Lürken, 1967). Of these 'unnatural' amino acids formed under alkaline conditions, lysinoalanine (N^{ϵ}-(D,L-2-amino-2-carboxyethyl)-L-lysine) has recently attracted considerable attention, mainly as a result of its association with the development of nephrocytomegaly in the rat (Woodard & Short, 1973), and the consequent concern that its presence in foods may present a human health hazard (Gould & MacGregor, 1977).

The exposure of edible proteins to hot alkaline conditions often occurs during commercial or home processing of foods containing protein. The alkali treatment of corn, a staple food of Central America, is a well-known practice used for centuries to improve digestibility and increase the availability of lysine, tryptophan, and niacin (Katz, Hediger & Valleroy, 1974). Purification of vegetable proteins is achieved commercially through steps involving alkali extraction and precipitation of the dilute protein solution (Horan, 1974; Saunders, Connor, Edwards & Kohler, 1975). Spun proteins, mainly from soya, are prepared by extrusion through spinnerets of a thick alkali solution of the previously alkali-extracted soya isolate (Rosenfield & Hartman, 1974; Thulin & Kuramoto, 1967). Other processes which use alkali include: modification of the functional properties (such as solubility and stability in solution, foaming, emulsifying) of proteins intended as food ingredients (Betschart, 1974; Circle & Smith, 1972; Tannenbaum, Ahern & Bates, 1970), specific treatments designed to destroy toxic contaminants, such as aflatoxin in peanuts (Sreenivasamurthy, Parpia, Srikanta & Shankar, 1967) or to shorten the cooking time of beans (Rockland & Metzler, 1967). A variety of commercial foods, including some heated under non-alkaline conditions, have been shown to contain lysinoalanine (Sternberg, Kim & Schwende, 1975).

To define further the extent of lysinoalanine formation to be expected in common food proteins under various conditions of pH, temperature and time, and to determine the influence of amino acid composition and structural location within the protein, we have investigated the formation of lysinoalanine in three common food proteins (lactalbumin, casein and soya isolate) and in a number of purified proteins and polyamino acids.

*Reference to a company and/or product named by the Department is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

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EXPERIMENTAL

Materials. Soya protein (Promine D; 90% protein, 4% ash, 1% fat, 0·5% fibre and 4·5% moisture) was purchased from Central Soya Chemical Division, Chicago, IL. Alcohol-extracted lactalbumin (82% protein, 1·9% fat, 0·6% ash, 0·4% fibre and 10% moisture) and vitamin-free casein (90% protein, 1·1% ash, 0·6% fat, 0·4% fibre and 5% moisture) were purchased from Nutritional Biochemicals Corp., Cleveland, OH. Poly-L-lysine hydrobromide (Lot no. 034C5300) and poly-L-serine (Lot no. 095C5018), two synthetic polypeptides, and glutathione, in both oxidized and reduced forms, were purchased from Sigma Chemical Co., St. Louis, MO. Chemically synthesized lysinoalanine·2 HCl (purity, 99·5%) was purchased from Miles Laboratories, Inc., Elkhart, IN; a sample was also kindly provided by Dr. J. Finley of the USDA, Western Regional Research Center. Purified milk proteins αs_1-casein, β-casein, and κ-casein, isolated using the method of Zittle & Custer (1963) and β-lactoglobulin isolated by the method of Aschaffenburg & Drewry (1957), were kindly provided by Dr. Hansen of Ohio State University.

Alkaline treatment conditions. Proteins (25 g) were dispersed in 300 ml of deionized water in 500 ml Erlenmeyer flasks with vigorous stirring. The flasks were stirred in a water bath within $\pm 0{\cdot}2$°C of the specified temperature for each treatment. Protein solutions were brought to the desired pH by addition of 2 N-NaOH solution. The pH was checked every 15 min and corrected if necessary by adding NaOH solution. At the specified sampling times 25 ml of each protein solution were pipetted into aluminium cups containing about ten drops of 2 N-HCl to neutralize the protein. The samples were immediately frozen by dipping the aluminium cups in a dry ice–acetone mixture. Samples were taken after 5, 10, 25, 40 and 60 min of the alkali treatment and every hour thereafter up to 6 hr of treatment. Each of the three proteins was tested under the following conditions: pH 12, 10, 8 and the unmodified pH (5·1 for lactalbumin and casein, 6·7 for soya isolate) at temperatures of 25, 60 and 90°C. At pH 12, 90°C (only) the lactalbumin concentration was reduced to 4% because higher concentrations formed a thick gel which was impossible to stir. A further treatment was to autoclave (120°C, 20 psi) a 15% solution of lactalbumin or casein at the above pHs for 20, 50, 90 and 150 min. During autoclaving the proteins were in aluminium cups with no stirring.

All frozen samples of proteins were freeze-dried. The proteins treated at pH 12, 60°C (only) were acid-precipitated, filtered and washed twice with ten volumes of distilled water after treatment, and subsequently freeze-dried. After drying, the treated proteins were ground before acid hydrolysis and subsequent amino acid analysis.

The experiments with the purified proteins and model peptides were performed in hydrolysis flasks which permitted treatment, hydrolysis and evaporation of the sample in a single vessel. To each flask were added soya isolate, alcohol-extracted lactalbumin, αs_1-, β- or κ-casein or β-lactoglobulin (40 mg), or poly-L-lysine (35 mg), with either poly-L-serine (25 mg) or glutathione in oxidized or reduced form (73 mg) and 3 ml 0·1 N-NaOH. The flasks were shaken at 150 cpm on a reciprocating shaker (4·7 cm stroke) at 60°C for 4 hr. Measurements of the pH were made at the beginning and end of treatment. At the end of 4 hr the contents of the flasks were neutralized by adding 3 ml 0·1 N-HCl and an equal volume of concentrated HCl was added to bring the concentration of HCl to 6 N.

Amino acid analysis. Proteins were hydrolysed in 6 N-HCl (10 mg protein/5 ml acid) at 110°C for 22 hr under nitrogen according to Blackburn (1968). Hydrolysed samples were dried in a rotary evaporator at

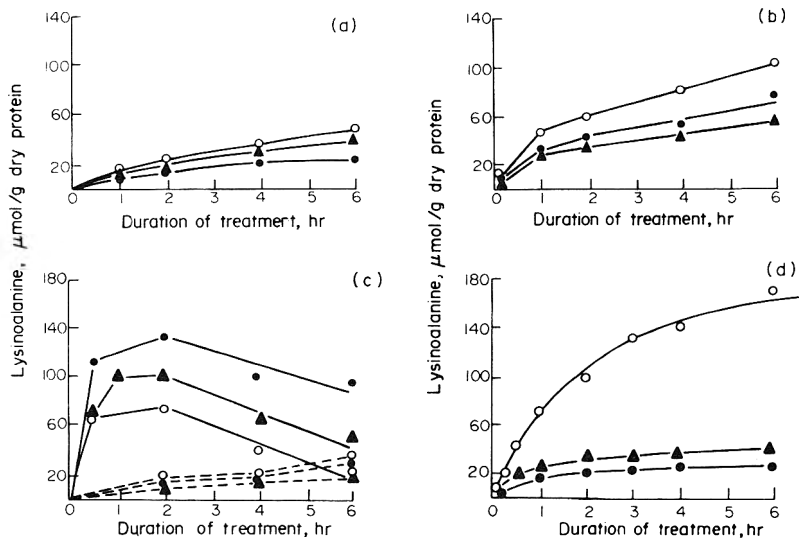

Fig. 1. Lysinoalanine formation in alkali-treated lactalbumin (○), casein (●) and soya protein (▲) under various conditions: (a) pH 10, 60°C; (b) pH 10, 90°C; (c) pH 12, 90°C (—) and 25°C (---); (d) 0·1 N-NaOH, 60°C. Under conditions a, b and c the total sample was neutralized and freeze-dried but under conditions d the samples were acid-precipitated and washed following treatment. All proteins were in a 7·7% suspension except the lactalbumin sample at pH 12, 90°C which was in a 4% suspension.

45°C, and were resuspended twice more in 4–5 ml of water and redried to ensure evaporation of all HCl.

For quantitative determination of the sulphur amino acids (cystine, cysteine and methionine), proteins were oxidized with performic acid to convert cysteine and cystine to cysteic acid and methionine to methionine sulphone according to the procedure described by Moore (1963).

Lysinoalanine, lysine and arginine were routinely separated by column chromatography on Dowex AG-50W-8 × 400 mesh resin on a 12 × 0·9 cm jacketed column maintained at 50°C and were determined on a Technicon Autoanalyzer by a modification of the ninhydrin method described by Quinn, Boisvert & Wood (1974). The column eluate, diluted with a stream of citrate buffer (pH 5·28), was mixed with the hydrindantin and the combined stream was heated to 90°C by passing through a jacketed heated coil, after which it was passed through a cooling coil and then through the colorimeter. The modified flow-rates that we used were (in ml/min): ninhydrin, 0·32; hydrazine, 0·03; nitrogen (reagent stream), 0·23; column eluate, 0·42; buffer diluent, 0·42; nitrogen (sample stream), 0·32; buffer to column, 0·42. The heating time was 5 min and the total time from column to photocell was 10 min.

Ninhydrin reagent was made as described by Quinn *et al.* (1974), and stored in an amber bottle open to air. Lithium acetate buffer was made as described by Moore (1968), except the pH was adjusted to 5·5. The hydrazine sulphate solution used was twice the concentration suggested by Quinn *et al.* (1974). Sodium citrate buffers were made as follows. For the pH 5·28 eluant buffer, 491 g citric acid, 288 g 97% NaOH, 136 ml concentrated HCl, 2 ml caprylic acid (as preservative) and 20 g BRIJ-35 (a detergent, Pierce Chemical Company, Rockford, IL) were dissolved in deionized water, to a final volume of 20 litres. For the pH 2·2 buffer used in diluting the protein hydrolysate, 21 g citric acid, 84 g 97% NaOH, 16 ml concentrated HCl, 0·1 ml caprylic acid, 20 ml thiodiglycol (to prevent oxidation of methionine) and 1 g of BRIJ-35 were dissolved in deionized water to a volume of 1 litre.

The elution times of the basic amino acids were established using known mixtures of pure amino acids. A colour constant for each amino acid was established by running measured quantities of known amino acids through the column. An internal standard of lysinoalanine was run with each determination.

For complete amino acid analysis (basic, neutral and acidic amino acids) the Durum model D-50 Amino Acid Analyzer (Durum Instrument Corporation, Palo Alto, CA) was used.

RESULTS AND DISCUSSION

Lysinoalanine yield from lactalbumin, casein and soya isolate

Figure 1 illustrates the lysinoalanine formation observed at five different combinations of pH and temperature as a function of time. At their unmodified pH in 7·7% solution (pH 5·1 for lactalbumin and casein; 6·7 for soya isolate), none of the proteins tested formed measurable quantities of lysinoalanine at temperatures ranging from 25°C to 120°C, after heating for up to 6 hr (not shown in Fig. 1). At pH 8 traces of lysinoalanine were detectable after heating for 2 hr at 90°C (detection limits were 0·2–0·4 μmol/g protein). After 6 hr at 90°C and pH 8, lysinoalanine levels were 8, 8 and 1 μmol/g protein, respectively, in soya protein, lactalbumin and casein.

At pH 10, 2–3 μmol of lysinoalanine/g protein were found in all three proteins after 6 hr at 25°C (data not shown), 5–10 μmol/g after a few minutes at 90°C and considerable quantities (50–100 μmol/g) after 6 hr at 90°C (Fig. 1b). At pH 12, lysinoalanine formation was rapid at elevated temperatures, and significant quantities were obtained even at 25°C (Fig. 1c).

Although the severity of renal lesions obtained in animals fed alkali-treated proteins is dependent on a number of factors in addition to the dietary lysinoalanine content (Gould & MacGregor, 1977; Feron, VanBeek, Slump & Beems, 1978), it is noteworthy that nephrocytomegaly has been observed in rats fed diets containing 100 ppm (0·43 μmol/g) free lysinoalanine (de Groot, Slump, Feron & VanBeek, 1976) or containing alkali-treated protein providing 1500 ppm lysinoalanine (Gould & MacGregor, 1977). These levels may be compared with the above observations that lysinoalanine levels in excess of 5000 ppm (21·4 μmol/g) are formed in only a few minutes at elevated temperature and pH; e.g. in about 20 min in lactalbumin at 90°C and pH 10 and in about 5 min in casein at 90°C and pH 12 (Fig. 1). Even at 25°C, 5000 ppm lysinoalanine was found after a few hours at pH 12.

Our failure to observe marked lysinoalanine formation at pH values of less than 8 is consistent with the findings of de Groot, Slump, VanBeek & Feron (1976), who found no lysinoalanine in soya protein treated at pH 7 or 8 for 4 hr at 40°C, but is in apparent contradiction to the findings of Sternberg & Kim (1977). These authors report finding 1000 ppm lysinoalanine in casein treated at pH 5·0 for 2 hr at 100°C and nearly 2000 ppm after 2 hr at 120°C at pH 5·0. Since our detection method was sensitive to at least ten times lower lysinoalanine levels than those reported by these authors, we cannot explain this discrepancy.

Although it was found that, in general, increasing lysinoalanine formation occurred with increasing alkalinity and temperature, under very severe treatment conditions (90°C, pH 12) it was found that lysinoalanine levels were maximal after about 2 hr and declined on more prolonged incubation (Fig. 1c). Under the conditions we used, lysinoalanine residues are apparently destroyed upon prolonged exposure, or are perhaps rendered unstable to the subsequent acid hydrolysis procedure. Provansal, Cuq & Cheftel (1975) report similar findings of lower lysinoalanine content after 5 or 16 hr at 80°C in 0·2 N-NaOH than after 1 hr under the same conditions. Friedman (1978) has also reported finding less lysinoalanine in wheat gluten after 8 than after 3 hr in 1 N-NaOH at 65°C. Provansal *et al.* (1975) report the partial (unspecified) destruction of pure lysinoalanine in 1 N-NaOH at 80°C for 16 hr. We found, however, that pure lysinoalanine was stable for 6 hr at 90°C and pH 12, indicating that the lysinoalanine loss from protein exceeded that expected from its chemical sta-

Table 1. *Lysinoalanine yield in lactalbumin and casein (15% aqueous suspensions) autoclaved at 120°C, 20 psi at different pH values*

Protein	pH	Duration of treatment (min)...	Lysinoalanine yield (μmol/0·16 g N) 15	120
Lactalbumin	8		5·7	7·0
Casein			12·5	12·4
Lactalbumin	10		6·5	8·1
Casein			19·2	14·8
Lactalbumin	12		18·5	12·5
Casein			32·5	33·5

bility under these conditions. This behaviour of lysinoalanine residues in protein at high pH and temperature probably explains why its presence in alkali-hydrolysed protein samples was not reported long ago, since the prolonged severe alkaline conditions that are used in standard alkaline protein hydrolysis (Blackburn, 1968) probably destroys the lysinoalanine initially formed. In agreement with this supposition, we found very little lysinoalanine in soya protein subjected to a standard alkali hydrolysis procedure (Blackburn, 1968). We have further noted approximately 10% loss of pure lysinoalanine subjected to our standard acidic protein hydrolysis procedure (6 N-HCl, 110°C, 22 hr, vacuum, 50 mg/ml lysinoalanine), with the commensurate appearance of three new peaks in the basic column ninhydrin chromatogram, one in the position of lysine and two others in a position indicative of alanine and/or serine. In view of the apparent difference in the stability of pure and protein-residue lysinoalanine under basic conditions, we did not consider it appropriate to correct our values for lysinoalanine obtained using acid hydrolysis of proteins for the small loss of pure lysinoalanine observed under the acid hydrolysis conditions used.

Heating the proteins in an autoclave at 120°C at pH 10 and 12 resulted in relatively poor lysinoalanine yields (Table 1). In this treatment the proteins were autoclaved without stirring or pH adjustment, whereas vigorous stirring in the open air and constant pH had been maintained in all previous treatments. It is not known whether the reduced lysinoalanine yield at 120°C was due to a more rapid destruction of lysinoalanine, such as that noted above (for further discussion, see Friedman (1978)), or to the reduced agitation and aeration. Whichever is the case, it is noteworthy that these conditions more closely approximate commercial canning than do those reported in Fig. 1.

Of the proteins investigated, lactalbumin yielded the highest amount of lysinoalanine at temperatures below 90°C. The higher lysinoalanine yield from lactalbumin during mild to moderate treatments (pH 10, 60°C) coincides with its higher maximum cystine content (145 μmol/0·16 g nitrogen compared with the casein and soya isolate values of 20 and 61 μmol/0·16 g nitrogen respectively). Assuming nearly complete conversion of cystine–cysteine to dehydroalanine, one of the lysinoalanine precursors which is the limiting factor under these conditions, casein and soya isolate under mild treatment conditions (pH 10, 60°C) yielded lysinoalanine in quantities (20 and 40 μmol/g, respectively) similar to their cystine–cysteine content.

The dramatic increase of lysinoalanine in the latter two proteins at 90°C, pH 12, (140 μmol/g casein, 100 μmol/g soya protein) could obviously not result from cystine residues, which would permit maxima of 20 and 61 μmol lysinoalanine/g, in casein and soya isolate, respectively, if it were the only source of dehydroalanine. The most obvious alternative source of dehydroalanine is serine (Mellet & Louw, 1965). It appears, therefore, that serine in the proteins studied requires more extreme conditions to undergo the β-elimination leading to the formation of dehydroalanine. One, therefore, would expect serine to become a major contributor to lysinoalanine formation in proteins at elevated temperature and pH, whereas cystine appears to be the most important contribution under mild conditions. This idea is supported by the observed loss of serine residues under relatively severe conditions of pH and temperature but not under milder conditions (Table 2; see also Friedman (1978), Provansal *et al.* (1975), and de Groot *et al.* (1976b)).

Lysinoalanine yield from purified proteins and model compounds

To elucidate the relative contribution of cyst(e)ine and serine to the formation of lysinoalanine in proteins and to provide some information on the type of crosslinking to be expected as a result of lysinoalanine formation, the proteins and model compounds given in Table 3 were treated with 0·1 N-NaOH at 60°C for 4 hr. αs_1-Casein, with the lowest cystine–cysteine content (as determined by analysis), yielded almost a hundred times as much lysinoalanine as could be accounted for by formation from the maximum available cystine. Similarly β-casein and κ-casein yielded more lysinoalanine than could be formed from their maximum cystine content alone. These results show clearly that residues other than cystine are. under these conditions, important contributor(s) to lysinoalanine formation. Presumably serine is the chief precursor of lysinoalanine in these proteins, in accord with the substantial lysinoalanine yields observed from the polyserine/polylysine mixture. Further, the formation of lysinoalanine from poly-serine and poly-

Table 2. *Ratio of serine:alanine in proteins subjected to various alkaline treatments*

Protein	Treatment	Serine:alanine ratio
Soya isolate	None	1·12
	8 hr, 60°, 0·1 N-NaOH	1·06
	2 hr, 90°, pH 12	0·54
Lactalbumin	None	0·76
	1·3 hr, 60°, 0·1 N-NaOH	0·74
	2 hr, 90°, pH 10	0·75
	2 hr, 90°, pH 12	0·25
Casein	None	1·53
	None*	1·48
	2 hr, 90°, pH 12	0·53
	15 hr, 80°, 0·2 N-NaOH*	0·44
Sunflower protein*	None	0·77
	1 hr, 55°, 0·2 N-NaOH	0·75
	1 hr, 80°, 0·2 N-NaOH	0·66
	5 hr, 80°, 0·2 N-NaOH	0·43

*Data for these conditions are taken from Provansal *et al.* (1975).

Table 3. *Lysinoalanine yielded after treatment with 0·1 N-NaOH at 60°C for 4 hr and cyst(e)ine content of some proteins and model peptides*

Protein or peptide	Lysinoalanine (μmol/g protein)	Cyst(e)ine (μmol/g protein)	pH	
			Initial	Final
Soya isolate*	78	122	12·1	11·6
Lactalbumin (alcohol-extracted)*	171	291	12·1	11·7
β-Lactoglobulin	118	284	12·1	11·6
αs_1-Casein	143	3·3	12·1	11·7
β-Casein	116	37·2	12·1	11·5
κ-Casein (crude)	87	76·8	11·7	11·6
κ-Casein (purified)	107	109	11·5	11·7
Poly-L-lysine + poly-L-serine	57	0	11·1	10·5
Poly-L-lysine + glutathione (oxidized)	47†	3257	8·5	8·2
Poly-L-lysine + glutathione (reduced)	0†	3257	7·8	7·6

*In separate experiments, the cyst(e)ine content of soya isolate treated under these conditions had dropped to 39 μmol/g protein after 8 hr of treatment, and that of lactalbumin had dropped to 107 μmol/g protein after 1·3 hr.
†μmol/g poly-L-lysine.

lysine demonstrates that serine need not be esterified with a 'good leaving group' before it can undergo the β-elimination (Patchornik & Sokolovsky, 1964a). It also clearly demonstrates that the formation of lysinoalanine by interpeptide crosslinks is possible, so that dehydroalanine need not be in a position adjacent to lysine on the same peptide chain as originally thought (Patchornik & Sokolovsky, 1964b). Dehydroalanine and lysine may be in different peptide chains and still be capable of forming lysinoalanine. As was pointed out above, 60°C was probably a suboptimal temperature for β-elimination of the serine residues in polyserine, so even greater lysinoalanine yields might be expected under optimal conditions.

Based on the above findings and those of others (de Groot *et al.* 1976b; Friedman, 1978; Provansal *et al.* 1975) both cystinyl and seryl residues (in the presence of lysyl residues) are potential precursors of lysinoalanine. The importance of the position of these residues in the peptide may vary with the treatment conditions. In dilute solutions and low temperatures intrapeptide reactions may be an important source of lysinoalanine as has been shown for ribonuclease (Patchornik & Sokolovsky, 1964b). Ribonuclease may be a special case however, because it contains two pairs of adjacent cystinyl–lysyl residues. This spatial proximity of potential reactants would be expected to favour an intrapeptide crosslink and minimize the probability of lysinoalanine formation through interpeptide reaction. Under more severe conditions amino acid sequence does not appear to play such a critical role in lysinoalanine production.

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BIOLOGICAL EFFECTS OF ALKALI-TREATED SOYA PROTEIN AND LACTALBUMIN IN THE RAT AND MOUSE*

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Abstract—Diets containing 20% alkali-treated soya protein and providing 1370–2630 ppm dietary lysinoalanine produced nephrocytomegaly in the *pars recta* of the proximal tubule in male Sprague-Dawley and Wistar rats. The frequency of abnormally large *pars recta* nuclei exceeded 33% in rats fed alkali-treated soya protein providing dietary lysinoalanine levels of 2080 or 2630 ppm. There were no quantitative or qualitative differences between the renal responses of Sprague-Dawley and Wistar rats fed the same diets. Swiss-Webster mice fed alkali-treated soya protein had slightly more than twice as many enlarged *pars recta* nuclei as did the control group, but the shift in size distribution was not significant at the 5% level. Sprague-Dawley rats developed only minimal karyomegaly on a diet of 20% alkali-treated lactalbumin, despite a dietary lysinoalanine level of 4970 ppm. Diets containing 8 or 10% untreated lactalbumin in addition to 12% alkali-treated soya protein or 10% alkali-treated lactalbumin did not induce nephrocytomegaly or karyomegaly, despite providing dietary lysinoalanine levels of 1753 and 2490 ppm, respectively. Sprague-Dawley and Wistar rats fed 20% alkali-treated soya protein showed elevated plasma levels of glutamic-pyruvic transaminase (GPT) and blood urea nitrogen (BUN), and had a significantly lower plasma protein content than control animals after 8 or 12 wk on the diet. Sprague-Dawley rats fed 20% alkali-treated lactalbumin had elevated GPT levels, but BUN and total plasma protein were normal; replacement of half the protein content of the diet with non-nutritive cellulose caused elevation of GPT, with no change in the BUN or plasma protein values. In rats fed 20% alkali-treated soya protein, approximately 33% of the lysinoalanine ingested daily was found in the faeces and 1·2% in the urine. No lysinoalanine was detected in liver or blood, but a quantity equivalent to about 0·6% of the daily intake was found in the kidneys. Higher urinary lysinoalanine excretion (2–7% of the intake) was found in animals fed alkali-treated lactalbumin.

INTRODUCTION

The fact that ingestion of alkali-treated proteins induced by alkali-treated protein (Gould & MacGregor, 1977); there are marked species variations in sensitivity to lysinoalanine-induced nephrocytomegaly (de Groot, Slump, Feron & van Beek, 1976) and the Woodard, Short, Alvarez & Reyniers, 1975), has raised concerns about the presence of lysinoalanine in human foods (Gould & MacGregor, 1977). Many commercial and common cooked foods contain lysinoalanine, in some cases at levels near, or even well above, those which produce nephrocytomegaly in rats (Sternberg & Kim, 1977; Sternberg, Kim & Schwende, 1975). However, a number of variables strongly modify the development in the rat of lesions induced by alkali-treated protein (Gould & MacGregor, 1977); there are marked species variations in sensitivity to lysinoalanine-induced nephrocytomegaly (de Groot, Slump, Feron & van Beek, 1976) and the relationship of the nephrocytomegaly to its possible health consequences is still unclear (Gould & MacGregor, 1977).

This report describes the results of experiments designed to investigate the biological effects of alkali-treated soya protein and alkali-treated lactalbumin in Wistar and Sprague-Dawley rats and in Swiss-Webster mice.

EXPERIMENTAL

Alkali treatment of soya protein and lactalbumin. Three batches of alkali-treated soya protein isolate, designated $ATSI_1$, $ATSI_2$, $ATSI_3$, were prepared at different times, using the conditions described by Woodard & Short (1973). The 0·1 N-NaOH solution was preheated to 60 ± 1°C. The soya protein (Promine D, Central Soya Chemical Division, Chicago, IL) was dispersed slowly with vigorous stirring to prevent formation of clumps. Water lost by evaporation during the 8-hr treatment period was replaced. The initial pH after suspending the protein was 12·1, and after the 8-hr treatment period the pH was 10·8. After

*Reference to a company and/or product named by the Department is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

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treatment, the pH was adjusted to 4·6 with 6 N-HCl until it remained stable for more than 15 min. The suspension was left to settle overnight in a coldroom at 2°C. The supernatant was siphoned off, the precipitate was remixed with ten volumes of distilled water, and again left to settle overnight at 2°C. After a total of three such washings with distilled water, the precipitate was drained overnight in perforated baskets lined with cheesecloth and was subsequently spread in trays, frozen, and freeze-dried. The protein recovery was 82–85%. The lysinoalanine contents were: $ATSI_1$, 0·68%; $ATSI_2$, 0·98%; $ATSI_3$, 1·27%.

A single batch of alkali-treated lactalbumin was prepared by dispersing 3·2 kg lactalbumin (alcohol extracted, Nutritional Biochemicals Corp., Cleveland, OH) in 93 litres of 0·1 N-NaOH at 60°C for a treatment time of 80 min. The initial pH after suspending the protein was 12·2, the pH after the alkaline treatment was 11·6. After treatment, the protein was precipitated by adding 2 N-HCl to achieve a pH of 3·9. The supernatant was discarded, the precipitate was washed once with 80 litres of distilled water and freeze-dried. The protein recovery was 89%. The lysinoalanine content of the final dried protein was 2·5%.

Protein hydrolysis and amino acid analysis. Protein hydrolysis and amino acid analyses were carried out as described previously (Karayiannis, MacGregor & Bjeldanes, 1979).

Diets. The diet used was similar to that used by Woodard (1969). It was composed of 20% protein, 64·4% sucrose, 10% corn oil, 5% salt mix, 0·35% vitamin mix and 0·3% DL-methionine. The salt mix was a modification of that described by Hegsted, Mills, Elvehjem & Hart (1941). It contained (in g/100 g salt mixture), K_2HPO_4, 32·2; $CaCO_3$, 30; NaCl, 16·7; $MgSO_4 \cdot H_2O$, 10·2; $CaHPO_4$, 7·5; $Fe(C_6H_5O_7)_2 \cdot 6H_2O$, 2·7; $MnSO_4 \cdot H_2O$, 0·5; KI, 0·08; $CuSO_4 \cdot 5H_2O$, 0·03; $ZnCl_2$, 0·025. The vitamin mix was similar to the commercial vitamin diet fortification mixture—1369 of ICN—Nutritional Biochemicals Corp., Cleveland, OH. It supplied (in mg/100 g diet) choline chloride, 165; ascorbic acid, 99; thiamine-HCl, 2·2; pyridoxine-HCl, 2·2; riboflavin, 2·2; menadione, 4·95; Ca-pantothenate, 6·6; nicotinic acid, 9·9; *p*-aminobenzoic acid, 11; inositol, 11; α-tocopherol, 12·1; folic acid, 0·20; biotin, 0·044; vitamin B_{12}, 0·003; and in IU/100 g diet: vitamin A-acetate, 2500; vitamin D_3, 600. The diets in which lactalbumin was the protein source were supplemented with 0·4% L-arginine HCl. In diets in which two different proteins were present, the total protein content of the diet was always 20%.

Animals. Male weanling rats (about 21 days old, 50–65 g) of two different strains, Sprague-Dawley and Wistar, and male weanling Swiss-Webster mice (10–11 g) were used in the 8-wk feeding studies. Animals were obtained from Simonsen Laboratories, Inc., Gilroy, CA. Food and tap-water were supplied *ad lib.* except for the pair-fed groups. Food consumption and body weight of the groups fed *ad lib.* were recorded weekly. Animals were examined daily for gross clinical abnormalities. Individually caged, pair-fed control animals were given a daily food allotment equal to the quantity consumed on the previous day by a paired rat receiving the experimental diet.

Tissue and sample collection. At necropsy, animals were anaesthetized with ether (mice and lactalbumin-fed rats) or 70 mg phenobarbital/kg body weight (groups fed soya protein). The chest cavity was opened and blood was drawn from the right ventricle for subsequent blood cell counts and plasma enzyme analyses. EDTA (3 mg/ml blood) was used as the anticoagulant for blood cell counts and potassium oxalate (2 mg/ml blood) for enzyme analyses. Animals were examined for signs of gross pathological change. Kidneys were removed and weighed. The left kidney was cut in half transversely (across the longer axis), and the right, longitudinally (parallel to the longer axis), such that two symmetrical pieces resulted. One half of each kidney was fixed in 10% buffered formalin and the other half was frozen for subsequent chemical analyses. The livers of the animals fed $ATSI_3$ and controls were immediately frozen between two slabs of dry ice for subsequent determination of *N*-demethylase activity.

In addition to the blood samples obtained at necropsy, blood samples were taken from the ventral tail artery of five Sprague-Dawley and five Wistar rats fed $ATSI_3$, and from the corresponding control groups, (Table 1, groups 4, 6, 8, 10) after 2, 11, 23 and 44 days on the test diets. These samples were immediately drawn from the hub of the needle used for the arterial puncture into a 40 μl capillary tube and were diluted into 20 ml of Isoton counting fluid (Coulter Electronics, Inc., Hialeah, FL). No anticoagulant was used. Red and white blood cell counts were performed as described below.

Urine and faeces were collected separately in metabolism cages. Animals were housed individually. A small crystal of thymol was placed in each urine collecting vial to inhibit bacterial growth. Samples were frozen immediately after collection. During the first 12 hr of urine collection no food was given, to avoid contamination of urine and faeces with lysinoalanine from the diet. Three rats from the $ATSI_3$-fed group were returned to a lysinoalanine-free control diet after this 12-hr period, and urine and faeces were then collected for a five-day period.

Histological measurements. The right kidney of each animal was sectioned as follows. The kidney was placed with the cut surface up and a razor blade was directed perpendicular to the cut surface parallel to the long axis of the kidney at a level just below the outer medullary stripe. The resulting corticomedullary wedge, after processing, was sectioned parallel to the plane of the last cut surface (frontal plane).

Kidney wedges were processed through ethanol, xylene and paraffin, and embedded in paraffin blocks (Luna, 1968). Frontal sections 5 μm thick were cut, mounted on microscope slides, dried overnight at 60°C, and stained with haematoxylin and eosin.

The size distribution of nuclei in the affected regions of the outer medullary stripe of the kidney was quantified as follows. The slides containing stained kidney sections were coded and randomized to avoid any subjective bias. The observer was not aware of the identity of the slides examined. The slide was scanned under a microscope which permitted either direct visual examination or projection of the image onto an 18 × 23 cm television monitor at a magnification of 1430×. The magnification was cali-

brated using a stage microscopic scale. One centimetre on the TV screen corresponded to 7 μm on the slide, permitting the diameter of nuclei to be measured to within 0·5 μm. Observation was started in the area of the outer medullary stripe judged to have the highest density of enlarged nuclei. Once measurements were begun, a predetermined and consistent scanning pattern was used. All *pars recta* nuclei in the projected area were measured and recorded. A total of eight screens, containing a total of 280–450 nuclei, were scored for each animal. Nuclei contained in collecting ducts, blood vessels, etc. were not included. Nuclei were classified into classes of 5·6–6·9, 7·0–8·3, 8·4–9·7, 9·8–11·1, 11·2–12·5 and 12·6–14·0 μm in diameter. In elongated nuclei the major and minor axes were averaged (Elias, Hennig & Schwartz, 1971). This method of scoring karyomegaly correlated well with subjective ranking of the severity of karyomegaly when extensive karyomegaly was present and, in addition, permitted the detection of relatively small shifts in the size distribution which were impossible to detect by subjectively ranking the degree of karyomegaly or nephrocytomegaly.

Blood cell counts, liver N-demethylase activity and plasma chemistries. Red and white blood cell counts were performed using a Model B Coulter Counter (Coulter Electronics, Inc., Hialeah, FL). Commercially available cell diluent (Isoton), cell-lysing agent (Zap-o-globin) and blood cell standards were obtained from Coulter Electronics, Inc.

For measurement of liver *N*-demethylase activity, frozen liver samples were chopped without thawing. Approximately 5 g liver and 20 ml ice-cold isotonic KCl (buffered with 0·01 M-phosphate buffer, pH 7·4) were weighed into a tared homogenizer tube and the liver was homogenized with a motor-driven teflon pestle and centrifuged at 10,800 **g** for 30 min. *N*-Demethylase activity in the supernatant was determined by measuring the rate of formaldehyde release from aminopyrine (Dalton & Di Salvo, 1972).

Blood samples were centrifuged at 8000 **g** for 30 min at 2°C immediately after collection. Plasma was pipetted into 3 ml plastic covered cups and frozen until analysis. All blood chemistries were performed on a Technicon Autoanalyzer II, using standard methods described in detail in the Technicon Manual (Technicon Instruments Corp., Tarrytown, N.Y.). The date and reference number of the technical bulletins describing each assay are: glutamic–pyruvic transaminase, AAII-22, April 1971; glutamic–oxaloacetic transaminase, AAII-10, June 1971; blood urea nitrogen, AAII-01, December 1970; total protein, AAII-14, October 1970; albumin, AAII-15, October 1970. Ornithine carbamoyl transferase was determined by the method of Strandjord & Clayson (1966).

Protein efficiency ratios (PER's) and digestibility tests were carried out using the standard AOAC method (Association of Official Analytical Chemists, 1975), using five weanling Sprague-Dawley rats per group. The diets consisted of 10% protein (calculated as $N \times 6{\cdot}25$), 20% corn starch, 8% corn oil, 5% USP salt mixture XIV supplemented with 0·055% $ZnSO_4 \cdot 7H_2O$ and 0·0023% $CoCl_2 \cdot 6H_2O$, 3% cellulose, 2% vitamin mix (see above) and dextrose to make 100%. All diets had an equal quantity of nitrogen, fat, fibre and water. The diets containing soya protein were supplemented with 0·3% DL-methionine. The untreated diet containing lactalbumin was supplemented with 0·4% L-arginine–HCl- and the alkali-treated lactalbumin diets with 0·49% L-arginine–HCl. Reference casein, approved by the Animal Nutrition Research Council supplied by Nutritional Biochemicals, Inc. (Cleveland, OH) was also tested.

RESULTS

Nephrocytomegaly, growth and feed utilization

The occurrence of nephrocytomegaly and growth effects observed in the groups of rats and mice fed the various diets containing alkali-treated proteins are summarized in Table 1. The diets containing 20% alkali-treated soya protein, and providing 1370 ppm to 2630 ppm dietary lysinoalanine, consistently produced nephrocytomegaly in rats. The histological appearance of the lesions was in accordance with that observed by Woodard (1969) and Woodard & Alvarez (1967) in Sprague-Dawley rats fed alkali-treated soya protein. Representative sections of the renal outer medullary stripe of Sprague-Dawley and Wistar rats fed alkali-treated or untreated soya protein, and of Sprague-Dawley rats fed alkali-treated lactalbumin are shown in Fig. 1.

The size distributions of nuclei in the affected regions of the outer medullary stripe are given in Fig. 2. In control rats of both strains, the frequency of nuclei exceeding 8.4 μm in diameter was less than 0·5%, while one-third or more of the nuclei exceeded this dimension in rats fed $ATSI_3$ or $ATSI_2$. There were no quantitative or qualitative differences in the responses of Wistar and Sprague-Dawley rats fed the same diet (Figs 1 and 2, Table 1).

Despite providing a dietary lysinoalanine level of 4970 ppm, the alkali-treated lactalbumin induced only mild karyomegaly when fed at 20% in the diet (Fig. 1 and 2). This protein was more mildly treated (see methods) and permitted greater growth than an equivalent amount of the alkali-treated soya protein. The 10% alkali-treated lactalbumin–10% untreated lactalbumin diet permitted normal growth and did not cause nephrocytomegaly (Table 1, Fig. 2), even though the dietary lysinoalanine level (2490 ppm) was roughly equivalent to that provided by the 20% $ATSI_3$ (2630 ppm) or the 20% $ATSI_2$ (2080 ppm) diet. Further, diet no. 19 (12% $ATSI_3$, 8% untreated lactalbumin) did not produce nephrocytomegaly, even though the dietary lysinoalanine (1753 ppm) exceeded that of the 20% $ATSI_1$ diet (1370 ppm).

Although the animals on the alkali-treated soya protein diets ate less and had a lower feed efficiency than did those on untreated protein, the lower food intake and lower protein availability did not, in themselves, induce nephrocytomegaly. Pair-fed groups (Table 1, groups 12, 13) and groups with the protein partly replaced by Alphacel (Table 1, groups 17, 18) did not develop nephrocytomegaly, even when the protein restriction reduced their growth below that observed with the alkali-treated protein. While this type of control is adequate for the overall reduction in protein availability which is known to result from alkali treatment (de Groot & Slump, 1969), it must be noted that alkali treatment could modify the balance of available amino acids by specifically reducing the

Table 1. *Nephrocytomegaly, growth and feed efficiency in Sprague-Dawley and Wistar rats and in Swiss-Webster mice fed diets containing alkali-treated proteins for 8 wk*

Group	No. in group, strain, species	Dietary protein*	Dietary lysinoalanine (ppm)	Final body weight (g)†	Average 8-wk feed efficiency (g gain/g feed consumed)	Nephrocytomegaly‡
1	5, SD, Rat	UTSI	0	360 ± 11	0·37	0 (5)
2	5, SD, Rat	$ATSI_1$	1370	293 ± 12	0·35	+ + (5)
3	5, SD, Rat	$ATSI_2$	2080	210 ± 4	0·25	+ + + (5)
4	10, SD, Rat	UTSI	0	355 ± 8	0·40	0 (6)
5	10, SD, Rat	$ATSI_3$	2630	229 ± 8	0·31	+ + + (7)
6	5, SD, Rat	UTSI§	0	406 ± 14	—	0
7	5, SD, Rat	$ATSI_3$§	2630	334 ± 14	—	+ + +
8	10, WIS, Rat	UTSI	0	364 ± 5	0·42	0 (7)
9	10, WIS, Rat	$ATSI_3$	2630	225 ± 9	0·31	+ + + (7)
10	5, WIS, Rat	UTSI§	0	439 ± 6	—	0
11	5, WIS, Rat	$ATSI_3$§	2630	309 ± 25	—	+ + +
12	5, SD, Rat	UTSI¶	0	319 ± 16	0·37	0 (5)
13	5, SD, Rat	$ATSI_3$	2630	270 ± 17	0·29	+ + + (5)
14	7, SD, Rat	UTL	0	371 ± 8	0·39	0 (7)
15	7, SD, Rat	ATL	4970	294 ± 9	0·35	+ (7)
16	7, SD, Rat	UTL + ATL (1:1)	2490	339 ± 8	0·33	0
17	7, SD, Rat	UTL + Alphacel (1:1)	0	253 ± 10	0·26	0 (5)
18	5, SD, Rat	UTL + Alphacel (2:3)¶	0	210 ± 13	0·26	0 (5)
19	5, SD, Rat	UTL + $ATSI_3$ (2:3)¶	1753	283 ± 16	0·40	0 (5)
20	7, SW, Mouse	UTSI	0	34 ± 1·2	—	0 (7)
21	7, SW, Mouse	$ATSI_3$	2630	30 ± 0·5	—	0 (7)
22	7, SW, Mouse	UTL + $ATSI_3$ (2:3)	1580	34 ± 1·3	—	0 (7)

UTSI = Untreated soya protein isolate
$ATSI_1$ = Alkali-treated soya protein isolate (containing 0·68% lysinoalanine)
$ATSI_2$ = Alkali-treated soya protein isolate (containing 0·98% lysinoalanine)
$ATSI_3$ = Alkali-treated soya protein isolate (containing 1·27% lysinoalanine)
SD = Sprague-Dawley WIS = Wistar SW = Swiss-Webster

*See text for detailed description. Diets contained 20% of the indicated protein or mixture (in the ratio given in parentheses). For example, the diet for group 18 contained 8% untreated lactalbumin and 12% Alphacel, by weight.

†Mean ± SEM.

‡Summary of results from Fig. 2. The symbol 0 indicates no nephrocytomegaly; + indicates an increase in nuclear size which is significant at the $P < 0·05$ level; + + indicates that at least 10% of nuclei in affected regions exceeded a diameter of 8·4 μm; + + + indicates that at least 25% of nuclei in affected regions exceeded a diameter of 8·4 μm. The number in parentheses is the number of animals from each group upon which the frequency distribution of *pars recta* nuclei in Fig. 2 was based. Groups not shown in Fig. 2 (6, 7, 10, 11, 16) were examined for nephrocytomegaly but complete frequency distributions were not measured for those groups.

§These groups were fed the experimental diets for 12 wk.

¶Pair-fed with group 13.

¶ Pair-fed.

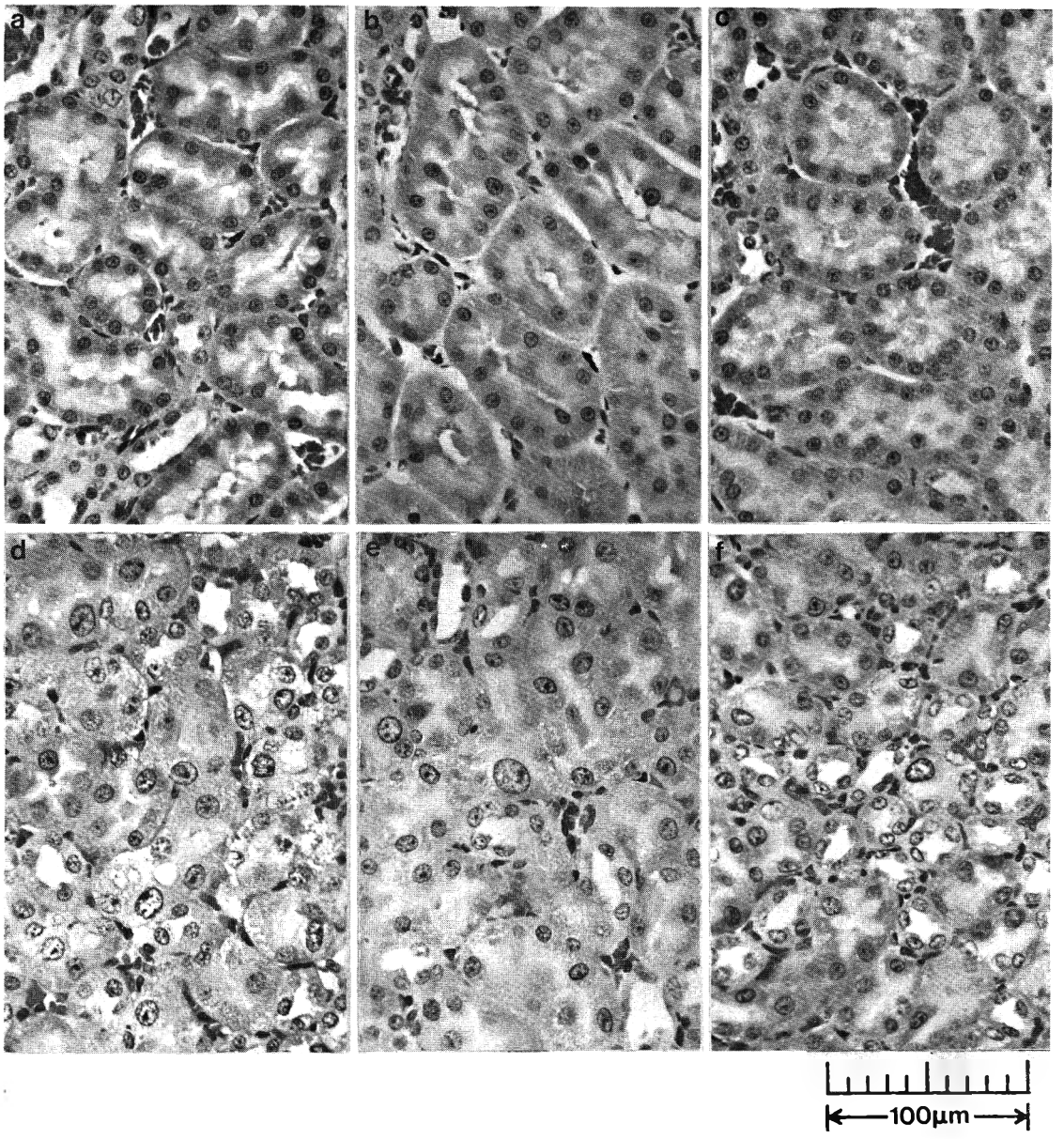

Fig. 1. Histological appearance of the renal outer medullary stripe of rats fed untreated or alkali-treated soya protein or lactalbumin for 8 wk (a) 20% untreated soya protein, Sprague-Dawley strain (b) 20% untreated soya protein, Wistar strain (c) 20% untreated lactalbumin, Sprague-Dawley strain (d) 20% alkali-treated soya protein ($ATSI_3$), Sprague-Dawley strain (e) 20% alkali-treated soya protein ($ATSI_3$), Wistar strain (f) 20% alkali-treated lactalbumin (ATL), Sprague-Dawley strain.

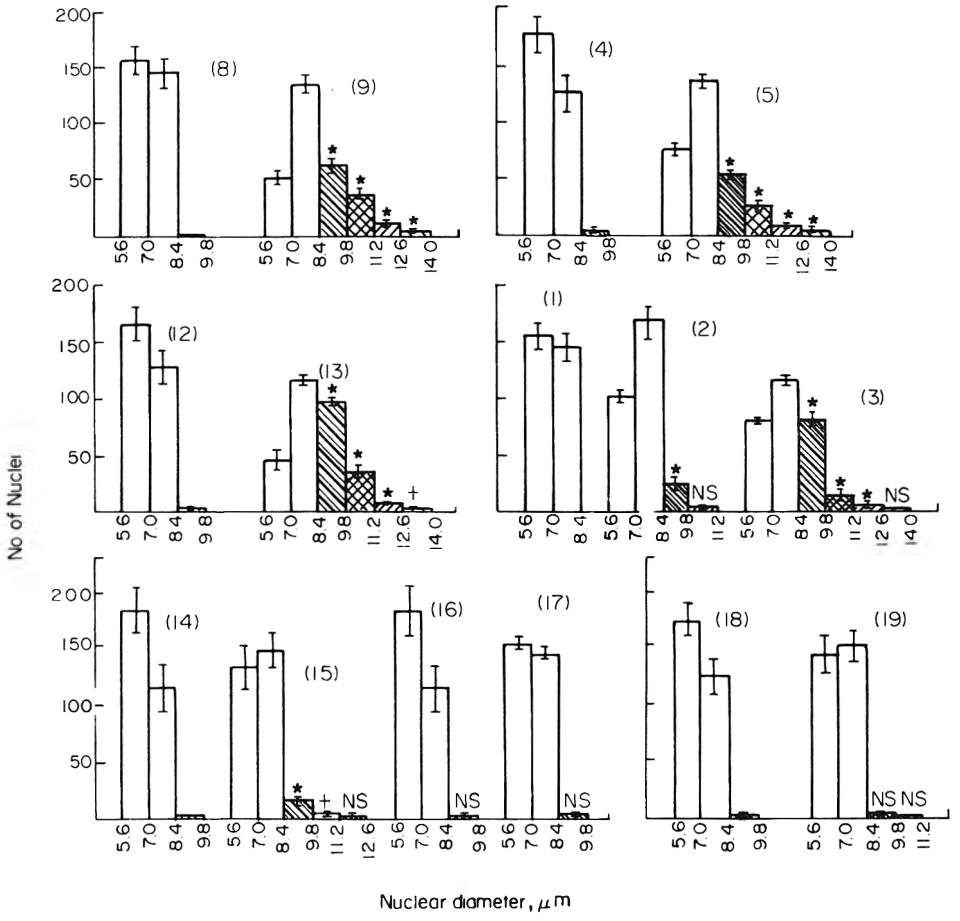

Fig. 2. Size distribution of *pars recta* nuclei in 5 μm kidney sections from rats fed 20% untreated or alkali-treated protein for 8 wk. Data are normalized to 300 nuclei/animal. The histograms correspond to the group numbers given in brackets. See Table 1 for details of each group. Bars indicate $\pm$ one standard error. Significant increases in the number of nuclei in size groups above 8·4 μm, compared with the same size group in the untreated controls, are indicated: $^{*}P < 0{\cdot}01$; $^{\dagger}P < 0{\cdot}05$ (nonparametric two-tailed comparison by rank-sum test (Wine, 1964)).

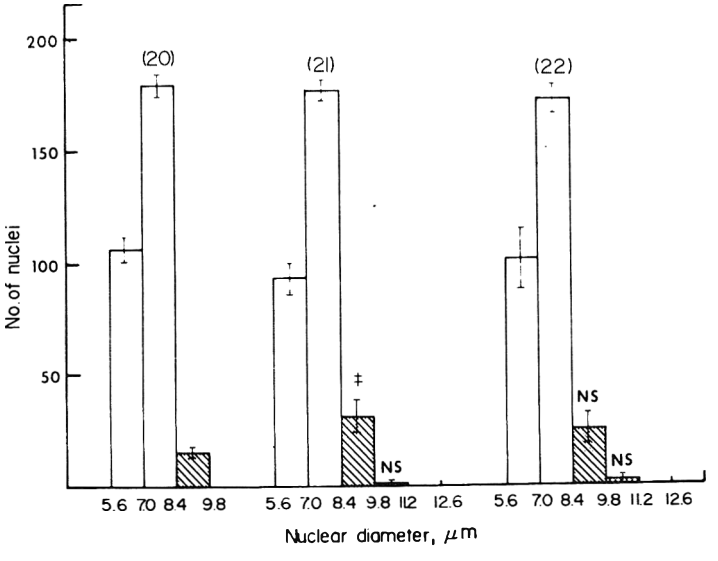

Fig. 3. Size distribution of *pars recta* nuclei in mice fed untreated soya protein, alkali-treated soya protein ($ATSI_3$), or a mixture of $ATSI_3$ with untreated lactalbumin for 8 wk. See Table 1 for details of the group numbers given in brackets. Bars represent one standard error. The result marked with a double dagger was not significantly different from control at $P < 0{\cdot}05$ by either nonparametric rank-sum test or by Student's *t*-test; however, $P < 0{\cdot}05$ in a one-tailed rank-sum test and $P \sim 0{\cdot}075$ by Student's two-tailed *t*-test.

Table 2. *Protein efficiency ratios and digestibility data for alkali-treated lactalbumin and soya isolate*

Dietary source of protein	Final body weight† (g)	Total feed consumption (g)	Protein efficiency ratio*		Digestibility* (%)	
			Actual	Adjusted	Diet	Nitrogen
ANRC protein	212 ± 15^{A}	430 ± 37^{A}	3·65 ± 0·06^{AB}	2·50	95	94
Lactalbumin	199 ± 13^{A}	375 ± 30^{A}	3·83 ± 0·05^{A}	2·62	95	90
ATL‡	107 ± 4^{B}	216 ± 10^{B}	2·42 ± 0·10^{C}	1·66	95	81
Soya isolate	201 ± 11^{A}	420 ± 26^{A}	3·47 ± 0·09^{B}	2·38	94	90
$ATSI_3$	108 ± 2^{B}	275 ± 11^{B}	1·92 ± 0·07^{D}	1·32	94	82

ANRC protein = Reference casein, approved by the Animal Nutrition Research Council
ATL = Alkali-treated lactalbumin $ATSI_3$ = Alkali-treated soya protein isolate containing 1·27% lysinoalanine)
Values were compared using Duncan's multiple comparison test. Means without a superscript letter in common are significantly different, $P < 0·01$.
*The protein efficiency ratio assay was 28 days. Digestibility data was from day 6 through day 13 of the test, means ± SEM.
†Five male weanling rats per group, Sprague-Dawley strain, initial age 21 days, mean initial weight = 55 grams.
‡Entire group had diarrhoea beginning on day 13.

availability of particular amino acids more than others. This would be very difficult, if not impossible, to mimic in control diets that are not alkali-treated.

In mice, the 20% $ATSI_3$ diet resulted in significantly ($P < 0·01$) reduced these indices in both lactalbumin and soya isolate. The PER of lactalbumin was (Fig. 3). The observed incidence of nuclei with diameters exceeding 8·4 μm was more than doubled over the group fed untreated soya protein however, and the significance level was less than 0·05 in a one-tailed, but not in a two-tailed comparison using the rank-sum test (Wine, 1964). It is noteworthy that Feron, van Beek, Slump & Beems (1977) report observing nephrocytomegaly in Swiss mice fed high levels of free lysinoalanine.

The feed efficiency of the animals fed alkali-treated proteins was always lower than that of the control animals (Table 1). PER and digestibility determinations (Table 2) showed that alkali treatment significantly ($P < 0·01$) reduced these indices in both lactalbumin and soya isolate. The PER of lactalbumin was reduced by 37% and that of soya isolate by 45%. The nitrogen digestibility of the proteins was reduced by the alkali treatment, from an initial value of 90%, to 81% and 82% for lactalbumin and soya protein, respectively. Alkali treatment reduced the PER value considerably more than the digestibility. This is in agreement with the finding of de Groot & Slump (1969) who reported that decreased net protein utilization was the earlier and more pronounced finding in alkali-treated proteins. The animals fed the alkali-treated soya protein test diet used in the pair-feeding experiment (Table 1, groups 12 and 13) excreted almost twice as much faecal nitrogen as did animals on the corresponding untreated soya protein diet (Table 3). These pair-fed groups had uncorrected nitrogen digestibility values of 89% and 94%, respectively.

Approximately 30% of the test animals developed diarrhoea, which began on the second day on diet, and improved after 2–3 wk. Animals fed the diets containing Alphacel did not develop diarrhoea. Diarrhoea was more severe in the animals fed alkali-treated lactalbumin than in those fed alkali-treated soya protein. It persisted up to the third to fourth week on the diet and became less severe thereafter.

Approximately 30% of the animals fed alkali-treated soya protein, but not those fed alkali-treated lactalbumin, suffered loss of hair (alopecia). Alopecia developed in the third week and ranged from severe (three quarters of the body surface) in some animals, to patches of less than one-fifth of the body surface. After 6–7 wk on the diet all animals had recovered and the hair had grown back. The distribution pattern of diarrhoea and alopecia among the cages and the pattern of occurrence in experiments performed at different times made the possibility of an infectious agent unlikely. Diarrhoea in rats fed alkali-treated soya isolate in diets similar to those we used has been reported (Van Beek, Feron & de Groot, 1974). Hair-loss similar to that observed has been reported in rats fed lysine-deficient diets (Gershoff *et al.* 1958). The onset of the hair-loss reported was at the third week as it was in our experiments. None of the animals on the alkali-treated lactalbumin diet suffered any hair-loss. The alkali-treated lactalbumin was of better nutritional quality than alkali-treated soya protein having a 17% higher PER value than the treated soya protein. Amino acid determination (Table 4) showed that alkali-treated lactalbumin contained higher amounts of most of the essential amino acids than $ATSI_3$. It also contained almost twice as much lysinoalanine. The difference in arginine content was eliminated by supplementing lactalbumin with 0·4% L-arginine–HCl. It appears that deficiency or imbalance of certain amino acids in the alkali-treated soya protein was poorly tolerated during the period of most rapid growth and became less important later, as evidenced by the recovery from alopecia. Test animals had a poor appearance, but no deaths or other abnormalities were observed. Gross necropsy findings were essentially negative.

Clinical chemistry and haematology

Animals on the alkali-treated protein diets exhibited significantly elevated levels of plasma GPT and of blood urea nitrogen (BUN, Table 5). The elevation occurred in both the Sprague-Dawley and Wistar strains. In the animals fed alkali-treated lactalbumin, there were no statistically significant changes in the GPT or BUN levels. In a group of three animals

Table 3. *Urinary and faecal lysinoalanine excretion and faecal nitrogen, fat and ash excretion in Sprague-Dawley rats ingesting alkali-treated soya protein or alkali-treated lactalbumin**

Parameter	20% Soya isolate†	20% Alkali-treated soya isolate†	20% Alkali-treated lactalbumin	10% Lactalbumin 10% Alkali-treated lactalbumin
Dietary LAL (ppm)	0	2630	4970	2475
LAL intake (mg/day)	—	41 ± 3·3 (5)‡	72§	40§
Urinary LAL excretion‖ (unhydrolyzed, mg/day)	—	0·50 ± 0·12 (3)	2·7 ± 0·70 (4)	0·37 (2)
Urinary LAL excretion (individual samples, before/after acid hydrolysis, mg/day)	—	—	4·7/5·0; 2·2/3·2	0·79/1·1; 0·78/0·98
Faecal LAL excretion (hydrolyzed, mg/day)	—	14 ± 2·3 (5)	—	—
Faecal nitrogen excretion (mg/day)	29 ± 4 (5)	55 ± 4 (5)¶	—	—
Faecal fat excretion (mg/day)	63 ± 6 (5)	52 ± 5 (5)	—	—
Faecal ash (mg/day)	199 ± 21 (5)	187 ± 16 (5)	—	—

LAL = Lysinoalanine

*Urinary excretion data were obtained after 36–37 days on the diet specified. Rats were approximately 8 wk old at this time. Faecal excretion data were obtained from five pair-fed rats per group from samples collected during the 38th to 48th day on the diet.

†Pair-fed groups.

‡Average intake during the 10-day period of faeces collection.

§Average intake during the 7 days preceding urine collection, determined on a group of seven rats including those from which urine was collected.

‖Measured during a 12-hr period following removal of the diet.

¶ $P < 0·002$ compared with group fed untreated soya isolate.

which was fed $ATSI_3$ for 8 wk and then returned to laboratory chow at week 9 the BUN returned to the control value by week 12, but the GPT value was still significantly higher at the $P < 0·05$ level (data not shown). Total plasma protein values were significantly lower in the $ATSI_3$ fed rats after 8 wk on the diet (Table 5). Plasma albumin levels were also generally lower in the groups receiving alkali-treated protein (Table 5), but these values were not statistically significantly different from control values, implying that the plasma globulin values (which were not measured) were also lower in these rats.

Plasma glutamic–oxaloacetic transaminase activity (GOT) values were not elevated in animals fed alkali-

Table 4. *Amino acid composition of soya isolate and lactalbumin before and after alkali treatment with 0·1 N-NaOH at 60°C*

	Amino acid content (μmol/0·16 g N)			
	Soya isolate		Lactalbumin	
Amino acid	Untreated	0·1 N-NaOH 60°C, 8 hr ($ATSI_3$)	Untreated	0·1 N-NaOH 60°C, 1 hr 20 min
Asp	964	935	944	964
Thr	327	312	480	470
Ser	535	505	485	473
Glu	1474	1356	1226	1174
Pro	513	478	473	453
Gly	584	563	326	320
Ala	477	476	640	638
Val	436	447	536	547
Met	117	101	181	159
Cys	122	39	291	107
Ileu	390	389	446	443
Leu	660	662	1026	1075
Tyr	222	223	241	244
Phe	347	347	249	242
LAL	—	57	—	122
His	170	160	141	115
Lys	450	356	686	541
Arg	479	448	197	133

Table 5. *Blood chemistry of Wistar and Sprague-Dawley rats fed alkali-treated soya protein isolate or alkali-treated or untreated lactalbumin at a dietary level of 20%*

Group no. †	Dietary protein	Weeks on diet	No. of rats/group	GPT (mU/ml)	BUN (mg % N)	Total protein (g%)	Albumin (g%)
				Wistar rats			
4	UTSI	8	10	$11 \pm 0{\cdot}5^{ef}$	$14 \pm 1{\cdot}03^{cd}$	$6{\cdot}9 \pm 0{\cdot}10^{bcd}$‖	$2{\cdot}9 \pm 0{\cdot}11^{abc}$
5	$ATSI_3$	8	10	$^{***}29 \pm 2{\cdot}0^{a}$	$^{**}19 \pm 0{\cdot}98^{a}$	$^{***}6{\cdot}0 \pm 0{\cdot}17^{e}$‖	$^{NS}2{\cdot}6 \pm 0{\cdot}09^{cd}$‖
6	UTSI	12	5	$15 \pm 1{\cdot}7^{def}$	$13 \pm 0{\cdot}92^{cd}$	$7{\cdot}6 \pm 0{\cdot}24^{ab}$¶	$3{\cdot}0 \pm 0{\cdot}13^{abc}$
7	$ATSI_3$	12	6	$^{***}27 \pm 1{\cdot}4^{ab}$	$^{NS}15 \pm 2{\cdot}16^{bcd}$	$^{NS}7{\cdot}0 \pm 0{\cdot}20^{abcd}$	$^{NS}2{\cdot}8 \pm 0{\cdot}04^{bcd}$
				Sprague-Dawley rats			
8	UTSI	8	10	$13 \pm 0{\cdot}6^{ef}$	$14 \pm 0{\cdot}83^{cd}$	$6{\cdot}7 \pm 0{\cdot}09^{cd}$	$2{\cdot}7 \pm 0{\cdot}07^{bcd}$
9	$ATSI_3$	8	10	$^{***}23 \pm 2{\cdot}4^{bc}$‡	$^{**}18 \pm 1{\cdot}12^{ab}$‡	$^{***}6{\cdot}1 \pm 0{\cdot}07^{e}$‡	$^{NS}2{\cdot}7 \pm 0{\cdot}10^{bcd}$
10	UTSI	12	5	$18 \pm 2{\cdot}1^{cd}$	$13 \pm 0{\cdot}96^{cd}$	$7{\cdot}4 \pm 0{\cdot}26^{ab}$	$3{\cdot}1 \pm 0{\cdot}08^{ab}$
11	$ATSI_3$	12	5	$^{*}28 \pm 2{\cdot}6^{a}$	$^{NS}16 \pm 0{\cdot}97^{abc}$	$^{NS}6{\cdot}4 \pm 0{\cdot}40^{de}$††	$^{NS}2{\cdot}7 \pm 0{\cdot}21^{bcd}$¶
12	UTSI§	8	5	$9 \pm 1{\cdot}1^{f}$	$11 \pm 1{\cdot}1^{d}$	$6{\cdot}5 \pm 0{\cdot}20^{de}$	$2{\cdot}9 \pm 0{\cdot}17^{bcd}$
13	$ATSI_3$	8	5	$^{***}19 \pm 0{\cdot}8^{cd}$	$^{*}16 \pm 1{\cdot}5^{abc}$	$^{NS}6{\cdot}0 \pm 0{\cdot}08^{e}$	$^{NS}2{\cdot}5 \pm 0{\cdot}11^{d}$
14	UTL	8	7	$12 \pm 0{\cdot}8^{ef}$	$14 \pm 0{\cdot}97^{cd}$	$7{\cdot}1 \pm 0{\cdot}23^{abc}$	$3{\cdot}1 \pm 0{\cdot}13^{ab}$
15	ATL	8	7	$^{NS}16 \pm 1{\cdot}6^{de}$	$^{NS}13 \pm 0{\cdot}90^{cd}$	$^{NS}6{\cdot}8 \pm 0{\cdot}12^{cd}$	$^{NS}3{\cdot}0 \pm 0{\cdot}10^{abc}$
17	UTL + Alphacel (1:1)	8	6	$^{***}21 \pm 1{\cdot}5^{cd}$	$^{NS}12 \pm 0{\cdot}86^{cd}$	$^{NS}7{\cdot}0 \pm 0{\cdot}25^{bcd}$	$^{NS}3{\cdot}3 \pm 0{\cdot}20^{a}$

GPT = Glutamic–pyruvic transaminase BUN = Blood urea nitrogen UTSI = Untreated soya protein isolate
$ATSI_3$ = Alkali-treated soya protein isolate (containing 1·27% lysinoalanine)
UTL = Untreated lactalbumin ATL = Alkali-treated lactalbumin

Values are means ± SEM. Letter superscripts indicate groups not significantly different by Duncan's Multiple Range Test. Means without a superscript in common differ at the 5% confidence level. Nonhomogeneous 'outlier' values are excluded from the table and indicated in the footnotes below. Significant differences of each group from its corresponding control group (fed the identical untreated diet for the same time) by the Student's *t*-test are indicated by the superscript to the left of the mean value: $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$; NS, not significantly different ($P > 0{\cdot}05$).

†See Table 1.

‡Data exclude one rat for which the GPT value was 115 mU/ml and the BUN value was 44 mg%. Total protein value of group 9 was based on eight samples.

§Pair-fed with group 13.

‖n = 9 (insufficient sample from one rat in group).

¶n = 4.

††n = 3.

treated protein, with the exception of a single Sprague-Dawley rat fed $ATSI_3$ for 8 wk, which had markedly elevated GOT, GPT, and ornithine carbamoyl transferase (OCT) values. With the exception of this single animal, OCT values did not differ significantly among test groups. No significant differences were noted in the liver *N*-demethylase activity between groups fed alkali-treated rather than untreated protein.

Red and white blood cells counts made on Sprague-Dawley and Wistar rats fed $ATSI_3$ or untreated soya isolate after 2, 11, 23, 44 and 56 days on the diet, and on pair-fed Sprague-Dawley rats fed the same diet for 56 days, did not reveal any differences among dietary groups. A lower value for the white blood cell count in Sprague-Dawley rats fed 20% $ATSI_2$ ($3{\cdot}6 \pm 0{\cdot}4 \times 10^3/mm^3$, n = 5) than in those fed untreated soya isolate ($6{\cdot}2 \pm 0{\cdot}4 \times 10^3/mm^3$, n = 5) had been observed in a previous experiment, but a similar difference was not observed in this, more extensive, experiment.

Urinary and faecal lysinoalanine excretion

A 10-day balance study was performed to determine the fate of ingested lysinoalanine in rats (Table 3). Animals fed alkali-treated soya protein were found to excrete more faecal nitrogen and slightly less fat than pair-fed controls. The nitrogen data are in agreement with the reduced PER and digestibility values determined in different groups of animals. Approximately 33% of the ingested lysinoalanine was excreted in the faeces. All faecal lysinoalanine was bound, since acid hydrolysis was required to release faecal lysinoalanine. The greatest part of the remaining 67% of the ingested lysinoalanine could not be accounted for.

Liver and blood analysis did not reveal any measurable concentration of lysinoalanine. In the kidneys of animals fed alkali-treated soya protein for 8 wk, however, a small storage of lysinoalanine was found, which accounted for approximately 0·6% of the lysinoalanine ingested in one day or 1·7% of the amount of lysinoalanine disappearing from the gut per day.

When some animals on the $ATSI_3$ diet were changed to a lysinoalanine-free diet and the urinary lysinoalanine was measured, it was found that lysinoalanine excretion continued up to 37–75 hr after the change (Fig. 4). The rate of excretion was very small after the tenth hour. The total quantity excreted accounted for 0·7–2·7% of the quantity disappearing from the gut daily.

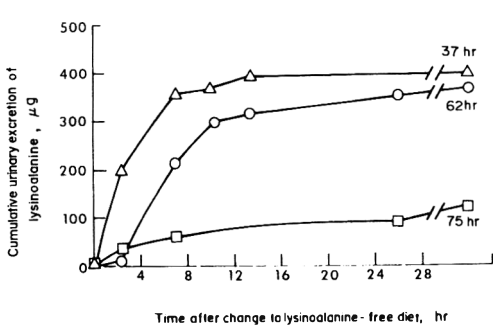

Fig. 4. Urinary excretion of lysinoalanine in Sprague-Dawley rats fed alkali-treated soya protein diet and then changed to a lysinoalanine-free diet. Each curve represents a different animal.

When the urinary lysinoalanine excretion was measured over a 12-hr period in rats fed diets containing alkali-treated lactalbumin, $ATSI_3$, or mixture of untreated and alkali-treated lactalbumin providing varying amounts of lysinoalanine, it was found that the amount of lysinoalanine excreted increased with dietary lysinoalanine levels (Table 3). Rats fed alkali-treated lactalbumin had the highest dietary lysinoalanine content and excreted the highest amount of lysinoalanine. This is noteworthy since it suggests that lysinoalanine is at least as well-absorbed from alkali-treated lactalbumin, which induced very little karyomegaly, as from alkali-treated soya protein. The quantity of lysinoalanine excreted accounted for 1–7% of that ingested per day. There was a wide variability in the amount of lysinoalanine excreted among the animals tested.

Most of the urinary lysinoalanine was free. A small increase in the urinary lysinoalanine was observed after acid-hydrolysis of the urine (Table 3) indicating that a portion of the urinary lysinoalanine was in a bound form. Evidence has been presented (Finot, Bujard & Arnaud, 1976) that some of the lysinoalanine is acetylated in the rat kidney before excretion.

Urinary and faecal excretion and storage in the kidney only accounted for approximately 35% of the ingested lysinoalanine. The remainder could be metabolized either in the animal's body or by the intestinal flora. Experiments with ^{14}C labelled lysinoalanine (Finot *et al.* 1976) indicated that the intestinal flora extensively metabolized lysinoalanine to CO_2 which was expired; and Leegwater (1978) has recently reported a previously unidentified major urinary metabolite.

DISCUSSION

The above results help to clarify a number of issues regarding the relationship between dietary lysinoalanine content and the development of nephrocytomegaly.

We, like Woodard and co-workers (Woodard, 1969; Woodard & Short, 1973; Reyniers, Woodard & Alvarez, 1974; Woodard *et al.* 1975) and Newberne and co-workers (Newberne, Rogers & Wogan, 1968; Newberne & Young, 1966), have consistently observed nephrocytomegaly in rats fed alkali-treated soya protein (containing 0·69% to 1·3% lysinoalanine) at a level of 20% in the diet. Until recently, de Groot and co-workers at the Central Institute for Nutrition and Food Research (CIVO), Zeist, The Netherlands, had consistently reported finding no nephrocytomegaly when similarly-treated soya protein of equal lysinoalanine content was fed to rats (de Groot & Slump, 1969; de Groot *et al.* 1976; de Groot, Slump, van Beek & Feron, 1976). A number of possible explanations for this discrepancy have been discussed in the literature (Feron *et al.* 1977; Gould & MacGregor, 1977; O'Donovan, 1976; Struthers, Dahlgren & Hopkins, 1977). In an attempt to resolve this discrepancy, we supplied Feron *et al.* with a sample of alkali-treated soya protein that we had found to produce nephrocytomegaly in our laboratory ($ATSI_3$, Table 1).

This sample contained no detectable free lysinoalanine (less than 10 ppm) and a very low level (approximately 60 ppm) of trichloroacetic-acid-extractable (low-molecular-weight) material (L. van Beek, personal communication, 1977). Their results with this protein ($ATSI_3$) that we supplied have been published (Feron *et al.* 1977). They fed this protein to CIVO Wistar-derived rats, incorporated into the diet routinely used at the CIVO and in a diet essentially identical with that which we used in the present work.

Nephrocytomegaly was observed in rats fed the latter diet, but only slight nephrocytomegaly in one of five rats fed this protein at the same level in the CIVO diet. Since both diets contained 2630 ppm lysinoalanine, supplied by the identical protein, it is clear that the composition of the diet was a major determinant of the development of nephrocytomegaly.

Feron *et al.* (1977) believe the dietary difference responsible for preventing the development of nephrocytomegaly to be the addition of 10% untreated casein to the CIVO diet. This idea is supported by our present findings with this same alkali-treated soya protein (Table 1, Fig. 2). We observed marked nephrocytomegaly. Further, the relatively high lysinoalanine yield from milder alkali treatment of lactalbumin at a dietary level of 12% of this protein, supplemented with an additional 8% untreated lactalbumin, provided 1752 ppm lysinoalanine in the diet but did not induce nephrocytomegaly, even though identically treated soya protein providing 1370 ppm did induce nephrocytomegaly. Further, the relatively high lysinoalanine yield from milder alkali treatment of lactalbumin permitted feeding a much better quality protein with a much higher lysinoalanine content than that of the alkali-treated soya protein. Only minimal *pars recta* karyomegaly was found in rats fed 20% alkali-treated lactalbumin providing 4970 ppm dietary lysinoalanine, while 10% alkali-treated lactalbumin with 10% untreated lactalbumin did not produce nephrocytomegaly despite a dietary lysinoalanine content of 2490 ppm. Thus, there is clearly no simple correlation between dietary lysinoalanine content and the degree of nephrocytomegaly observed. We believe all the above findings are consistent with the hypothesis that a diet with an adequately balanced protein source has a marked protective effect against the development of nephrocytomegaly from alkali-treated protein. It is not yet known if these findings extend to free lysinoalanine.

We think it unlikely that the lysinoalanine in the lactalbumin used was less available to the rat than the lysinoalanine in the soya protein because the nitrogen digestibility of alkali-treated lactalbumin and soya protein were comparable (Table 2), and the urinary lysinoalanine excretion was considerably higher in rats fed alkali-treated lactalbumin than in those fed alkali-treated soya protein (Table 3). The development of nephrocytomegaly, then, is dependent on a number of factors beyond simple available dietary lysinoalanine content. A number of these factors have previously been discussed in detail (Gould & MacGregor, 1977).

The strain difference between the CIVO Wistar-derived rats used by de Groot and co-workers and the Sprague-Dawley-derived rats used by Woodard and co-workers, by Newberne and co-workers, and by ourselves is apparently not an important factor in the divergent findings reported by these groups in the past. As mentioned above, Feron *et al.* (1977) found nephrocytomegaly in CIVO Wistar-derived animals when $ATSI_3$, supplied by us was incorporated into a diet essentially identical with that which we used; they did not, however, observe nephrocytomegaly when this same protein was incorporated into the CIVO standard diet used in the earlier negative studies at CIVO. The reason for the failure of the CIVO group to observe nephrocytomegaly in the past must therefore be attributed to the diet used and not to an insensitivity of CIVO rats. Further, we found an identical shift in the size distribution of *pars recta* nuclei in the affected outer medullary stripe* of Wistar and Sprague-Dawley rats when the same $ATSI_3$-containing diet was fed to both strains (Fig. 2). Feron *et al.* (1977) also compared the sensitivity to free lysinoalanine of Sprague-Dawley and two lines of Wistar-derived rats used at the CIVO and found no major differences.

Struthers *et al.* (1977) have reported finding a marked species difference between Sprague-Dawley and Wistar rats fed alkali-treated soya protein. There were certain differences in the method of preparation of the alkali-treated protein, and in the composition of the experimental diet, employed by these workers and that used by ourselves and others cited above. The description of the lesions observed by these workers is somewhat at variance both with the morphological appearance of the lesions we observed in these experiments and with those described by Woodard (1969) and Woodard & Alvarez (1967). Nonetheless, their results indicate that at least certain lines of a particular strain may vary in their sensitivity to alkali-treated protein-induced renal alterations. This, however, is almost certainly not the reason why the CIVO group failed to observe nephrocytomegaly in their earlier experiments.

Our negative findings in mice are consistent with the known insensitivity of mice to lysinoalanine-induced nephrocytomegaly (de Groot *et al.* 1976). It is noteworthy, however, that high dietary levels of free lysinoalanine do induce nephrocytomegaly in mice (Feron *et al.* 1977), and that the relatively sensitive method of scoring of karyomegaly which we used did reveal an incidence of nuclei with a diameter greater than 8·4 μm which was more than twice as high in the mice receiving alkali-treated soya protein as that in the mice receiving the untreated protein. The probability that a variation of this size could have occurred by chance was very close to the 5% conventionally accepted as statistically significant. It may be that a very slight degree of karyomegaly was in fact produced in the mice, and that it could be observed as statistically significant if larger groups of animals were used, or if more cells were scored per animal. It should be noted that the method by which we score the degree of karyomegaly is very much more sensi-

*Note that the most widely accepted term for the zone of the kidney in which the *pars recta* predominates is the outer stripe of the outer medulla, or the outer medullary stripe (Tisher, 1976; Trump & Bulger, 1968; Woodard *et al.* 1975). This zone has been referred to by some authors as the inner stripe of the cortex.

tive than the subjective comparisons used by others in the past. Further, the feeding time we used in the experiments with mice was arbitrarily set at the time of the maximum expression of karyomegaly in the rat, and is not necessarily the optimal time to observe this response in the mouse. It would be informative to examine larger groups of mice and record the results as a function of the time during which the alkali-treated protein diet was fed using the method of scoring karyomegaly used in these experiments.

The reduced feed efficiency and poor growth performance of the animals fed alkali-treated soya protein is consistent with previously reported findings (de Groot & Slump, 1969), but the altered blood-chemistry values we observed have not previously been noted. The significance of the elevated GPT and BUN values and the decreased plasma protein in the animals fed alkali-treated soya protein is not entirely clear. Elevated GPT can be indicative of liver or kidney damage, but is not specific (Mattenheimer, 1971). Restricting food to one half the *ad lib.* level has been reported to result in increased GPT in rats (Schwartz, Tornaben & Boxill, 1973). We observed an elevation of GPT similar to that observed in the rats fed alkali-treated soya protein when half of the dietary protein was replaced with Alphacel (Table 5, Group 17), but not in animals pair-fed untreated soya protein at a dietary intake equal to that of the *ad lib.* quantity ingested by the rats fed alkali-treated soya protein (Table 5, Group 12). Neither of these two groups had elevated BUN or decreased plasma protein values. Nevertheless, it is possible that these changes are due to the altered nutritional balance of the diets used rather than to organ-specific effects of lysinoalanine or other compounds formed in the alkali-treated protein.

The nature of the decreased nutritional quality of the alkali-treated soya protein, and the relationship of the development of nephrocytomegaly to the available amino-acid balance in the diet, are not well understood. The particular amino acid or combination of amino acids responsible for the prevention of the kidney abnormalities, and how it relates to the mechanism of induction of the lesion attributed to lysinoalanine, remains to be established. Newberne & Young (1966), and Newberne *et al.* (1968) showed that supplementation with methionine, choline and vitamin B_{12} could prevent similar kidney lesions produced by Alpha Protein, a protein we now know to have contained lysinoalanine and to have had a nutritionally inadequate amino acid composition. Woodard & Alvarez (1967) could not confirm these findings with alkali-treated soya protein, nor could van Beek (personal communication, 1977) demonstrate a reduction in the severity of the nephrocytomegaly induced by free lysinoalanine by supplementing with these nutrients.

Chemical analyses of our alkali-treated soya protein and alkali-treated lactalbumin (Table 4) showed that the alkali-treated lactalbumin had higher lysine, leucine, methionine, threonine, valine, isoleucine and tyrosine than did the alkali-treated soya protein. In addition to these differences, the animals fed alkali-treated lactalbumin had a higher food intake than did the animals fed the alkali-treated soya protein, thereby accentuating the higher nutrient intake. Hair loss, which has been reported to result from lysine deficiency (Gershoff *et al.* 1958), was observed in the animals fed alkali-treated soya protein, but not in those fed alkali-treated lactalbumin. Chemical analysis, however, is not always a good indicator of nutritional damage. For example, de Groot & Slump (1969) found the nutritional damage from hot alkali treatment of soya protein to be greater than could be accounted for by either losses in essential amino acids or decreased overall nitrogen digestibility. Nonetheless, the above amino acids which differ markedly in the alkali-treated soya protein and alkali-treated lactalbumin should be systematically examined to determine whether the balance of these amino acids modulates the development of nephrocytomegaly, and therefore explains the marked difference in the severity of the lesions obtained with these two proteins. We have already found that supplementation of the diet used in this study with a combination of 0·8% L-lysine, 0·55% L-arginine and 0·45% L-threonine did not significantly diminish the severity of the karyomegaly after 8 wk on the 20% alkali-treated soya protein diet (unpublished results).

It should also be mentioned explicitly that we do not consider it proved that the lysinoalanine formed in the alkali-treated protein is the sole, or necessarily even the principal, factor responsible for the induction of nephrocytomegaly. The present evidence does, however, favour the hypothesis that lysinoalanine is the factor in alkali-treated protein that induces nephrocytomegaly, but that nutritional factors strongly modulate the development of the lesions. Recent findings, however, demonstrate that lysinoalanine is not unique among the potential products of alkaline treatment in its ability to induce *pars recta* lesions with a similarity to the lesions induced by alkali-treated proteins. Ornithinoalanine (Feron *et al.* 1977), fructoselysine (Erbersdobler, von Wangenheim & Hänichen, 1978) and D-serine (D. H. Gould & J. T. MacGregor, unpublished data) all produce lesions of the *pars recta* with certain features in common with the lysinoalanine-induced lesion. As discussed previously (Gould & MacGregor, 1977), other products of alkali treatment could also play a role in the development of nephrocytomegaly. The problem is obviously complex, and it may be some time before a full appreciation of all the factors governing the development of nephrocytomegaly in animals ingesting alkali-treated proteins is realized.

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ACUTE TOXICITY OF PATULIN AND ITS INTERACTION WITH PENICILLIC ACID IN DOGS

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Abstract—The acute toxicity of iv-administered patulin and its interaction with ip-injected penicillic acid were investigated in male dogs. The LD_{50} of patulin was estimated to be 10·4 mg/kg (95% confidence limits 7·2–15·1 mg/kg). Patulin had severe effects in dogs, particularly on the lungs. Lethargy, anorexia, haematemesis, diarrhoea, pulmonary haemorrhages and oedema, and luminal haemorrhages of the gastro-intestinal tract were common features in dogs given patulin in a dose of 10 mg/kg or more. Sublethal doses (7·5 mg/kg or less) did not have drastic pulmonary effects. Enhancement of patulin toxicity by penicillic acid was indicated by the occurrence of deaths in dogs exposed simultaneously to sublethal doses of both mycotoxins, by the presence in these dogs of toxic signs and lesions resembling those elicited by a single lethal dose of patulin given alone, and by the presence of pulmonary histopathology in dogs receiving both toxins at levels that, given alone, produced no such lesions. Elevated serum alkaline phosphatase in dogs given the mycotoxin combination also suggested possible interaction between the two toxins.

INTRODUCTION

Patulin and penicillic acid, secondary fungal metabolites produced by members of the genus Penicillium and Aspergillus, were originally isolated as antibiotics but proved too toxic for clinical use (Stansfield, Francis & Stuart-Harris, 1944). Subsequently, both have been found in mouldy feeds (Wilson, 1976) and in cheese stored at 5°C (Bullerman, 1976). In addition, patulin has been identified in rotten fruits, apple juice and mouldy bread (Wilson, 1976), while penicillic acid occurs in blue-eyed diseased corn (Kurtzman & Ciegler, 1970). The striking similarity between the two mycotoxins (Broom, Bulbring, Chapman, Hampton, Thomson, Ungar, Wein & Woolfe, 1944; Murnagan, 1946) with regard to their interaction with sulphydryl groups and in the kinetics of enzyme inhibition (Ashoor & Chu, 1973a,b; Chan, Phillips & Hayes, 1979; Phillips & Hayes, 1977) suggested the need to investigate their toxic interaction.

Dogs are extremely sensitive to mycotoxins, including aflatoxin B_1 (Wilson, Teer, Barney & Blood, 1967), ochratoxin A (Szczech, Carlton & Tuite, 1973), citrinin (Kitchen, Carlton & Tuite, 1977) and rubratoxin B (Hayes & Williams, 1977). Although several studies have suggested an interaction between mycotoxins in the dog (Hayes, Unger & Williams, 1977; Hayes & Williams, 1977; Kitchen *et al.* 1977), no significant interaction was reported between penicillic acid and rubratoxin B (Hayes *et al.* 1977). Studies of mycotoxin–mycotoxin interactions in other animals have been reported but are limited (Lillehoj & Ciegler, 1975). Since dogs frequently come into contact with moulded rations and with dog food made from contaminated cereal grains, this study was initiated to characterize the toxic effects of patulin in the dog and to investigate possible interactions of this mycotoxin with a sublethal dose of penicillic acid.

EXPERIMENTAL

Animals. Nineteen male dogs of mixed breeding (mean body weight 9·3 kg) were obtained locally and quarantined for 1 wk prior to the beginning of the experiment. During that time routine prophylactic measures were taken. Dogs were housed in air-conditioned concrete-floor isolation pens containing a run. Water was freely available and animals were fed a commercial ration (35 g feed/kg) each morning.

Mycotoxins. Patulin was produced by *Penicillium urticae* NRRL 5256 grown in stationary culture at 25°C in 2·8-litre Fernback flasks containing 500 ml potato-dextrose broth (Norstadt & McCalla, 1969). The supernatant fluids from several 14-day fermentations (16 litres) were combined and concentrated to 1 litre in a pilot-plant evaporator. The concentrate was extracted three times with an equal volume of ethyl acetate, and the solvent extracts were pooled, dried with anhydrous sodium sulphate and evaporated to an oily residue. The residue was transferred to an activated Florisil (60–100 mesh) column (Sigma Chemical Co., St. Louis, MO) and the column was developed with methylene chloride. The eluate was evaporated to dryness and the residual solids were

refluxed with a minimal volume of ether. Patulin crystallized from the ether when it was cooled and left to stand overnight. After three recrystallizations from the same solvent, toxin purity (98·6%) was determined by melting point and spectroscopic analyses.

To produce penicillic acid, 15 litres of a modified Raulin-Thom medium (Bentley & Keil, 1962) was distributed in aliquots of 500 ml per 2·8-litre Fernback flask. The flasks were inoculated with *P. cyclopium* NRRL 1888 and incubated statically at 25°C for 12 days. The mycelium was removed by filtration, and the filtrate was concentrated to 0·5 litre in a pilot-plant vacuum evaporator. The concentrated supernatant fluid was extracted twice, using 1 litre chloroform for each extraction. The chloroform extracts were combined and the solvent was removed by flask evaporation. The residual oily liquid was added to 20 vols cold pentane–hexane and the precipitate was recovered by filtration, crystallized twice from benzene and then crystallized once after refluxing in hexane to which a small amount of benzene had been added. Purity (99%) of the fine white needles was determined by melting point and infra-red and nuclear magnetic resonance spectroscopy. High resolution mass spectral analysis confirmed compound identity in both cases.

The toxins were dissolved in saline (pH 7·2) immediately before use. Patulin was injected iv and penicillic acid ip, as outlined in Table 1. The dosage schedule used for the latter was based on the study of Hayes *et al.* (1977) who reported that 20 but not 10 mg/kg was lethal to dogs. The dosage schedule for patulin was derived from a 14-day iv LD_{50} determination carried out early in the experiment.

Experimental procedures. Daily observations were made for anorexia, somnolence and general appearance and activity. Additional data obtained each day included body weight, rectal temperature and the quantity of food consumed. Normal values for each animal were established by collecting blood samples from the femoral vein once on days 7 and 1 before administration of the toxin(s), and samples were collected from each dog also on days 1, 3, 7 and 14 after the injection. At each sampling time, 12 ml blood was taken and 5 ml of this was added to a tube containing one drop of dipotassium ethylenediaminetetraacetate. White and red blood cell counts, haemoglobin concentration, packed-cell volume (PCV), mean corpuscular volume (MCV) and mean corpuscular haemoglobin concentration (MCHC) of erythrocytes were determined with a Coulter counter, Model S. Differential leucocyte counts were estimated by standard methods (Schalm, 1975). No anticoagulant was added to the remaining 7 ml of blood, the serum from which was analysed for total protein, albumin, calcium, inorganic phosphate, cholesterol, uric acid, creatinine, BUN, total bilirubin, glucose, alkaline phosphatase (EC 3.1.3.1), creatinine phosphokinase (EC 2.7.3.2; CPK), lactic dehydrogenase (EC 1.1.1.27; LDH) and glutamic–oxalacetic transaminase (EC 2.6.1.1; SGOT) by means of a sequential, multiple analyser (SMA-18, Technicon Instruments Corp., Tarrytown, NY). Serum potassium, CO_2, chlorine and sodium were also determined.

Collection of tissues. All the dogs were autopsied. Survivors were killed at the end of 14 days with an overdose of sodium pentobarbital. Animals were examined for gross abnormalities. Tissues (heart, liver, lung, stomach, intestine and kidney) collected for histological examination were fixed in Lavdowsky's solu-

Table 1. *Effects of patulin and penicillic acid given in saline to male dogs and the relationship between dose and survival time*

Dog no.	Initial body weight (kg)	Dose (mg/kg)		Survival time (hr)*	Body-weight† loss (kg)	Weekly food consumption (kg)			Pulmonary lesions‡
		P	PA			Before treatment	After treatment		
							Wk 1	Wk 2	
1	9·98	20	—	8	—	2·1	—	—	Oedema
2	11·34	15	—	4	—	2·4	—	—	Oedema
3	8·60	15	—	5	—	1·8	—	—	Oedema
4	9·07	15	10	24	0·2	1·8	—	—	Oedema
5	7·26	15	10	5	—	1·4	—	—	Oedema
6	11·11	10	—	25	0·2	2·2	—	—	Oedema, atelectasis
7	8·16	10	—	5	—	2·1	—	—	Oedema
8	9·52	10	—	T	0·2	1·1	0·9	1·2	Atelectasis
9	9·07	10	10	5	—	1·6	—	—	Oedema
10	7·48	10	10	5	—	1·2	—	—	Oedema
11	9·30	10	10	T	1·1	2·2	0·2	1·7	Focal pneumonia
12	6·35	7·5	—	T	0·9	1·6	—	0·6	Atelectasis
13	5·90	7·5	—	T	0·5	1·8	0·7	1·2	Atelectasis
14	8·84	7·5	10	5	—	1·6	—	—	Oedema
15	11·79	7·5	10	T	0·5	2·3	1·2	1·5	Oedema
16	12·24	5	—	T	0·2	2·7	1·7	2·1	Atelectasis
17	11·34	5	—	T	0·5	NM	NM	NM	Atelectasis
18	11·56	5	10	T	0·5	2·7	1·7	2·4	Haemorrhage
19	9·30	—	10	T	—	2·1	1·8	2·0	Atelectasis

P = Patulin PA = Penicillic acid NM = Not measured

*Dogs surviving to and killed on day 14, when the experiment was terminated, are indicated by 'T'.

†Values were obtained just before death or at termination.

‡Liver lesions were present only in dogs 1 (centrilobular glycogen depletion), 6 (congested sinusoids and compressed cell cords), 10 (congested sinusoids) and 19 (congested sinusoids).

tion (formaldehyde, ethanol and acetic acid). Other sections of liver were fixed in 10% aqueous formaldehyde for the preparation of frozen sections and staining with Sudan black for fat. Tissues were embedded, sectioned at 8 μm and stained with haematoxylin and eosin or with PASH (Periodic acid–Schiff reagent method plus haematoxylin). The latter method used with and without prior hydrolysis with amylase or diastase permitted demonstration of cytoplasmic glycogen in hepatocytes (Williams, 1951). Cytoplasmic basophilia of the hepatocytes, stained by toluidine blue with and without prior ribonuclease hydrolysis, was used to show RNA (Lillie, 1965).

RESULTS

Patulin

Data presented in Table 1 indicate that a dose of 15 mg patulin/kg was lethal, whereas 7·5 mg/kg or less was not. The 14-day iv LD_{50} value for patulin was 10·4 mg/kg, with 7·2–15·1 mg/kg as the 95% confidence limits (Weil, 1952). Death was limited to the 25-hr period immediately following administration of patulin.

Dogs receiving 10 or 15 mg patulin/kg showed haematemesis and diarrhoea within a few hours of dosing. They stopped eating and became lethargic and laterally recumbent. Within a few hours they developed tachypnoea, with increasing serosanguineous nasal discharge. These dogs had cold extremities and a subnormal rectal temperature and they appeared in shock just before death. In the single dog that recovered (dog 8), these signs disappeared by 24 hr.

All the dogs exposed to 5 or 7·5 mg patulin/kg survived the 14-day experiment. Those given 7·5 mg/kg were anorexic and lethargic; they vomited but showed only slight tachypnoea and minimal nasal-fluid discharge. Animals exposed to the lowest dose (5 mg/kg) appeared normal. Although not drastic, weight loss did occur in all treated animals (Table 1).

Plasma alkaline phosphatase levels were not affected by exposure to patulin (Table 2); however, increases in the activity of SGOT, LDH and CPK occurred at 24 hr in the single survivor receiving 10 mg/kg and of LDH in one of two dogs and CPK in both dogs receiving 7·5 mg patulin/kg (Table 2). A mild depression of total serum protein in all patulin-treated dogs and of serum albumin in dogs given 7·5 mg/kg or more were noted (data not presented). Other serum constituents were not affected. All doses of patulin, however, caused a slight increase in erythrocyte counts, haemoglobin and circulating neutrophils and a concomitant decrease in lymphocytes 24 hr after exposure (data not presented). These values returned to normal in 3–7 days.

Autopsy of dogs that succumbed to patulin treatment consistently revealed accumulation of serosanguineous fluid in the thoracic cavity. Microscopically, massive fluid accumulation was seen also in the lungs. The trachea was filled with a frothy fluid. Congestion and paint-brush haemorrhages of the serosal surfaces of the abdominal organs, luminal (mucosal) haemorrhaging of the stomach and large intestines and patchy haemorrhages of the small intestines were common. The dog that survived 10 mg patulin/kg and was killed 14 days after treatment (dog 8) had no fluid in the respiratory system. No remarkable liver lesions were seen in dog 7 (10 mg/kg), which died within 5 hr. The liver of dog 6, which died 25 hr after receiving 10 mg patulin/kg, showed central venous and sinusoidal congestion, compression of parenchymal cell cords and limited early necrosis. These changes were absent from the liver of dog 8, which survived this dosage. Sublethal doses of patulin left serosal congestion of the abdominal organs and atelectasis as residual effects at 14 days.

Penicillic acid

The dog (19) that received 10 mg/kg of penicillic acid showed slight initial inappetance but did not lose weight (Table 1). As reported earlier (Hayes *et al.* 1977), no remarkable changes were observed in the blood chemistry or haematology except for a marginal depression of LDH (Table 2) and a mild decrease in serum protein and albumin (data not presented). The liver from this dog (19) was dark red with sinusoidal congestion. The serosal lining of the abdominal organs was also congested. Tissues were histologically normal.

Combination of patulin and penicillic acid

Patulin in a dose of 10 mg/kg given alone or in

Table 2. *Changes in serum enzyme activity in dogs 24 hr after exposure to patulin (iv) and/or penicillic acid (ip)*

Dog no.	Dose (mg/kg)		AP			GOT			LDH			CPK		
	P	PA	C*	T†	D‡ (%)	C*	T†	D‡ (%)	C*	T†	D‡ (%)	C*	T†	D‡ (%)
8	10	—	32	31	−3	44	180	+309	223	439	+97	82	318	+288
11	10	10	44	53	+20	48	53	+10	303	501	+65	218	518	+138
12	7·5	—	30	29	−3	72	30	−58	244	240	−2	250	474	+90
13	7·5	—	84	65	−23	50	60	+20	181	498	+175	207	703	+240
15	7·5	10	60	179	+198	54	60	+11	113	307	+172	242	216	−11
16	5·0	—	28	25	−11	46	35	−24	195	245	+26	198	132	−33
18	5·0	10	34	32	−6	43	40	−7	181	123	−32	127	406	+220
19	—	10	51	50	−2	38	40	+5	108	69	−36	126	94	−25

P = Patulin PA = Penicillic acid AP = Alkaline phosphatase GOT = Glutamic-oxalacetic transaminase
LDH = Lactic dehydrogenase CPK = Creatinine phosphokinase C = Control value
T = Test value D = Deviation from control

*Control value (mU/ml) is the mean of the results of tests performed twice before the dogs were treated.
†Test value (mU/ml) is the result of the test performed 24 hr after treatment.
‡Percentage deviation from control = [(test − control value)/control value] × 100.

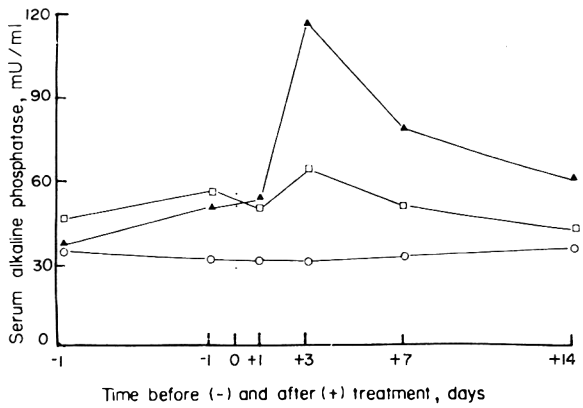

Fig. 1. Effects of 10 mg patulin/kg given iv (○——○), 10 mg penicillic acid/kg given ip (□——□), or both mycotoxins each in a dose of 10 mg/kg (▲——▲) on serum alkaline phosphatase in dogs. Arrows indicate the day of administration of the toxin(s).

combination with penicillic acid killed, in each case, two of three animals (Table 1). The third of the dogs given both toxins at 10 mg/kg (dog 11) recovered, despite the severity of the effects. Although no deaths were caused by 7·5 mg patulin/kg, simultaneous administration of 10 mg penicillic acid/kg and 7·5 mg patulin/kg killed one of the two animals. Patulin in a dose of 5 mg/kg alone or in combination with 10 mg penicillic acid/kg was not lethal.

Reactions in dogs given 10 mg patulin/kg or more alone or in combination with penicillic acid were similar. A dose of 7·5 mg patulin/kg given in combination with penicillic acid elicited effects resembling those seen in dogs given a dose of 10 mg patulin/kg or more.

Although no interaction of the compounds was reflected in most of the serum enzymes, clinical chemistry or haematology parameters measured, a marked elevation of serum alkaline phosphatase was noted on day 3 in the dog receiving a combination of 10 mg/kg of patulin and penicillic acid (Fig. 1) and on day 1 in the dog given a combination of 7·5 mg/kg of patulin and 10 mg/kg of penicillic acid (not shown in Fig. 1). Neither mycotoxin alone had an effect at these dose levels. Penicillic acid appeared to antagonize the increase in serum CPK caused by higher (7·5 or 10 mg/kg) doses of patulin and at the same time caused an increase in this enzyme when given with 5 mg patulin/kg, a dose that, given alone, had no effect (Table 2).

Pathology resulting from the simultaneous administration of penicillic acid with 10 mg patulin/kg or more was essentially that caused by the same dose of patulin alone. Only atelectasis was seen in lungs of the dogs given 7·5 or 5 mg patulin/kg alone, whereas fluid accumulation in the thoracic cavity, overt pulmonary oedema and haemorrhages in the intestinal and gastric lumen, which occurred in dogs exposed to 10 mg patulin/kg or more alone, were noted with the combination of 7·5 mg patulin/kg and 10 mg penicillic acid/kg. Pulmonary haemorrhages were present in the dog simultaneously exposed to 5 mg patulin/kg plus 10 mg penicillic acid/kg.

Hepatic sinusoidal congestion in the liver of dog 19 (10 mg penicillic acid/kg) in this study and in dogs exposed to similar doses of penicillic acid in another study (Hayes *et al.* 1977) was not observed in the livers of the dogs given both mycotoxins, except in dog 9 given both toxins at levels of 10 mg/kg.

DISCUSSION

Patulin had drastic effects in dogs, including emesis and diarrhoea and extensive gastro-intestinal haemorrhages. The lungs were the only site of major histopathology clearly related to treatment. All doses of patulin, alone or in combination with penicillic acid, produced pulmonary damage, chiefly atelectasis (all doses), emphysema and oedema (10 mg/kg or more). Patulin at doses of 10–15 mg/kg produced massive pulmonary oedema and death within as brief an interval as 4–5 hr, whereas 7·5 mg/kg or less caused minimal lung damage (atelectasis) and no death.

Broom *et al.* (1944) reported signs of right-sided heart failure (hydroperitoneum and congestion of the liver) in addition to lung oedema in mice given lethal iv doses of patulin. Central vasopressor, peripheral vasodilator and mild cardiac depressant actions of patulin were also demonstrated. As described in our results, the dogs that died within 24 hr of a dose of patulin of 10 mg/kg or more showed no remarkable liver lesions, while death at 25 hr (dog 6) revealed sinusoidal congestion. Pulmonary oedema was seen in all dead dogs irrespective of the time of death, suggesting that this lesion was the probable cause of death. Sadek & Pfitzer (1975) demonstrated a good correlation between the increase in serum CPK activity 6–12 hr after chemical exposure and the induction and the degree of myocardial damage. Although dogs exposed to 7·5 or 10 mg patulin/kg had increased serum CPK activity, neither gross nor microscopic cardiac damage was evident. This evidence may not preclude cardiac failure but may suggest direct pulmonary vascular damage as the most likely effect of patulin leading to pulmonary oedema.

Garza, Swanson & Branen (1977) reported a decrease in serum alkaline phosphatase in monkeys fed 5 mg patulin/kg/day for several weeks; no decrease in this enzyme was observed in our study. This may be the result of the different routes of administration, the duration of exposure and/or the species used.

Hayes *et al.* (1977) observed sinusoidal congestion in the liver on day 14 after treatment of dogs with 10 mg penicillic acid/kg. One dog in our study given the same dose of penicillic acid also showed hepatic sinusoidal congestion. However, this hepatic lesion was absent in most dogs that received both mycotoxins, irrespective of survival time. Interaction of penicillic acid with the higher doses of patulin (7·5 or 10 mg/kg) was antagonistic to the serum CPK activity, the effect of 7·5 mg patulin/kg being completely abolished; however, the death of the dogs receiving this mycotoxin combination was associated with pulmonary oedema. This evidence further supports the idea that direct pulmonary vascular damage was responsible for lung oedema. With 5 mg patulin/kg, penicillic acid caused a 220% increase in this enzyme, however, suggesting a complex interaction pattern depending upon the dose of each mycotoxin. This aspect of patulin toxicity and its interaction with penicillic acid needs confirmation and further study.

Although Hayes & Williams (1977) did not claim a synergistic interaction between aflatoxin B_1 and rubratoxin B, they speculated on an aetiologic role for both mycotoxins in canine hepatitis. They postulated that each mycotoxin could sensitize renal cells to the action of the other, thus leading to the ultimate renal lesion. Kitchen *et al.* (1977) suggested a synergistic interaction between ochratoxin and citrinin because of an increase in mortality and in severe renal lesions in dogs given both mycotoxins in comparison to those given either alone. In our study, 10 mg penicillic acid/kg caused deaths and increased the severity of toxic signs and tissue lesions in dogs also exposed to sublethal (7·5 mg/kg) doses of patulin. The demonstration of lung oedema in these dogs (nos 14 and 15) either at the time of death or after 14 days (Table 1) and the lack of such lesions in dogs given either 7·5 mg patulin/kg or 10 mg penicillic acid/kg alone strongly suggests a synergistic effect. In addition, the 5-mg/kg dose of patulin, which when given alone produced atelectasis, resulted in pulmonary haemorrhages when given simultaneously with 10 mg penicillic acid/kg. A clear potentiation was noted also in the elevation of serum alkaline phosphatase in animals exposed simultaneously to both toxins. The results of our study suggest, therefore, the possibility of a synergistic interaction between patulin and penicillic acid when these mycotoxins are simultaneously administered to dogs.

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QUANTITATIVE AND MORPHOLOGICAL ASPECTS OF CUTANEOUS IRRITATION BY TRICHOTHECENE MYCOTOXINS

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Abstract—The cutaneous responses to irritation by two trichothecene mycotoxins, T-2 toxin and diacetoxyscirpenol (DAS), were examined in rats and rabbits. Sequential gross and histopathological observations on cutaneous lesions produced by low doses of T-2 toxin revealed a non-specific acute dermal inflammatory reaction, characterized by hyperaemia, oedema and neutrophil exudation, with variable degrees of necrosis of the epidermis. Reactions increased in intensity until 48 hr, after which the inflammatory response rapidly diminished. Inflammatory reactions to T-2 toxin were histologically similar to reactions to croton oil. Lesions developed similarly in each species, but the intensity of reactions to a given dose was higher in rabbits than in rats. Wide variation in the intensity of reactions among either rabbits or rats prevented an accurate prediction of dose on the basis of a quantitative scoring of inflammation. A reliable assay technique was designed in which concentrations of solutions of T-2 toxin (2 μl in volume) were estimated directly by assessing the intensity of reaction relative to reactions to a graded series of standard solutions of T-2 toxin applied to the same rat. By this simple and sensitive procedure, concentrations of T-2 toxin in the range of 5–60 μg/ml could be measured accurately and precisely. Measurements obtained were independent of the degree of sensitivity of test animals.

INTRODUCTION

Trichothecene mycotoxins, the major one of which is T-2 toxin, are toxic metabolites produced by various fungi, including Fusarium and Stachybotrys (Bamburg & Strong, 1971; Mirocha, Pathre & Christensen, 1978). These fungi may develop on cereal crops in the field, and under cold moist conditions the toxic metabolites may be produced (Joffe, 1971). Because trichothecenes are cytotoxic to germinal cells of the bone marrow, lymphoid tissues and intestinal mucosa (Bamburg & Strong, 1971), they have been considered to cause various mycotoxicoses characterized by pancytopaenia and haemorrhagic diathesis, including alimentary toxic aleukia of man (Joffe, 1971 & 1978), mouldy-corn poisoning of cattle (Hsu, Smalley, Strong & Ribelin, 1972) and stachybotryotoxicosis of horses and cattle (Rodricks & Eppley, 1974).

Fusariotoxicosis, a mycotoxic syndrome with signs of stomatitis, dermatitis, gastritis (or proventriculitis), food refusal and vomiting has been described in poultry and swine (Greenway & Puls, 1976; Wyatt, Harris, Hamilton & Burmeister, 1972a), and can be produced experimentally with pure trichothecene mycotoxins (Chi & Mirocha, 1978; Chi, Mirocha, Kurtz, Weaver, Bates & Shimoda, 1977; Wyatt, Weeks, Hamilton & Burmeister, 1972b). The major effects observed have been attributed to potent emetic and irritant activities of the trichothecenes (Palyusik & Koplik-Kovacs, 1975; Wyatt *et al.* 1972b).

Detection and measurement of trichothecene mycotoxins in feedstuffs by physico-chemical methods has been difficult (Pathre & Mirocha, 1977). The best results have been achieved with a computer-assisted mass spectrometer and gas chromatograph (Mirocha, Pathre, Shauerhamer & Christensen, 1976). The analytical difficulties have promoted the use of bioassay systems, the one most commonly used being based on the skin-irritation activity of the trichothecenes (Eppley, Stoloff, Trucksess & Chung, 1974). Several skin-irritation bioassay methods have been described (Gilgan, Smalley & Strong, 1966; Marasas, Bamburg, Smalley, Strong, Ragland & Degurse, 1969; Ueno, Ishikawa, Amakai, Mikajima, Saito, Enomoto & Ohtsubo, 1970; Wei, Smalley & Strong, 1972). These have been designed as detection assays, with as little as 10 ng T-2 toxin being detectable by one method (Chung, Trucksess, Giles & Friedman, 1974). Such assay systems, which are at best semi-quantitative, can distinguish the trichothecenes specifically from other mycotoxins, although they do not distinguish between members of the trichothecene group (Eppley, 1975).

In spite of the widespread use of cutaneous irritation in bioassay methods for the detection of trichothecenes in foodstuffs, little is known of the pathogenesis and dose-response relationship of the reactions produced by these mycotoxins. Descriptions of the histopathological changes in the skin after the application of pure trichothecenes (Marasas *et al.* 1969; Ueno *et al.* 1970) or crude extracts (Joffe, 1971; Schoental & Joffe, 1974) are limited to changes observed after 2 days. Furthermore, the reactions described were produced with extremely high levels of toxins, well in excess of the amounts that produce the mild reactions observed frequently after applications of positive test extracts from feed samples (Chung *et al.* 1974).

Using two trichothecene mycotoxins, T-2 toxin and diacetoxyscirpenol (DAS), we examined the nature of

the irritant activity in an attempt to determine the relationship between irritation and systemic toxicity. This report describes morphological and dose-response studies on cutaneous reactions to these trichothecenes, and a sensitive, quantitative bioassay for the measurement of the irritant toxicity of trichothecenes.

EXPERIMENTAL

Animals and care. Female Wistar rats (weighing approximately 150 g) and young female white New Zealand rabbits (approximately 1·5 kg) from Canadian Breeding Farms and Laboratories, St. Constant-Laprairie, Quebec, were used in these studies. Fur growth on the back was usually inactive in animals of these weights. Animals were housed in conventional screen-bottomed steel cages with pelleted laboratory animal diets and water supplied *ad lib.* All treatments and management procedures conformed to the guidelines of the Canadian Council on Animal Care. The fur over the back was clipped with a No. 40 surgical clipper blade (Oster Corp. Milwaukee, WI, USA) within the 24 hr preceding application of solutions of the toxins. To identify application sites, grid lines were marked on the skin with a felt-tip pen containing insoluble ink which did not produce a cutaneous reaction.

Toxins. Crystalline T-2 toxin and DAS were used. T-2 toxin was supplied initially by Dr. H. R. Burmeister, Northern Regional Research Laboratory, Peoria, IL, USA (Burmeister, 1971). Further supplies of T-2 toxin and the DAS were purchased from Makor Chemicals, Jerusalem, Israel. Toxins were dissolved in reagent-grade ethyl acetate (J. T. Baker Co., Phillipsburg, NJ, USA) and applied directly to the skin using a variable-dose microsyringe (CR700-20, Hamilton Co., Reno, NV, USA). All applications were applied randomly and were appraised under incandescent light without knowledge of the treatment given.

Design of morphological studies (trials I and II)

These experiments were conducted to examine the nature and pathogenesis of the skin lesions caused by trichothecenes.

Trial I. Nine rats were divided into three groups. T-2 toxin (80 μg/ml) was applied in volumes of 3 μl to one group at intervals of 3, 6, 9, 12, 24 and 36 hr before autopsy, to another 15, 18, 21, 24, 30 and 48 hr before autopsy, and to the third 3, 6, 9, 12 and 14 days before autopsy. Eight treatment sites on each rat were used, allowing three separate applications for each time, with one site left untreated. A similar experiment was conducted on six rabbits using 2-μl applications of T-2 toxin (80 μg/ml) at intervals of 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 36, 42 and 48 hr before autopsy.

Trial II. To compare the nature of the irritant effects of T-2 toxin with that of croton oil (Sigma Chemical Co., St. Louis, MO, USA), T-2 toxin was dissolved in ethyl acetate–maize oil (1:9, v/v) to a concentration of 50 μg/ml. This was applied to the skin of rats in volumes of 2 μl, while croton oil was applied in volumes of 4 μl, each at intervals of 2, 4, 6, 8, 10, 12, 15, 18, 21, 24, 27, 30, 33, 36, 42, 48, 60, 72 and 84 hr before autopsy.

In both trial I and trial II, the macroscopic appearance of each test site was evaluated before the animals were killed either by iv sodium pentobarbital or by ether inhalation. The treated areas of skin were removed, stapled to wooden tongue depressors and fixed by immersion in 10% neutral buffered formalin. Blocks of skin taken through the diameter of the application site were embedded in paraffin, sectioned at 8 μm and stained with haematoxylin and eosin for microscopic examination.

Design of dose-response studies (trials III–V)

Two different methods, already described, were used to evaluate the dose-response relationship of the irritant effect. A third method, suitable for use in a quantitative bioassay, was designed and evaluated.

Trial III. In this experiment, the relationship between intensity of reaction and concentration of toxin was examined. Six solutions of T-2 toxin at concentrations of 10, 20, 40, 80, 160 and 320 μg/ml were applied randomly in volumes of 2 μl to each of ten rats and eight rabbits. The reactions were scored at 24, 48 and 72 hr by the method of Draize, Woodard & Calvery (1944), both hyperaemia and oedema being scored on a 0–4 scale of increasing intensity. The two scores were added to give the Draize score (maximum score 8). Mean scores for reactions to each dose were determined and analysed by regression against the logarithm of the concentration of toxin applied. Dose-response relationships at the three times of evaluation were compared, as were the responses between the two species.

Trial IV. The relationship between concentration of toxin and the frequency response was measured using the method described by Kligman & Wooding (1967). T-2 toxin in volumes of 2 μl and at concentrations of 2·5, 5, 10, 15, 20, 40 and 80 μg/ml was applied to each of ten rats. The response was appraised quantally (i.e. present or absent) after 24 hr and 48 hr, and the frequency response to each dose level was determined. A similar trial was conducted using levels of 5·3, 8, 12, 18, 26·7, 40, 60 and 90 μg/ml of both T-2 and DAS, each on separate groups of ten rats. Using normograms adapted from Litchfield & Wilcoxon (1949) to measure median effective dose as described by Kligman & Wooding (1967), the median irritant dose (ID_{50}) and slope function of the probit-plotted frequency response against the logarithm of concentration were determined for each toxin. Irritancy of T-2 toxin and DAS were compared, and the degree of variation in response between individuals, as indicated by the slope of the response curves, was assessed for both 24- and 48-hr readings.

Trial V. Because the previous trials had demonstrated marked variation in the intensity of cutaneous reactions to a given dose of toxin, both among animals and within the same individual, the following assay system was designed to allow quantitative evaluation of the dose applied, by minimizing the effects of such variation. Rats were used because variation in sensitivity from one to another was lower than in rabbits.

A 6 × 3 grid of square areas each approximately 2 × 2 cm was marked with insoluble ink on the

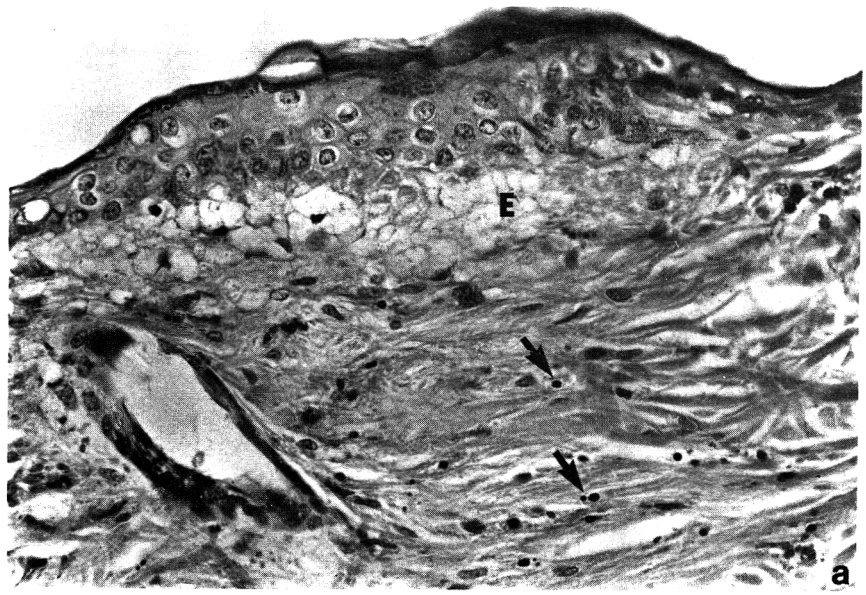

Fig. 1. Microscopic appearance of skin of a rat 12–48 hr after application of 0.24 μg T-2 toxin in 3 μl ethyl acetate: (a) after 12 hr, showing sub-epidermal oedema (E) and necrosis of dermal fibroblasts (arrowed); (b) after 24 hr, showing dense dermal infiltration by neutrophils, particularly in the deeper regions, and focal necrosis of the epidermis, associated with neutrophilic exudation; (c) after 48 hr, with neutrophil exudation concentrated below the epidermis, which is intact and hyperplastic. Haematoxylin and eosin (a) × 100, (b) and (c) × 40.

shaved skin of the back of each rat. Along one randomly selected row, in either direction, six standard concentrations of T-2 toxin (10, 20, 30, 40, 50 and 60 μg/ml) in 2 μl were applied in sequence. The remaining 12 sites on each rat were used for test applications. Each test solution was randomly applied at least ten times, with no more than four applications of the same test solution being applied to any one rat.

At 24 and 48 hr after application, the inflammatory reactions at test sites were compared with the responses to the known toxin standards. The concentration applied to each test site was estimated directly by comparison with the intensities of reactions in the standard scale. The average of all estimates of the concentration of the unknown was the assayed concentration, measured as equivalent concentration of T-2 toxin.

To examine the dose-response relationship of irritation using this method, solutions of T-2 toxin in ethyl acetate at various concentrations close to the minimum effective concentration were assayed. Solutions were masked and assayed as unknowns, and the assayed concentrations were compared with the actual concentrations. In series A, ten solutions, with concentrations of 8, 14, 20, 26, 30, 36, 42, 50, 54 and 70 μg/ml were assayed at both 24 and 48 hr, each test solution being applied three times to each of four rats. In series B, twelve solutions with concentrations of 4, 7, 8, 11, 14, 16, 22, 28, 32, 44, 56 and 64 μg/ml were each applied twice to each of five rats and assayed at 24 and 48 hr. In series C, the same set of unknowns used in series B was reassayed, with readings only at 48 hr. In series D, five solutions with concentrations of 6, 12, 24, 36 and 48 μg/ml were each divided into ten duplicate samples, all of which were coded and assayed at 48 hr. All reactions were read by two different people, neither of whom knew the range of the concentrations of the samples. Precision of the assay was assessed from the distribution of ten assayed values, and the accuracy was evaluated by comparing measured concentrations with actual concentrations.

Studies of factors affecting the irritant response (trials VI–VIII)

In order to define further the conditions under which the quantitative bioassay should be performed, the variation in response by an individual animal, and the influence of solvent, concentration and dose, were examined.

Trial VI. Because the bioassay depended on individual rats exhibiting both a constant intensity of reaction to the same dose and a graded response to different dose levels, consistency of each of these aspects was examined. Three dose levels of DAS (10, 15 and 30 μg/ml) in 2 μl volumes were each applied seven times along the rows of a 7 × 3 grid on each of ten rats. Reactions were assessed at 24 and 48 hr and scored on a 0–4 scale for hyperaemia according to the method of Draize *et al.* (1944). Within each row of seven identical applications, those reactions scored above or below the most common score were counted, and the overall frequency of such atypical reactions was determined to measure inconsistency of response by a rat to a given dose. In addition, each panel of three side-by-side sites was examined for the presence of a response graded with dose. The frequency of correct differentiation of the doses was determined to measure the consistency of the dose-response relationship.

Trial VII. To examine the effect of the vehicle on the skin irritation response, 2-μl volumes of solutions of T-2 and DAS in either ethyl acetate, methanol, DMSO or maize oil, at a constant concentration (100 μg/ml) were each applied once to six rats. The intensity of the reactions at 24 and 48 hr was determined and compared for each vehicle.

Trial VIII. To determine whether the concentration of toxin or the absolute amount applied was responsible for the production of a reaction, T-2 toxin was applied twice to each of nine rats in three ways: (i) 0·03 μg in 2 μl, (ii) 0·03 μg in 4 μl, and (iii) 0·06 μg in 4 μl. Thus applications (i) and (iii) were the same in concentration, whereas (i) and (ii) were the same in quantity. The reaction frequencies determined for each at 24 and 48 hr were compared.

RESULTS

Morphological studies

Trial I. Reactions to T-2 toxin applied topically to rats and rabbits became visible by 12 hr as flat hyperaemic plaques, which subsequently increased in redness to a maximum at 48 hr. Plaques became swollen and centrally pale by 18 hr and were covered with moist exudation by 24–30 hr. After 48 hr, redness subsided and the plaques became covered with a dry friable exudate. Scaley superficial flakes were frequently present over the reaction sites at 72 hr, but these broke away by 6 days leaving a small smooth pink hairless spot which gradually diminished over the next week to be virtually unnoticeable by day 14.

Microscopically, neutrophils were sequestered in dermal capillaries and at the luminal margins of venules by 3 hr. Between 6 and 12 hr nuclei of fibroblasts in the interfollicular dermis were pyknotic and karyorrhectic (Fig. 1a). Neutrophils had infiltrated throughout the dermis by 12 hr and into the outer layers of the epidermis by 24 hr (Fig. 1b), and were concentrated in and below the epidermis at 48 hr (Fig. 1c), but few were observed at 72 hr or later.

Changes in the epidermis and hair follicles were variable during the first 24 hr. Isolated pyknotic nuclei were found infrequently in the basal layer of the epidermis. Epidermal spongiosis and sub-epidermal oedema were evident in some sites (Fig. 1a). By 24 hr, focal areas of coagulation necrosis of epidermis were found in the more severe reactions (Fig. 1b), but in milder reactions, the epidermis overlying the dermal inflammatory response was intact. Hyperplasia of the epidermis, which began after 24 hr, became prominent by 48 hr and persisted throughout the 14-day observation period. Sub-epidermal fibroplasia appeared by day 6 and subsequently increased.

Trial II. A similar sequence of gross and microscopic changes was observed for reactions produced by T-2 toxin in the maize-oil vehicle, but reactions were less intense than in trial I, in which ethyl acetate was used. These weaker reactions faded after 24 hr, and neutrophil exudation through the epidermis was minimal, in contrast to the strong reactions which

were observed in trial I and which intensified between 24 and 48 hr.

The sequential development of reactions to croton oil was essentially similar to that observed for T-2 toxin. However, croton oil produced more severe reactions and a more pronounced hyperplastic response in the epidermis. Focal coagulation necrosis of the epidermis was more frequently observed in sites treated with croton oil.

Dose-response studies

Trial III. Mean Draize scores for both rabbits and rats increased with increasing concentration of T-2 toxin, except at 24 hr, when no relationship with dose was evident in rabbits (Fig. 2). At 48 hr, significant dose-response regressions were evident in rabbits ($P \leqslant 0{\cdot}05$) and rats ($P \leqslant 0{\cdot}01$) but, for a given dose, scores were greater in rabbits, indicating that they were more sensitive to the toxins. Scores were higher at 48 hr than at 24 and 72 hr for both species. Variation in scores among rabbits, indicated by the standard deviations, was greater than among rats (Fig. 2).

Trial IV. The median effective concentration (ID_{50}) values (Table 1), measured quantally by the method of Kligman & Wooding (1967), demonstrated that the skin of rats was extremely sensitive to both T-2 toxin and DAS. Marked variation in sensitivity among individual rats was evident in the relatively flat dose-response gradients (Fig. 3) and high slope function values (Table 1). Most rats responded to concentrations above 15 μg/ml. It was not possible to distinguish between the irritancy of T-2 toxin and DAS by this method because confidence intervals of the ID_{50} and slope function were wide (Table 1).

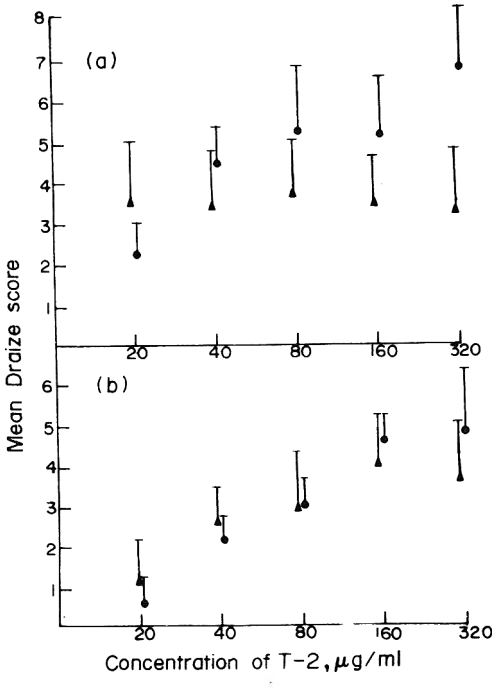

Fig. 2. Comparison of dose-response relationship of cutaneous irritation by T-2 toxin, measured quantitatively in (a) the rabbit and (b) the rat, according to Draize *et al.* (1944). Points are mean scores, with standard deviation bars, measured 24 (▲) and 48 (●) hr after application of the toxin (trial III).

Trial V. In each series of bioassays, the reactions to the range of standard solutions of T-2 toxin were graded with dose, except that several rats were insensitive to most of the concentrations of the standards and many rats exhibited one or two reactions that appeared to be out of sequence in the standard range. Where such atypical reactions were encountered, they were ignored, and estimations of unknowns were made by comparison with the rest of the reactions in the standard range. Gradation of reactions to standards was less evident at 24 hr than at 48 hr because more atypically intense reactions to low concentrations were visible at 24 hr.

Intensities of reactions to all test solutions used in series A, B and C, as measured in equivalent concentrations of T-2 toxin, increased in significant linear fashion with increasing dose ($P \leqslant 0{\cdot}001$ by t tests on regression coefficients). However, at 48 hr, measured concentrations (M) were more closely related to actual concentrations (A) than at 24 hr. The regression equations, derived from all measurements in series A, B and C were:

At 48 hr: $M = 7{\cdot}2 + 0{\cdot}74A$
coefficient of determination = 0·86
At 24 hr: $M = 10{\cdot}4 + 0{\cdot}65A$
coefficient of determination = 0·73

In Series A, only nine applications for each of the test solutions were measured because three of the ten rats were rejected due to low sensitivity to the standard range of solutions. Measurement errors in series A, B and C were consistently small, except that concentrations of all solutions greater than 55 μg/ml were underestimated by more than 10 μg/ml (Fig. 4). This plateau effect was brought about by difficulties in assessing dose-equivalent measurements for reactions that exhibited similar or greater intensity than was produced by the strongest standard concentration (60 μg/ml). Accuracy of assayed concentrations, as indicated by standard deviations from the expected regression lines and by 95% confidence limits was greater when samples at the upper end of the range (greater than 55 μg/ml) were excluded (Table 2).

The duplicate samples in series D were assayed precisely and accurately; the largest error among the 50 assays was 11 μg/ml (Table 3). Standard deviations of assayed concentrations were not more than 5·0 μg/ml. Precision was sufficient to distinguish between solutions differing by 12 μg/ml, but there was considerable overlap in the ranges of concentrations assayed for the two solutions differing by only 6 μg/ml. Means of assayed concentrations of each set of ten duplicate samples were within 4·6 μg/ml of the actual concentrations (Table 3).

Factors affecting irritant response

Trial VI. Reactions to each dose of DAS were highly consistent for individual rats, in contrast to the previously observed variation in the reactions of different rats. Atypical responses were uncommon at both 24 and 48 hr (Table 4). For each time of examination, approximately 20% of reactions were scored either above or below the most common score (Table 4).

Correct gradation of reaction with dose occurred in 52 of the 70 test panels at 24 hr and in 30 of the

Table 1. *Median irritant concentration (ID_{50}) and slope functions for topically applied T-2 toxin and diacetoxyscirpenol measured by quantal assay in trial IV*

Toxin	Time of examination (hr*)	ID_{50} (μg/ml)	95% confidence limits	*S*	95% confidence limits
T-2	24	11·0	8·1–15·0	1·64	1·30–2·07
T-2†	24	7·4	4·3–12·7	2·31	1·46–3·68
DAS	24	11·0	7·9–15·4	1·74	1·34–2·26
T-2	48	13·5	10·6–17·3	1·49	1·25–1·77
T-2†	48	8·5	5·6–13·0	1·84	1·27–2·67
DAS	48	14·5	9·3–22·6	2·04	1·44–2·90

S = Slope function DAS = Diacetoxyscirpenol

*Hours after application of test material.

†Second test on T-2 toxin.

The irritant response was determined according to the method of Kligman & Wooding (1967), for frequency response of skin irritation to multiple levels of test irritants exposed to each test individual. The two tests on T-2 toxin and that on DAS were each conducted in separate groups of ten rats.

panels at 48 hr. The lower frequency at the latter time was largely due to a higher frequency of negative responses (Table 4).

Trial VII. Reactions were similarly frequent and intense at both 24 and 48 hr for ethyl acetate and methanol solvents. The reaction intensities and frequencies were much lower when maize oil was used as the vehicle. In DMSO, DAS produced no reactions and T-2 toxin produced very mild reactions.

Trial VIII. The same frequency of reactions (14/18) to 0·03 μg T-2 toxin occurred at 24 hr irrespective of the concentration, but the larger quantity of T-2 toxin (0·06 μg in 4 μl) evoked 17/18 reactions. At 48 hr 10/18 reactions were evident for each application of 0·03 μg T-2 toxin, whereas the larger quantity of T-2 toxin (0·06 μg in 4 μl) evoked 14/18 reactions.

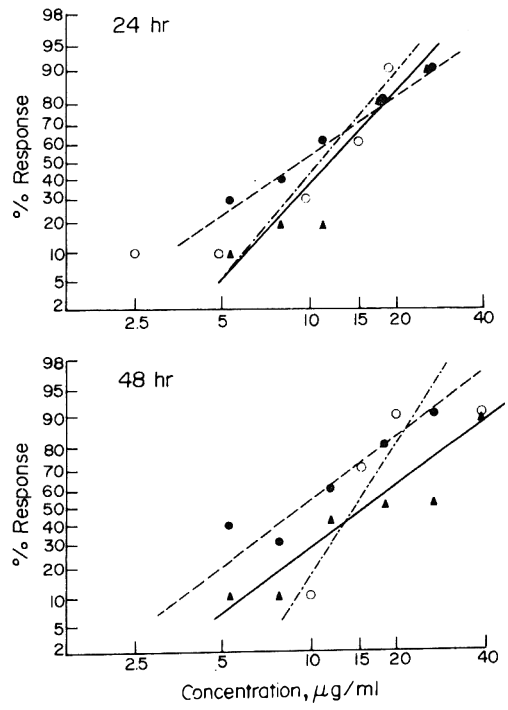

Fig. 3. Frequency response relationships of cutaneous irritation of rats exposed to graded topical doses of T-2 toxin (▲——▲ and ●- -●) and to diacetoxyscirpenol (○——○), determined at 24 and 48 hr (trial IV).

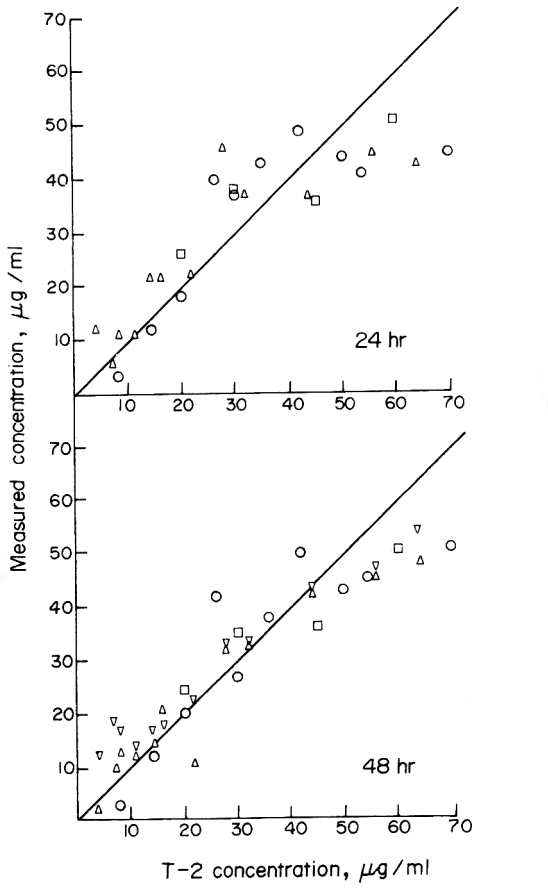

Fig. 4. Relationship between measured concentration and assayed concentration of solutions of T-2 toxin used in trial V. Different symbols represent different series of assays. Measurements were more accurate (i.e. closer to the expected regression line) when measured at 48 hr. Higher concentrations were consistently underestimated.

Table 2. *Accuracy of measurement of concentrations of T-2 toxin by the quantitative dermal irritation bioassay in trial V*

Test series	Concn of samples assayed (μg ml)	No. of replicate applications	24-hr readings SD* (μg/ml)	24-hr readings 95% CL† (μg/ml)	48-hr readings SD* (μg/ml)	48-hr readings 95% CL† (μg/ml)
A	8; 14; 20; 26; 30;	9	12·0	±23·5	9·5	±18·6
	36; 42; 50; 54; 70	9‡	8·5	±16·7	9·1	±17·8
B	4; 7; 8; 11; 14; 16; 22;	10	9·3	±18·1	7·5	±14·7
	28; 32; 44; 56; 64	10‡	6·3	±12·3	5·1	±10·1
C	4; 7; 8; 11; 14; 16; 22;	10	—	—	6·8	±13·3
	28; 44; 56; 64	10‡	—	—	6·0	±11·8
A.B.C§			9·8	±19·2	8·1	±15·9
			7·1	±14·0	6·4	±12·5

*Standard deviation of measured values from actual concentrations.
†95% confidence limits (= ±1·96 SD).
‡Measurements for samples above 55 μg/ml have been excluded from the calculations.
§Combined results.

Table 3. *Performance of bioassay in measuring concentrations of T-2 toxin in ethyl acetate*

Actual concn (μg/ml)	Mean assayed concn* (μg/ml)	Range (μg/ml)	SD (μg/ml)	SEM (μg/ml)	CV (%)
48	49·1	43–59	5·0	1·6	10·2
36	40·6	35–46	4·2	1·3	10·3
24	25·9	20–30	3·8	1·2	14·7
12	13·1	9–15	3·5	1·1	26·7
6	8·3	4–14	3·1	1·0	37·3

SD = Standard deviation SEM = Standard error of the mean
CV = Coefficient of variation
*Of ten duplicate samples.

DISCUSSION

The microscopically observed changes in the dermis after topical exposure to T-2 toxin were consistent with those described in the inflammatory responses of skin to a variety of physical and chemical injuries (Rostenberg, 1957; Steele & Wilhelm, 1966 & 1970). Qualitatively, the inflammatory changes were similar to those evoked by topical application of croton oil, although the epidermal proliferative response to this irritant appeared more pronounced. In mild reactions, T-2 toxin caused minimal damage in the epidermis, but evoked an intense dermal inflammatory reaction, indicating that the effect of T-2 toxin on the skin was not necrotizing or corrosive at low concentrations. However, in stronger reactions, necrosis of both dermis and epidermis occurred. In another reported investigation with rats, high topical doses of T-2 toxin resulted in severe necrosis, with sloughing of epidermis and dermis after several days (Marasas *et al.* 1969). Necrotizing lesions have also been described in the skin of guinea-pigs 2 and 3 days after application of moderately high doses of other trichothecene mycotoxins, namely diacetoxyscirpenol, fusarenon-X and nivalenol (Ueno *et al.* 1970), and in the skin of rabbits and mice after applications of crude extracts of *Fusarium poae* and *F. sporotrichioides* (Joffe, 1971; Schoental & Joffe, 1974), which are now known to produce high levels of T-2 toxin (Yagen & Joffe, 1976).

Severe necrotizing lesions produced by high doses of trichothecenes were still evident 8 days after application (Joffe, 1971). Although clearly illustrating the irritant potency of the toxins, such lesions bear little resemblance to the mild reactions frequently encountered in routine use of dermal irritation as a bioassay to screen feed extracts for trichothecene toxins

Table 4. *Summary of inconsistencies in the responses of rats to multiple applications of diacetoxyscirpenol at constant doses (trial VI)*

Parameter	Response at 24 hr	Response at 48 hr
Frequency of reactions	104/210	74/210
No. of atypical reactions	7	11
No. of reactions with atypical, increased intensity	10	9
No. of reactions with atypical, decreased intensity	8	5
Frequency of correct differentiation of doses	52/70	32/70
Frequency of poor differentiation of doses		
(a) all negative	8	20
(b) similar scores	10	15
(c) incorrect	0	3

(Chung *et al.* 1974; Eppley *et al.* 1974). In our studies, reactions to low doses of T-2 toxin or DAS (10–30 μg/ml) were often greater at 24 hr than at 48 hr, by which time some reactions had disappeared. For slightly higher doses between 30 and 100 μg/ml, reactions generally increased to a peak intensity at 48 hr and were more clearly graded according to dose. Similar differences in dose-response were observed for test extracts applied to rabbits by Chung *et al.* (1974), who suggested that the fading of mild reactions after 24 hr indicated a non-specific irritant, whereas a continuing increase in inflammation up to 48 hr was more likely to indicate the presence of trichothecene toxins in feed extracts. Since we observed both types of reactions in response to T-2 toxin, it appears that this difference in reactions can be due solely to differences in the concentration of the irritant applied, and that the reactions that intensified for 48 hr did so because of secondary dermal and epidermal damage associated with more severe purulent inflammation. No morphological changes in any way specific for T-2 toxin were found in irritated skin.

Mean Draize scores for a given dose of T-2 toxin were consistently higher in rabbits than in rats (Fig. 2) indicating that rats were less sensitive to T-2 toxin. This was to be expected since rabbits are generally recognized as being more sensitive than other laboratory animals to most topical irritants (Draize *et al.* 1944; Marzulli & Maibach, 1975). Although some rats had reacted at 24 hr to as little as 5 μg/ml, the majority did not respond to concentrations less than 15 μg/ml, in agreement with observations of Wei *et al.* (1972) who found that most rats would exhibit reactions at 24 hr to quantities of T-2 toxin above 25 μg/ml. Chung *et al.* (1974) concluded, after comparing the sensitivity of Fisher rats and rabbits to topical T-2 toxin and observing minimum sensitivities of 20 and 5 μg/ml for each species, respectively, that rabbits were superior to rats in bioassays for trichothecenes because of their greater sensitivity. However, in our studies, variation in sensitivity between individuals was greater in rabbits than in rats, with rabbits demonstrating virtually no dose-response relationship for reactions appraised at 24 hr (Fig. 2). For this reason, and because they are more conveniently managed, rats appear to be a more suitable animal for quantitative assays.

The quantal dose-response relationships of reactions by rats to T-2 toxin and DAS demonstrated a wide variation in sensitivity among different rats. Slopes of response lines were not sufficiently high or precise to allow precise measurement of irritancy on the basis of the frequency response. Thus it was necessary to devise an assay that avoided influences of variation in sensitivity of test rats.

Reactions to multiple applications of the same dose were found to be highly consistent in any one rat, although some atypical reactions occurred (Table 4). The dose-response relationship was also consistent. Reactions were dependent on the type of vehicle used (trial VII) and on the dose applied (trial VIII). The assay procedure was therefore designed to use constant volumes of both standards and test solutions in the same solvent.

The quantitative bioassay method described proved to be an accurate and precise means of measuring the irritant activity of T-2 toxin. Even though rats were less sensitive than rabbits, concentrations of T-2 toxin as low as 4 μg/ml were accurately measured (Fig. 4). This compares favourably with the detection limit of approximately 5 μg/ml using rabbits (Chung *et al.* 1974). Measurements of reaction were independent of variations in the intensity of inflammatory reactions among rats. By using a total of 18 application sites on each rat, thus allowing the series of standard solutions to be applied to each rat, test reactions were appraised in units of concentration of toxin, rather than by the subjective measurement of intensity of inflammation used in other systems (Chung *et al.* 1974; Joffe, 1971; Ueno *et al.* 1970; Wei *et al.* 1972). Because measurements were independent of the absolute intensity of inflammation, readings from different rats were additive. Accordingly, this assay should produce consistent results even with different strains of rat, provided the range of concentrations of the standards corresponds to the lowest effective concentrations for the rats used.

A further advantage of the described assay was that only mild reactions were produced by the effective range. Severe reactions are undesirable both for humane reasons and because they can lead to self-mutilation, making the intensity of reactions unrelated to the dose applied. Also, since the mild reactions quickly subside, rats may be re-used after 3–4 wk.

The accuracy of measuring the concentration of T-2 toxin by averaging the readings from nine or ten replicate applications was consistently high across most of the test range used, and was greater when readings were made at 48 hr (Table 2). The tendency to underestimate the equivalent concentration for test reactions stronger than those observed in the standard range resulted in errors greater than 10 μg/ml. However, when measurements from test solutions over 55 μg/ml were excluded, accuracy improved (Table 2). The degree of precision was high for a bioassay, and was sufficient to allow differences in concentration of the order of 10 μg/ml to be detected. The mean concentrations of ten assayed duplicates were closer to the actual concentrations, indicating that both accuracy and precision are likely to improve when more than ten applications per assay are used.

The assay described here was designed to quantify dermal irritancy of pure trichothecene toxins, but it could be used to measure the irritancy of other chemicals. It could also be readily adapted to measure the toxicity of feed extracts believed to contain trichothecenes, although cutaneous reactions to trichothecene toxins in unpurified extracts of feedstuffs are weaker than to the same amount of toxin in a purified extract (Pathre & Mirocha, 1977). Such interference might be due to suppression of cutaneous reactions by some anti-inflammatory component in crude feed extracts, or to some alteration of the characteristics of the vehicle by the impurities. Thus, the sensitivity of the assay for crude extracts would be lower, but, because variation in the sensitivity of the skin does not appear to have a great influence on the accuracy of measurements obtained, this assay method could still be used quantitatively, provided the extracts are not heavily pigmented, and provided the standards are made in a similar extract. The range of standard concentrations would need to be adjusted accordingly.

The non-specific nature of the dermal response to trichothecene toxins requires that the presence of trichothecene mycotoxins in irritant test extracts be confirmed by other tests. Trichothecenes produce typical, but not pathognomonic, cellular injury in the intestinal crypts and lymphoid tissues (Sato & Ueno, 1977), so the presence of these toxins may be tentatively confirmed by administration of test extracts to mice.

Identification of trichothecenes can be achieved only by physico-chemical methods, and usually after much difficulty. Thin-layer chromatography methods are most widely used and will qualitatively identify T-2 toxin and other trichothecenes (Pathre & Mirocha, 1977). Such methods are not quantitative (Eppley, 1975) and require at least partial purification of test extracts (Pathre & Mirocha, 1977). Interfering substances may also complicate the identification and quantitation of trichothecenes by gas–liquid chromatography (GLC) methods (Pathre & Mirocha, 1977). At present, definitive identification of some trichothecenes in feed extracts can be achieved by gas chromatography–mass spectrometry using selected ion monitoring (Mirocha *et al.* 1976).

To interpret the significance of trichothecenes in feedstuffs associated with disease, it is necessary to have a measurement of toxicity of the feed. The complex extraction and purification processes needed for physico-chemical detection and measurement of trichothecenes may reduce recovery of the toxins resulting in an underestimation of their levels (Hsu *et al.* 1972; Mirocha *et al.* 1976). Furthermore, because there are many trichothecenes with different polar characteristics, it is not possible to measure simultaneously more than a few members of the group (Mirocha *et al.* 1976). For these reasons, measurement of the biological toxicity of the feedstuff, using minimal extraction, is a necessary complement to physico-chemical analysis.

Cutaneous irritancy may be a useful measurement of biological toxicity, but, at present, the relationship between dermal irritancy and systemic toxicity is not clear. Toxins such as T-2 toxin, HT-2 toxin and DAS, which have low acute LD_{50} values, are also extremely irritant, but two other trichothecenes, nivalenol and fusarenon-X, were apparently much less irritant in spite of having low acute LD_{50} values (Sato & Ueno, 1977; Ueno *et al.* 1970). The relationship between the irritancy and subacute toxicity of these compounds is also unknown, but there is evidence that the problems in fusariotoxicosis in poultry and swine are largely due to the irritancy of trichothecenes in the mouldy diets (Chi & Mirocha, 1978; Wyatt *et al.* 1972b). Thus, although such correlative information is largely unavailable at present, measurements of irritancy would provide readily-obtainable estimates of the toxicity of feedstuffs containing trichothecenes.

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TRANSFER OF VARIOUS POLYCHLORINATED BIPHENYLS TO THE FOETUSES AND OFFSPRING OF MICE

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Abstract—Seven polychlorinated biphenyls (PCBs), 2,4,4′-trichlorobiphenyl (2,4,4′-tri-CB), 2,5,3′,4′-tetra-CB, 2,4,5,2′,5′-penta-CB, 2,4,5,2′,4′,5′-hexa-CB, 2,3,4,2′,4′,5′-hexa-CB, 2,3,4,5,6,2′,5′-hepta-CB, and 2,3,4,5,2′,3′,4′,5′-octa-CB were administered together in the diet to female adult mice for 18 days either before or after mating. Dams, foetuses and offspring were analysed for the individual PCBs. All the PCBs given to the dams were transferred to their foetuses and offspring through the placenta and milk. The amount of each compound transferred through milk was however much larger than the amount transplacentally transferred. The 2,5,3′,4′-tetra-CB and 2,3,4,5,6,2′,5′-hepta-CB, were rapidly eliminated by dams and offspring. Small amounts (1–3%) of 2,4,4′-tri-CB and 2,4,5,2′,5′-penta-CB, adminstered during pregnancy, were transferred to the offspring. Larger quantities (15–53%) of the 2,4,5,2′,4′,5′-hexa-CB, 2,3,4,2′,4′,5′-hexa-CB and 2,3,4,5,2′,3′,4′,5′-octa-CB were transferred to the offspring when they had been administered either before or during pregnancy.

INTRODUCTION

Foetuses from mothers with Yusho, polychlorinated biphenyl (PCB) poisoning, and a baby breast-fed by a mother who was poisoned after the delivery of the baby showed some of the characteristic symptoms of Yusho (Kikuchi, Hashimoto, Hozumi, Koga, Oyoshi & Nagakawa, 1969; Taki, Hisanaga & Amagase, 1969; Yoshimura, 1974). This indicates that PCBs are transferred from mothers to foetuses through the placenta and to babies through the milk. Our animal experiments (Masuda, Kagawa, Tokudome & Kuratsune, 1978) also showed this but the transfer of PCBs via the milk was considerably greater than the transplacental transfer. Since the biological activities of different PCBs are known to vary greatly (Brandt, Bergman & Wachtmeister, 1976; McKinney, Chae, Gupta, Moore & Goldstein, 1976; Yoshimura, Ozawa & Saeki, 1978), their transfer to foetuses and to offspring may also vary depending on their structure. We investigated this possibility using seven different PCBs which were administered orally to the dams either before or during pregnancy and the results are outlined below.

EXPERIMENTAL

Materials and apparatus. Ethanol, *n*-hexane, water, sodium hydroxide, anhydrous sodium sulphate, and silica gel were purified for PCB analysis as described previously (Masuda, Kagawa & Kuratsune, 1974; Masuda *et al.* 1978). The PCB isomers used in this experiment were purchased from Analabs, Inc., North Haven, CT, USA. The gas chromatograph used was a Shimadzu GC-4BM with an electron-capture detector of Nickel 63 and a glass column (3 mm × 2 m) containing Chromosorb W AW DMCS (60–80 mesh) coated with 5% SE-30. The column temperature was isothermal at 180°C.

Animals and diet. Male and female ddN mice were supplied by the animal centre of Kyushu University. The test diet was prepared by the method used in the previous experiment (Masuda *et al.* 1978). Powdered feed (Oriental M) was well mixed with an ethyl ether solution containing all of the following PCBs: 2,4,4′-trichlorobiphenyl (2,4,4′-tri-CB), 2,5,3′,4′-tetra-CB, 2,4,5,2′,5′-penta-CB, 2,4,5,2′,4′,5′-hexa-CB, 2,3,4,2′,4′,5′-hexa-CB, 2,3,4,5,6,2′,5′-hepta-CB, and 2,3,4,5,2′,3′,4′,5′-octa-CB, and the solvent was then evaporated. The mixture was thoroughly kneaded with water and soluble starch dissolved in hot water to make a dough. Pieces of dough were pelleted and dried at 60°C overnight. The dried pellets were analysed for the added PCB isomers by gas chromatography, finding the mean levels derived from triplicate determinations 0·32, 0·42, 0·42, 0·44, 0·44, 0·16 and 0·23 ppm, respectively.

Experimental procedure. Ten-week-old female mice were divided into six groups of 10–15 each. Two of these groups were caged individually, fed the test diet for 18 days, and then mated; insemination was confirmed by the presence of a vaginal plug. The pregnant mice were raised on the normal diet until day 18 of pregnancy, when one group of mice was killed, and the other was kept alive on the normal diet until their offspring were 5 wk old. The other two groups that had been given the normal diet were also mated and the pregnant mice were caged individually and fed the

test diet until day 18 of pregnancy, when one of the two groups was killed while the other was kept alive on normal diet for 5 wk. The remaining two groups served as controls, consuming only the normal diet throughout the experiment. After insemination, one group of mice was killed on day 18 of pregnancy, and the other was kept alive until the offspring were 5 wk old.

The pellets of test diet in each cage were weighed every day and their consumption was calculated. The amount of each isomer in the foetuses, the liver, and the remainder of the body excluding the digestive tract and its contents were determined for each dam killed on day 18 of pregnancy. One or two offspring from each litter were killed at weekly intervals from 1 wk after birth for whole-body analysis. The remaining dams were also killed at 5 wk and the liver and the rest of the body apart from the digestive tract were analysed separately for the PCB isomers.

Analytical procedure. Extraction and separation procedures of samples were essentially the same as those described in the previous paper (Masuda *et al.* 1978). Samples were first homogenized with 100–200 ml *n*-hexane and 20 g anhydrous sodium sulphate in a Waring blender. The separated *n*-hexane solutions were combined and evaporated to dryness, yielding fatty residues which were saponified with 1 N-NaOH in 50–100 ml ethanol. For the liver samples, the saponification was carried out without extraction. The *n*-hexane extracts of the NaOH solution were combined, concentrated, and then chromatographed on a column of silica gel (2 g) eluting with 150 ml of *n*-hexane. The eluate was concentrated and subjected to gas chromatography. Quantitative estimates of individual PCBs were made by comparing the peak areas of their gas chromatograms with those of the authentic PCB isomers which showed retention times of 3·8, 5·8, 6·6, 10·3, 11·9, 14·2 and 29·5 min, respectively, in the order of PCB isomers described above.

RESULTS

All the pregnant mice fed the test diet before or during pregnancy or fed the normal diet grew well throughout pregnancy, the mean body weights of these three groups on day 18 of pregnancy being 44·5, 42·4 and 44·6 g respectively. There was no significant difference in body-weight gain among these groups. The mean numbers of offspring produced by each dam were 6·5, 7·3 and 7·6 in these groups respectively. For the three groups, the mean body weights of each pup at 1 wk of age were 5·1, 5·1 and 5·0 g, respectively, and at 5 wk of age were 26·7, 27·3 and 27·4 g, respectively. There were no significant differences in body weight gain among these groups. No abnormality in appearance was observed in any group.

The control groups showed low levels of PCBs (Tables 1 and 2), although they had not been given the test diet. These PCBs were probably attributable to pollutants in the normal diet.

Table 1. *Concentration of PCBs in pregnant mice and their foetuses after oral administration of PCB isomers for 18 days either before or during pregnancy*

Type of PCB	PCB feeding regime	PCB level (ppb) in		
		Whole body	Liver	Foetuses
a	Control	0·09 ± 0·05	0·34 ± 0·09	0·03 ± 0·02
	BP	0·88 ± 0·73*	1·7 ± 1·8	0·09 ± 0·07*
	DP	79 ± 78**††	4·7 ± 3·3**†	1·3 ± 0·89**††
b	Control	0·14 ± 0·07	0·43 ± 0·12	0·04 ± 0·01
	BP	0·23 ± 0·06**	0·39 ± 0·13	0·13 ± 0·04**
	DP	14 ± 13**††	1·0 ± 0·6*††	0·34 ± 0·22**††
c	Control	0·18 ± 0·11	0·82 ± 0·30	0·03 ± 0·01
	BP	2·4 ± 0·95**	1·3 ± 0·45*	0·15 ± 0·04**
	DP	85 ± 49**††	6·0 ± 2·8**††	1·3 ± 0·74**††
d	Control	0·63 ± 0·30	2·0 ± 0·61	0·03 ± 0·01
	BP	250 ± 50**	190 ± 28**	6·9 ± 3·1**
	DP	360 ± 46**††	160 ± 56**	8·3 ± 4·3**
e	Control	0·33 ± 0·15	5·0 ± 1·6	0·02 ± 0·01
	BP	170 ± 36**	1520 ± 180**	6·9 ± 3·0**
	DP	330 ± 58**††	650 ± 76**††	8·9 ± 4·7**
f	Control	ND	0·17 ± 0·36	ND
	BP	0·17 ± 0·22	ND	ND
	DP	1·4 ± 1·0††	0·63 ± 0·42	0·03 ± 0·04
g	Control	0·03 ± 0·03	0·05 ± 0·03	ND
	BP	57 ± 16**	26 ± 8·5**	1·5 ± 0·64
	DP	81 ± 38**†	88 ± 29**††	1·2 ± 1·1

a = 2,4,4′-tri-CB b = 2,5,3′,4′-tetra-CB c = 2,4,5,2′,5′-penta-CB d = 2,4,5,2′,4′,5′-hexa-CB
e = 2,3,4,2′,4′,5′-hexa-CB f = 2,3,4,5,6,2′,5′-hepta-CB g = 2,3,4,5,2′,3′,4′,5′-octa-CB
BP = Before pregnancy DP = During pregnancy ND < 0·01 ppb

§Numbers of dams in the control, before pregnancy and during pregnancy groups were 7, 12, and 12 respectively.

Values are means ± SD for the numbers of dams given; those marked with asterisks differ significantly by Student's *t* test from the corresponding controls (*$P < 0.05$; **$P < 0.01$) and those marked with daggers differ significantly from the corresponding group given the PCB diet before pregnancy (†$P < 0.05$; ††$P < 0.01$).

Table 2. *Concentrations of PCBs in dams and offspring of mice after oral administration of isomers for 18 days either before or during pregnancy*

Type of PCB	PCB feeding regime§	PCB level (ppb) in Offspring aged (wk) 1	2	3	4	5	Dams (5 wk after delivery) Whole body	Liver
a	Control	0·10 ± 0·09	0·05 ± 0·09	0·21 ± 0·10	0·06 ± 0·02	0·10 ± 0·03	0·14 ± 0·06	0·50 ± 0·66
	BP	0·56 ± 0·50*	0·40 ± 0·21**	0·14 ± 0·08	0·13 ± 0·06*	0·06 ± 0·03**	0·11 ± 0·07	0·30 ± 0·09
	DP	7·9 ± 8·4**††	11·4 ± 7·6**††	0·94 ± 0·58**††	0·13 ± 0·08	0·09 ± 0·03	0·57 ± 0·37**††	0·35 ± 0·32
b	Control	0·19 ± 0·10	0·10 ± 0·12	0·38 ± 0·14	0·14 ± 0·05	0·17 ± 0·04	0·21 ± 0·07	0·83 ± 0·89
	BP	0·39 ± 0·21*	0·43 ± 0·20**	0·24 ± 0·09*	0·20 ± 0·11	0·11 ± 0·05*	0·15 ± 0·06	0·56 ± 0·17
	DP	0·50 ± 0·20**	0·55 ± 0·16**	0·31 ± 0·12	0·14 ± 0·05	0·15 ± 0·04	0·31 ± 0·11*††	0·42 ± 0·21
c	Control	0·25 ± 0·18	0·14 ± 0·15	0·39 ± 0·13	0·13 ± 0·03	0·22 ± 0·06	0·22 ± 0·06	0·84 ± 0·84
	BP	1·3 ± 0·87**	1·1 ± 0·31**	0·51 ± 0·22	0·31 ± 0·16*	0·25 ± 0·12	0·21 ± 0·10	0·53 ± 0·19
	DP	8·4 ± 4·1**††	6·1 ± 3·1**††	1·1 ± 0·57**††	0·29 ± 0·13**††	0·30 ± 0·14	0·47 ± 0·22**††	0·49 ± 0·30
d	Control	0·86 ± 0·49	0·55 ± 0·34	0·89 ± 0·33	0·38 ± 0·07	0·50 ± 0·08	0·46 ± 0·14	1·3 ± 0·77
	BP	260 ± 120**	190 ± 38**	110 ± 38**	60 ± 31**	33 ± 16**	4·6 ± 3·7**	18 ± 14**
	DP	330 ± 110**	280 ± 130**†	110 ± 18**	50 ± 17**	37 ± 18**	4·8 ± 1·5**	2·8 ± 1·3*††
e	Control	0·52 ± 0·27	0·47 ± 0·29	0·95 ± 0·38	0·31 ± 0·07	0·39 ± 0·08	0·34 ± 0·20	3·3 ± 1·5
	BP	200 ± 94**	170 ± 36**	100 ± 35**	47 ± 22**	29 ± 17**	3·9 ± 2·2**	38 ± 20**
	DP	320 ± 110**†	300 ± 160**†	100 ± 16**	50 ± 18**	33 ± 14**	6·3 ± 2·8**†	30 ± 19**
f	Control	ND	ND	ND	ND	ND	0·01 ± 0·04	0·20 ± 0·37
	BP	ND	ND	ND	ND	ND	ND	1·5 ± 1·8
	DP	0·27 ± 0·26	0·21 ± 0·17	0·01 ± 0·02	ND	ND	ND	ND
g	Control	0·01 ± 0·01	0·02 ± 0·01	0·05 ± 0·02	0·01 ± 0·01	0·02 ± 0·01	0·03 ± 0·04	0·11 ± 0·14
	BP	81 ± 21**	33 ± 23**	23 ± 13**	21 ± 16**	12 ± 8·1**	8·6 ± 8·3**	5·5 ± 3·4**
	DP	120 ± 92**	130 ± 71**††	33 ± 12**	21 ± 8·7**	14 ± 5·6**	6·5 ± 4·6**	4·6 ± 3·5**

a = 2,4,4′-tri-CB b = 2,5,3′,4-tetra-CB c = 2,4,5,2′,5′-penta-CB d = 2,4,5,2′,4′,5′-hexa-CB
e = 2,3,4,2′,4′,5′-hexa-CB f = 2,3,4,5,6,2′,5′-hepta-CB g = 2,3,4,5,2′,3′,4′,5′-octa-CB
BP = Before pregnancy DP = During pregnancy ND < 0·01 ppb

§Numbers of dams in the control, before pregnancy, and during pregnancy groups were 8, 12, and 12 respectively.

Values are means ± SD for the number of dams given; those marked with asterisks differ significantly (Student's *t* test) from the corresponding controls (*$P < 0.05$; **$P < 0.01$) and those marked with daggers differ significantly from the corresponding group given the PCB diet before pregnancy (†$P < 0.05$; ††$P < 0.01$).

Table 3. *Mean levels* of PCBs accumulated in mice as a percentage of the total PCB intake*

Type of PCB	PCB feeding regime	PCB intake by dams killed at wk 18† (μg)	PCB level (percentage of intake) in			PCB intake by dams killed 5 wk after delivery (μg)	PCB level (percentage of intake) in						
			Whole body	Liver	Foetuses		Offspring aged (wk)					Dam (5 wk after delivery)	
							1	2	3	4	5	Whole body	Liver
a	BP	17 ± 0·9	0·13	0·02	0·006	17 ± 1·6	0·11	0·14	0·07	0·11	0·06	0·02	0·006
	DP	27 ± 3·4	7·3	0·03	0·03	25 ± 2·0	1·2	2·9	0·37	0·08	0·07	0·06	0·004
b	BP	23 ± 1·2	0·03	0·004	0·004	23 ± 2·1	0·06	0·12	0·10	0·12	0·08	0·04	0·004
	DP	35 ± 4·5	1·0	0·006	0·006	33 ± 2·6	0·05	0·10	0·09	0·07	0·09	0·02	0·003
c	BP	23 ± 1·2	0·27	0·01	0·004	23 ± 2·1	0·19	0·26	0·21	0·19	0·17	0·03	0·004
	DP	35 ± 4·5	6·0	0·03	0·02	33 ± 2·6	0·94	1·2	0·33	0·13	0·18	0·04	0·003
d	BP	24 ± 1·3	27	1·6	0·22	24 ± 2·2	40	44	41	34	21	0·47	0·12
	DP	37 ± 4·7	25	0·84	0·12	34 ± 2·7	35	49	31	23	21	0·35	0·01
e	BP	24 ± 1·3	18	13	0·23	24 ± 2·2	31	39	37	27	19	0·40	0·27
	DP	37 ± 4·7	23	3·2	0·14	34 ± 2·7	34	53	30	23	19	0·50	0·13
f	BP	8·7 ± 0·5	0·05	—	—	8·7 ± 0·8	—	—	—	—	—	—	0·02
	DP	13 ± 1·7	0·27	0·008	0·002	13 ± 1·0	0·08	0·10	0·08	—	—	—	—
g	BP	13 ± 0·7	12	0·41	0·09	12 ± 1·2	25	15	16	18	16	1·8	0·08
	DP	19 ± 2·5	12	0·89	0·03	18 ± 1·4	25	44	18	18	15	0·89	0·04

a = 2,4,4′-tri-CB b = 2,5,3′,4′-tetra-CB c = 2,4,5,2′,5′-penta-CB d = 2,4,5,2′,4′,5′-hexa-CB
e = 2.3,4,2′,4′,5′-hexa-CB f = 2,3,4,5,6.2′,5′-hepta-CB g = 2,3,4,5,2′,3′,4′,5′-octa-CB BP = Before pregnancy
DP = During pregnancy

*Mean of 12 experiments.
†Mean ± SD.

Table 1 shows the concentrations of individual PCB isomers in the whole body, liver and foetuses of the pregnant mice that were fed the PCBs for 18 days before or during pregnancy and killed on day 18 of pregnancy. The concentrations in foetuses were much lower than those in the whole bodies of dams, their ratios ranging from 1:70 to 1:10 except in the case of 2,5,3′,4′-tetra-CB, that had been administered before pregnancy. The concentrations in foetuses were also lower than those in the livers of dams. The ratios of these concentrations, however, varied even more, ranging from 1:220 to 1:3.

Table 2 lists the concentrations of individual PCB isomers in offspring aged from 1 to 5 wk and those in the whole bodies and livers of dams that were fed the PCBs for 18 days before or during pregnancy and nursed their offspring for 5 wk. The concentrations of PCBs in 1-wk-old offspring were very high compared with those of the corresponding isomers in foetuses shown in Table 1. The levels in 2-wk-old offspring were similar to those in 1-wk-old offspring. However, the levels of PCBs in offspring decreased during the period from 3 to 5 wk after birth, since weaning took place 2 or 3 wk after birth. The levels in 5-wk-old offspring were similar to the corresponding values in their dams.

The levels of PCBs in foetuses and offspring were very different depending on the PCB structure. The concentrations of 2,5,3′,4′-tetra-CB and 2,3,4,5,6,2′,5′-hepta-CB were relatively low, <0·01–0·5 ppb in foetuses and offspring both when their dams were fed the PCBs before and during pregnancy. The levels of 2,4,4′-tri-CB and 2,4,5,2′,5′-penta-CB were comparatively high, 1·3 ppb in foetuses and 8 ppb in 1-wk-old offspring when their dams were fed the PCBs during pregnancy, while they were lower, 0·09–0·15 ppb in foetuses and 0·56–1·3 ppb in offspring when the PCBs were administered before pregnancy. By contrast 2,4,5,2′,4′,5′-hexa-CB, 2,3,4,2′,4′,5′-hexa-CB, 2,3,4,5,2′,3′,4′,5′-octa-CB were retained by the foetuses (1·2–8·9 ppb) and even more so by offspring (81–330 ppb) under both feeding conditions.

Table 3 summarizes (as percentages of the total intake by the dam) quantities of individual PCB isomers retained in the tissues of dam, foetuses and litter of offspring. The total quantities of PCBs per litter were estimated from the number of offspring in the litter and their PCB concentrations. Very small amounts of PCB isomers were detected in the foetuses, less than 0·3% of the total intake by a dam for all the PCB isomers tested, and about 1% of the amount determined in their dam except in a few cases. However, quantities of PCBs were much larger in the offspring killed during the lactation period (about 2 wk after birth), and in most cases were greatest in 2-wk-old offspring. The values for the bodies of offspring aged 3–5 wk gradually decreased, as they were weaned and started to take the normal diet. In contrast with these offspring, only small amounts of the PCBs remained in the bodies of dams that had nursed them for 5 wk.

The amounts accumulated in dams, foetuses and offspring vary greatly depending on the particular PCB tested. The amounts of 2,5,3′,4′-tetra-CB and 2,3,4,5,6,2′,5′-hepta-CB retained by the dams, foetuses, and 2-wk-old offspring were very small, being 0·03–1%, less than 0·006%, and less than 0·12%, respectively, of the total intake of the individual PCBs both when PCBs were fed before and during pregnancy. 2,4,4′-Tri-CB and 2,4,5,2′,5′-penta-CB accumulated in the tissues of dams (6–7%) and in offspring during the lactating period (1–3%) when the PCBs

were given during pregnancy, while they accumulated less when they were fed before pregnancy. The hexa-CBs and octa-CB were retained to a much greater extent, 12–27% of the total intake being found in the body of the dam and 15–53% of the ingested PCBs by dam being transferred to 2-wk-old offspring through the milk.

DISCUSSION

All the PCB isomers tested on mice were transferred to foetuses through the placenta in small amounts, but their transfer from dams to offspring in the milk was considerably greater. These transfer properties are similar to the results of previous experiments with Kanechlor 500 using mice (Masuda *et al.* 1978). The transfer properties, however, varied considerably between individual PCBs, just as Orberg (1977) had reported using 2,5,4′-tri-CB and 2,4,5,2′,4′,5′-hexa-CB and mice. Biphenyls with relatively low numbers of chlorine atoms such as 2,4,4′-tri-CB, 2,5,3′,4′-tetra-CB and 2,4,5,2′,5′-penta-CB were eliminated from the bodies of mice and less transferable to their offspring through milk, while highly chlorinated compounds such as 2,4,5,2′,4′,5′-hexa-CB, 2,3,4,2′,4′,5′-hexa-CB and 2,3,4,5,2′,3′,4′,5′-octa-CB were accumulated in the tissues and were more transferable to offspring. However, 2,3.4.5.6,2′,5′-hepta-CB was the most easily eliminated and the least transferable of all the PCBs tested despite being a highly chlorinated compound. The transferability of PCBs cannot be explained by the numbers of substituted chlorine atoms on the biphenyl alone. The positions of the chlorine atoms is also relevant. Of the PCB isomers investigated, PCBs having adjacent hydrogen atoms at 3 and 4 positions in an aromatic ring, such as 2,5,3′,4′-tetra-CB, 2,4,5,2′,5′-penta-CB and 2,3,4,5,6,2′,5′-hepta-CB, were accumulated less in dams and offspring of mice. 2,4,4′-Tri-CB was an exception to this showing relatively low accumulation, although it did not have the adjacent hydrogen atoms; this may be attributable to its very low number of chlorine atoms.

In certain PCBs such as 2,4,5,2′,4′,5′-hexa-CB and 2,3,4,2′,4′,5′-hexa-CB, about half the quantity consumed by the dam was transferred to the offspring through the milk. These isomers have been found to be accumulated in the human body (Jensen & Sundström, 1974; Kuroki & Masuda, 1977). These PCBs are presumably transferred from mothers to infants via the milk in large quantities.

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EFFECTS OF CADAVERINE ON HISTAMINE TRANSPORT AND METABOLISM IN ISOLATED GUT SECTIONS OF THE GUINEA-PIG

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Abstract—The effects of cadaverine on histamine transport and metabolism in the intestine were studied *in vitro* with isolated sections of guinea-pig gut. [Ring-2-^{14}C]histamine diluted with non-radioactive histamine was placed in the gut sac, with and without cadaverine and the solution outside the gut sac was analysed at intervals, for 3 hr. No latent period was observed before the transport of radioactivity began. Cadaverine increased the rate of transport of radioactivity across the gut wall. The relative effect of cadaverine on transport of radioactivity decreased as the ratio of cadaverine:histamine approached 1:2. Cadaverine produced only small changes in the relative levels of histamine and histamine metabolites that appeared outside the gut sac. These findings are discussed in relation to a proposed mechanism of cadaverine potentiation of histamine toxicity *in vivo*.

INTRODUCTION

Histamine has been at the centre of attention in studies of the causative agent(s) of scombroid poisoning. Histamine causes reactions similar to those observed in scombroid poisoning, and its elevated level in fish has been used as an indication of spoilage. However, the exact nature of the toxin(s) involved in scombroid poisoning is still not clear (Arnold & Brown, 1978; Foo, 1975). Histamine has a relatively low oral toxicity in humans (Weiss, Robb & Ellis, 1932). Other substances such as diamines may be involved in the poisoning.

Diamines in the series $NH_2(CH_2)_nNH_2$, especially those with short chains ($n < 5$) have pronounced effects on histamine biological activity. The short-chain diamines are substrates for histaminase, they inhibit histaminase competitively, and they potentiate various effects of histamine (Mongar, 1957). Many antihistaminases potentiate histamine-induced contraction of guinea-pig ileum, trachea, and uterus, and their effectiveness as potentiators correlates with their antihistaminase activity (Arunlakshana, Mongar & Schild, 1954). These activities were greatest for putrescine and cadaverine.

Whereas putrescine and cadaverine have similar effects on histamine-induced reactions *in vitro*, their effects on histamine toxicity in whole animals show important differences (Bjeldanes, Schutz & Morris, 1978; Parrot & Nicot, 1966). First, cadaverine exhibits a maximum effect on histamine toxicity following simultaneous administration of the two compounds. A maximum potentiation effect was observed with putrescine when it was administered 40 min prior to administration of histamine. Second, the relative levels of cadaverine:histamine observed to yield a toxic effect are similar to the ratios in toxic fish (Kim & Bjeldanes, 1979; Mietz & Harmas, 1977); whereas the relative levels of putrescine:histamine required for toxicity are much higher than the levels in toxic fish.

In an effort to understand the mechanism of cadaverine potentiation of histamine toxicity, we have studied the effects of cadaverine on histamine absorption and metabolism in isolated gut sections of the guinea-pig.

EXPERIMENTAL

Preparation of gut segments. Female guinea-pigs weighing 600–900 g were used. The animals were placed in separate cages and fasted for 24 hr prior to the experiments. The animals were killed by decapitation and the intestines were removed, cleaned with warm Ringer's solution and placed in cold Ringer's solution. A 30-cm piece from each end of the intestine was removed, and the remaining portion was cut into 5-cm pieces. Segments were closed with thread at one end.

Preparation of test solutions. Freshly made and gassed Ringer's solution was used to prepare test solutions. Solutions were 0·1 ml in volume, containing 1 mg of histamine dihydrochloride. Various amounts (0·10, 0·25, 0·40, 0·50 and 1·0 mg) of cadaverine dihydrochloride (Calbiochem, La Jolla, CA) were added to this solution for each group of experiments.

Transport of radioactivity across the gut wall. [Ring-2-^{14}C]histamine dihydrochloride (Amersham Corp., Arlington Heights, IL) with 0·5 μCi of activity was added to a syringe containing 0·1 ml of test solution including 1 mg of histamine dihydrochloride (Sigma Chemical Co., St. Louis, MO). This solution was placed in the gut-sac lumen through the open end. The open end was closed with thread as the syringe was withdrawn. The prepared gut sac was placed in a flask containing 40 ml of Ringer's solution. The flask was placed on a water-bath shaker (Eberbach Corp., Ann Arbor, MI) and incubated at 37°C under an atmosphere of 95% O_2–5% CO_2 (Airco Inc., Madison, WI).

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To determine the rate of transport during the initial 20 min, 2-ml aliquots of bath solution were removed at 5-min intervals and counted for radioactivity. To determine the rate of transport for longer periods, aliquots of bath solution were removed every 20 min. After 3 hr, incubation was stopped and sacs were removed from the flask. The remaining portion of the outside solution was kept for metabolite analysis.

As a routine, 14 ml scintillation liquid was added to each vial containing the 2-ml aqueous sample solution. Each vial was closed, shaken vigorously until the solution became clear, and counted for radioactivity in a liquid scintillation counter (Beckman CPM 100 Liquid Scintillation System, Beckman Co., Fullerton, CA) for 1 min. All counts were corrected for counting efficiency and quenching effect before further calculations.

Analysis of metabolites. The solutions remaining after the removal of 2-ml portions were concentrated *in vacuo* for metabolite analysis. If the solutions were turbid, a few drops of 20% trichloroacetic acid (TCA) solution were added. The samples were then refrigerated for 2 hr, filtered through Whatman no. 1 filter and dried *in vacuo*. The residue was dissolved in a small volume of acidic methanol (pH 1) and filtered. The supernatant was again dried and dissolved in 1 ml methanol. The mixture was filtered through glass fibre filter paper to remove excess salt, and the filtrate was diluted to 1 ml with methanol.

Levels of 1-methylhistamine, imidazoleacetic acid, 1-methyl-4-imidazoleacetic acid, acetylhistamine and histamine in concentrated sample solutions were determined by an adaption of the methods described by Snyder, Axelrod & Bauer (1964).

Histamine and methylhistamine. A 10-μl portion of each sample solution was diluted with 2 ml 0·4 N-perchloric acid in glass-stoppered centrifuge tubes. Then, 0·3 ml 5 N-NaOH saturated with NaCl was added to each tube. Histamine and methylhistamine were extracted with a solution of *n*-butanol–chloroform (3:2) and 1-methylhistamine was extracted with chloroform. The former solvent extracted 80% of histamine and 1-methylhistamine, and the latter extracted 70% of 1-methylhistamine. Aqueous layers were then transferred to vials for counting. Organic layers were washed with 2 ml 0·1 N-NaOH saturated with NaCl, transferred to scintillation vials and evaporated to dryness under a stream of nitrogen. After addition of 2 ml water and 14 ml scintillation fluid, vials were covered and shaken until the solutions became clear. Radioactivity of each vial was counted for 5 min.

The amounts of histamine and 1-methylhistamine are presented as percentages of the total counts. Total counts are the sum of the counts of the organic and aqueous phases.

$$H\ (\%) = (\%\ BCE/0{\cdot}8) - MH$$

$$MH\ (\%) = (\%\ CE/0{\cdot}7)$$

where H is histamine, % BCE is the percentage of the counts in the butanol–chloroform extract, MH is 1-methylhistamine, and % CE is the percentage of the counts in the chloroform extract.

Imidazoleacetic acid, 1-methyl-4-imidazoleacetic acid, and acetylhistamine. A 5-μl portion of the sample solution was spotted on Whatman no. 1 paper together with authentic compounds, and with the standard solution. The standard solution was prepared by adding standard compounds of histamine, 1-methylhistamine, 1-methyl-4-imidazoleacetic acid, imidazoleacetic acid (Calbiochem, La Jolla, CA) and acetylhistamine (Aldrich Chemical Co., Milwaukee, WI) to Ringer's solution and processed in the same way as the sample solution. The paper was developed by descending technique in a solution of *tert*-butanol–formic acid–water (70:15:15). The areas with authentic compounds and standard solution were separated from sample chromatograms, rendered visible by spraying with 1% *p*-nitrobenzenediazonium tetrafluoroborate (Eastman Chemical Co., Rochester, NY) in acetone, followed by spraying with 1% KOH in water. The areas corresponding to authentic imidazoleacetic acid, 1-methyl-4-imidazoleacetic acid, and acetylhistamine on the sample chromatogram were cut into pieces and placed in vials containing 2 ml of distilled water. The remaining portion of sample chromatograms was cut into pieces, placed in vials, and eluted with water. Fourteen ml of scintillation liquid was added to each vial. Vials were covered and shaken until the solutions became clear. Each vial was counted for 5 min in a liquid scintillation counter.

1-Methylhistamine was found to have very similar R_F values to 1-methyl-4-imidazoleacetic acid in the standard solution. Therefore, the area cut from 1-methyl-4-imidazoleacetic acid contained both 1-methylhistamine and 1-methyl-4-imidazoleacetic acid. This, however, would not interfere with our analysis, because methylhistamine was determined by the extraction method. Thus, it was possible to determine 1-methyl-4-imidazoleacetic acid by difference. The amount of each metabolite is expressed as the percentage of the total counts.

Statistical analysis. For each set of data, the mean and the standard error of the mean were estimated. The differences between means of different groups were tested for statistical significance by Student's *t*-test. Results of test groups (with added cadaverine) were compared with the control group (without added cadaverine). Any results with $P > 0{\cdot}05$ were considered to be statistically insignificant.

RESULTS AND DISCUSSION

The rate of transport of total radioactivity from radiolabelled histamine, represented by the slope of the curves in Fig. 1, is increased by cadaverine. The combination of 1 mg of cadaverine dihydrochloride with 1 mg of histamine dihydrochloride increased the transport rate to about 68 dpm/min from 42 dpm/min with histamine alone. These rates correspond to $5{\cdot}2 \times 10^{-4}$ μmol/min and $3{\cdot}1 \times 10^{-4}$ μmol/min, respectively, as histamine dihydrochloride and result in a 45% increase in transport of histamine and metabolites after 3 hr.

The investigation of total radioactivity transported across the gut wall during the initial 20 min showed that transport of radioactivity begins almost immediately after placing radiolabelled histamine in the gut sac. The amount of radioactivity which appeared in the solution outside the gut sac after 5 min was 7% of that present after 3 hr, and increased to 18% after

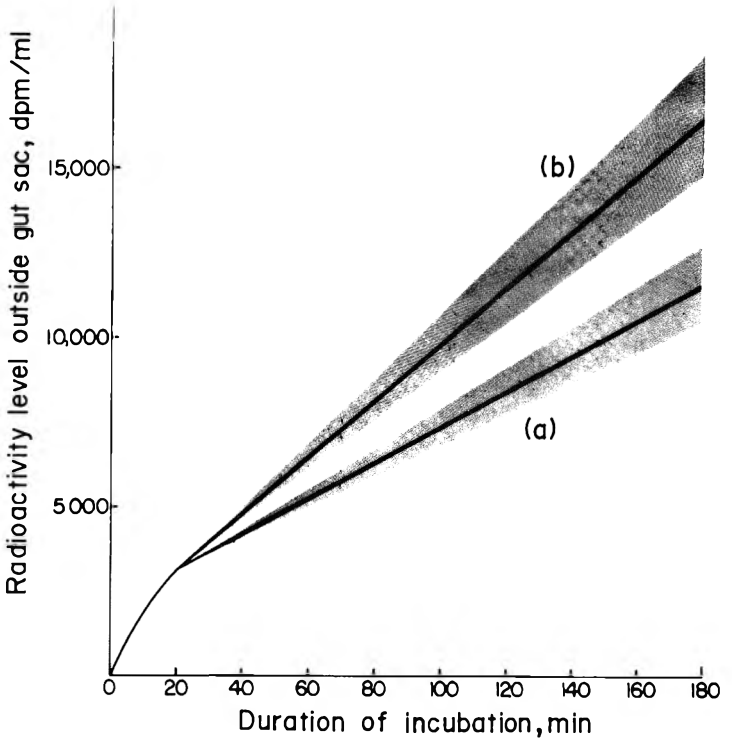

Fig. 1. The rate of radioactivity transport. (a) 1 mg of histamine dihydrochloride was placed in the gut sac (five replicates). (b) 1 mg of histamine dihydrochloride and 1 mg of cadaverine dihydrochloride were placed in the gut sac (four replicates). The curves represent the mean slopes for each experiment. The shaded areas include the slopes for all the replicates of each experiment. The slopes of all replicates indicated in (a) are significantly different ($P < 0.01$) from the slopes of all replicates indicated in (b).

20 min. Cadaverine had no apparent effect on this initial period of transport of radioactivity. Latent periods of from 10 to 60 min, depending on the dose, have been reported for transport of histamine across the gut wall of guinea-pigs (Parrot & Nicot, 1966; Dworetzky & Code, 1951). It is possible that this apparent latent period was due to the relatively insensitive analytical method used for histamine analysis.

The effect of cadaverine concentration on the amount of radioactivity transported after 3 hr is presented in Fig. 2. A ratio of amounts of cadaverine to histamine of 1:10 resulted in a 17% increase over controls of transport of radioactivity. The effect of cadaverine was nearly at a maximum when the ratio of cadaverine to histamine was 1:2. At this relative level, transport was increased by 29% over that of the controls. Higher relative concentrations of cadaverine beyond this level did not appreciably increase the amount of radioactivity appearing outside the gut segment. When histamine alone was placed inside the gut sac, 41·2% was transported across the gut wall as histamine and metabolites. The percentage increased significantly to 48% by adding cadaverine in the ratio (cadaverine:histamine) 1:10, to 52·8% at the level of 1:2, and to 53·6% at the level of 1:1.

The results of metabolite analysis indicate that cadaverine had a minor effect on the overall scheme of histamine metabolism. The percentage of free histamine appearing outside the gut sac increased from 30·7% when histamine alone was present to 35·2 and 37·2% when cadaverine was added to the gut sac in ratios to histamine of 1:2 and 1:1, respectively. Imidazoleacetic acid, which is produced by histaminase-mediated oxidative deamination of histamine, constituted the highest percentage among all the metabolites examined. The level of imidazoleacetic acid was increased slightly, but significantly, with cadaverine.

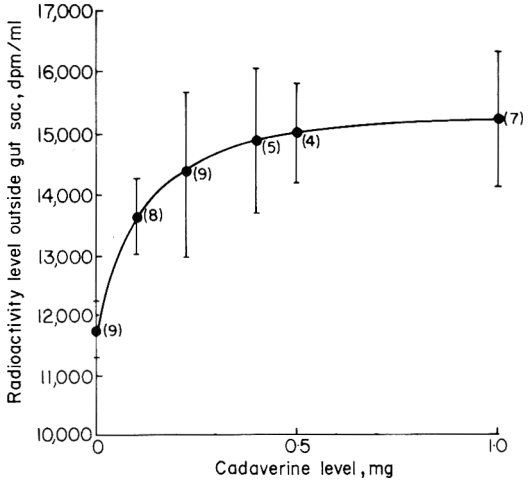

Fig. 2. The effect of cadaverine on radioactivity transport. Each gut sac contained 1 mg of histamine dihydrochloride, 0·5 μCi of [Ring-2-^{14}C]histamine, and the specified amount of cadaverine dihydrochloride. Numbers in parentheses indicate the number of samples for each point. The bar at each point indicates the range of one standard error above and below the mean value.

but this increase was not related to the dose of cadaverine used. The percentage was increased from 26·6% when no cadaverine was added to 30·3% by the presence of cadaverine at the relative level of 1:1. Levels of 1-methylhistamine accounted for less than 10% of total metabolites and were unaffected by cadaverine. Levels of 1-methyl-4-imidazoleacetic acid were negligible. Acetylhistamine comprised 4·8% of the total metabolites when histamine was placed alone in the gut sac. With cadaverine, the proportion of acetylhistamine appearing outside the gut sac was approximately doubled for all groups, regardless of the cadaverine dose.

Whereas cadaverine exhibited a marked influence on the rate of transport of histamine and metabolites across the gut wall, the proportions of each metabolite were not changed appreciably. Thus, cadaverine potentiation of histamine toxicity may result from induction of increased rates of absorption of histamine and metabolites. The established antihistaminase activity of cadaverine does not appear to play a significant role.

Parrot & Nicot (1966) have suggested that mucin may be important in opposing the passage of histamine across the gut wall and that the role of enzymatic detoxification in the gut is only secondary in this regard. The presence of the plateau in the curve indicating the effect of cadaverine concentration on the transport of radioactivity (Fig. 2) is consistent with a proposed role of substances such as mucin in maintaining a barrier to histamine transport. If cadaverine potentiates histamine toxicity by binding to mucin, as has been suggested for putrescine (Parrot & Nicot, 1966), then the relative effect of various amounts of cadaverine on histamine transport would depend on the quantity of unbound mucin in the gut. In our experiments, approximately 0·5 mg of cadaverine provided a nearly maximum effect on transport of histamine and metabolites. This also might be the quantity of cadaverine required for optimum binding of available mucin in the gut segment.

Our results and those obtained by Parrot & Nicot (1966) suggest that putrescine, cadaverine, and possibly other substances may effect an increase in histamine toxicity by related mechanisms. The marked differences in effects of putrescine and cadaverine on histamine toxicity observed *in vivo* may depend on the degree of binding of these potentiators to mucin or related substances.

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PERCUTANEOUS ABSORPTION OF DODECYL-TRIMETHYLAMMONIUM BROMIDE, A CATIONIC SURFACTANT, IN THE RAT

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Abstract—The route and rate of excretion of the cationic surfactant [^{14}C]dodecyltrimethylammonium bromide (DTB) given by parenteral injection were investigated in rats, and the percutaneous absorption of the surfactant through non-occluded rat skin and the resulting blood levels were also studied. The surfactant was applied to the intact clipped skin of rats in aqueous solution or in a hair-rinse formulation. Elimination by the rat after parenteral administration was rapid and was effected primarily via the urine, more than 80% of the radioactivity being eliminated within 24 hr of application. Percutaneous absorption of the surfactant was low. Application in a cream hair-rinse preparation under user conditions resulted in the absorption of about 0·1% of the administered radioactivity in 48 hr. No measurable radioactivity was present in the blood. Application of the surfactant at a higher concentration in aqueous solution gave a somewhat higher absorption (0·6% in 72 hr). Some radioactivity (equivalent to less than 100 ng unchanged DTB/g) was found in the blood after application of the surfactant to the skin without subsequent rinsing.

INTRODUCTION

Surface-active quaternary ammonium compounds are cationic and are widely used as disinfectants, laundry additives and sanitizing agents. The cationic surfactants are used, together with anionic, nonionic and amphoteric surfactants, in cosmetics (Quack, 1975). Because of their high affinity for proteins, which accounts for their bactericidal or bacteriostatic action, cationic surfactants are frequently applied in hair-care formulations. The concentrations of quaternary ammonium compounds in the different formulations (hair-rinse preparations, hair-bleaching or colouring preparations, hairdressing creams, conditioning shampoos and other shampoos) generally lie in the 0·1–4% range.

The possibility of percutaneous absorption of these cationic surfactants from cosmetic formulations into the circulation raises the question of the systemic toxicity of these compounds. It is known from acute toxicity studies that cationic surfactants are 10–100 times more toxic when administered by the iv route than when given orally, indicating that these compounds are only poorly absorbed by the intestines. This was confirmed by Isomaa (1975), who reported that after oral administration of radioactive cetyltrimethylammonium bromide, only small amounts of radioactivity were found in the urine and in the blood plasma. There was no appreciable enterohepatic circulation of the radioactivity.

The studies reported here were designed to investigate the distribution and excretion of cationic surfactants in the rat after iv and sc application and their percutaneous absorption. In addition, we were interested in measuring the blood concentration of the topically applied surfactant following percutaneous absorption of the quaternary ammonium compound.

Cationic surfactants used in hair cosmetic formulations often have one alkyl group with a chain length of 12 or more carbon atoms and three methyl groups. In our studies, we used ^{14}C-labelled *N*-dodecyl-*N*,*N*,*N*-trimethylammonium bromide (DTB) to achieve greater accuracy in determining the extent of percutaneous absorption.

EXPERIMENTAL

Materials. Dodecyl-[1-^{14}C]trimethylammonium bromide ([^{14}C]DTB) was obtained from Farbwerke Hoechst AG, Frankfurt, Germany. The specific activity of the compound was 7·6 mCi/mmol (24·7 μCi/mg). Radiochemical purity was established by thin-layer chromatography on a silica gel SiF plate (Riedel de Haen, Seelze), using *n*-butanol–*n*-propanol–ammonia solution (sp.gr. 0·91)–water (7:5:7:2, by vol.) and subsequent radioautography.

The hair-cream formulation used for some experiments consisted of a mixture of a fatty alcohol and a fatty alcohol polyoxyethylene (0·90%), paraffin oil (1%), fatty acid ester (3%), preservative (0·20%), cetyltrimethylammonium chloride (0·50%), gelling agent (0·60%), polyvalent alcohol (9%) and perfume/water (to 100%).

Animals. Male Wistar rats weighing 200–230 g were used in all the experiments. They were allowed free access to water and food (Altromin GmbH, Lage).

Experimental design

Intravenous administration of [^{14}C]DTB. A 0·023% solution of DTB (500 μl) in 0·9% (w/v) aqueous NaCl was injected into the tail vein of each of two rats (0·822 mg and 0·767 mg DTB/kg body weight). The animals were kept in metabolism cages for 24 hr, during which urine and faeces were collected separately. Expired air was monitored by bubbling the air from the cages through traps containing ethanolamine. The animals were killed 24 hr after the injection. The

organs were removed and their content of radioactivity was determined. Urine and faeces were checked for metabolites by thin-layer chromatography.

To three further rats, DTB solutions (50 μl) in 0·9% (w/v) NaCl (0·135–0·174% DTB) were administered via a jugular cannula (Upton, 1975). Two of the rats were killed 15 min after treatment, and the organs were removed for radioactivity determinations. The radioactivity in the blood of the remaining rat was determined 3, 9, 15, 30, 60, 120 and 300 min after administration of DTB, a 200-μl sample of blood being withdrawn via the jugular cannula at each interval and replaced by 200 μl heparinized saline.

Subcutaneous administration of [^{14}C]DTB. A solution of [^{14}C]DTB in 0·9% (w/v) aqueous NaCl was used, 1 ml of the solution, containing 0·29 mg [^{14}C]DTB, being administered sc in the dorsal region of three male rats. The animals were kept separately for 48 hr in metabolism cages and urine and faeces were collected daily. The animals were then killed, the area around the point of injection was removed and the carcass was homogenized.

Cutaneous application of an aqueous solution of [^{14}C]DTB with rinsing. The dorsal hair of each animal was closely clipped one day before application. Only rats with uninjured skin were chosen for the experiment. Before treatment, the animals were anaesthetized with a short-acting narcotic (Ketanest, Parke Davis & Co. Detroit, MI, USA). A 200-μl sample of a 1% solution of DTB in water was applied to a marked 10-cm^2 area (30 × 33 mm) of clipped skin, lathered for 3 min with a glass rod and left there for 15 min. The treated area was then rinsed with 100 ml water (at 37°C), the rinsings were collected and the animals were lightly dabbed dry with absorbent tissue. The treated skin was covered with a specially designed light glass cap, which was fitted with small holes to avoid occlusive conditions and was glued to the skin with a special adhesive (Sicomet 99®, Sichel, Hannover), care being taken to avoid contamination of the treated area with the adhesive.

The animals were held for 72 hr in individual metabolism cages, urine and faeces being collected separately and removed every 24 hr. After 72 hr, the rats were killed and the treated skin, the liver and the kidneys were removed. Radioactivity was determined in the urine, faeces and rinsings, the kidneys and liver, the skin removed from the application site, the homogenized carcass, and the absorbent tissue and glass cap.

Cutaneous application of [^{14}C]DTB in a hair-rinse preparation. To determine the percutaneous absorption of DTB from a hair-rinse preparation under user conditions, an amount of the preparation roughly equivalent to that used in practice was applied to the 8-cm^2 area of the clipped skin of rats. The whole area of the human scalp is approximately 600 cm^2, so that assuming the use of 20 ml shampoo in shampooing, 33 μl shampoo/cm^2 is applied under normal conditions of use.

One day before the experiment, the radioactively labelled hair-rinsing application was prepared by dissolving 25 mg [^{14}C]DTB in 5 g of the hair-rinsing preparation (to provide a 0·5% DTB solution). The real concentration according to the measured radioactivity was 0·42%. On the following day, 261–293 mg of this solution was applied to the clipped dorsal skin of five anaesthetized rats, lathered for 3 min with a glass rod and left there for 5 min. The treated area was rinsed with 100 ml water (at 37°C), the rinsings were collected and the animals were lightly dabbed dry with absorbent tissue. The treated area was covered with a glass cap. The animals were kept singly for 48 hr in metabolism cages and urine and faeces were collected separately every 24 hr.

At 0·5, 1, 3, 5, 22, 26, 30, 46 and 50 hr after application, blood was withdrawn from the tail vein and the level of radioactivity was determined. After the animals had been killed, the treated skin was removed. Radioactivity was determined in the urine, faeces, rinsings, homogenized carcass, absorbent tissue and glass cap.

Cutaneous application without rinsing. To ensure measurable radioactivity in the blood after penetration of DTB through the rat skin, the surfactant was applied in these experiments at a higher concentration than before and the skin was not rinsed. A 3% DTB solution in water was applied in a volume of 240 μl to an 8-cm^2 area of the clipped dorsal skin of three anaesthetized rats. After the treated skin area had been covered with a glass cap, the animals were placed in individual metabolism cages for 48 hr. Urine and faeces were collected separately every 24 hr. Blood samples were taken from two rats via a jugular cannula during the periods 0–7·5, 22–30 and 46–50 hr after application at time intervals of 30 or 60 min. Radioactivity was determined in the urine, faeces, rinsings, blood samples, removed treated skin, homogenized carcass and glass cap.

Carbon-14 analyses. All samples were counted in a Tri-Carb 2425 liquid scintillation spectrometer (Packard Instrument Co., Frankfurt/M). Counting efficiencies were determined by a channels ratio technique or by the addition of an internal standard for quenching correction. Faecal samples were lyophilized and ground to a powder and three 200-mg aliquots were prepared for liquid scintillation counting by combustion in a Sample Oxidizer, Model 306 (Packard Instruments). Urine and ethanolamine-CO_2 were placed directly in scintillation fluid (Unisolve-1, from Koch-Light Laboratories Ltd., Colnbrook, Bucks, England).

Skin samples were solubilized in TS-1 (Koch-Light Laboratories Ltd.), and an aliquot of the solution was treated with a special scintillation cocktail (Neame, 1975). The radioactivity was determined in the liquid scintillation counter. The carcass was homogenized in a mincer after addition of sawdust (10% of the carcass weight) and a lyophilized aliquot was combusted in the sample oxidizer after the sample had been pulverized in a coffee-mill. The organs were either combusted *in toto* after lyophilization in the sample oxidizer, or an aliquot of the homogenized sample was used. The minimum detectable count rate was considered to be twice the background level.

RESULTS

Elimination of [^{14}C]DTB after iv and sc doses

The fate of the radioactivity administered by iv or sc injection of [^{14}C]DTB is shown in Table 1. Most of the radioactivity was eliminated from the body within the first 24 hr and was mainly excreted in the

Table 1. *Mean rate of excretion of radioactivity in rats given an iv or sc dose of [^{14}C]DTB*

Time after injection (hr)	Recovery (% of radioactivity administered)			
	In urine	In faeces	Tissue/carcass residues	Total
		Intravenous dose*		
0–24	58·9 ± 1·06	11·6 ± 0·42	15·3 ± 2·55	85·8 ± 1·06
		Subcutaneous dose†		
0–24	63·4 ± 13·7	11·1 ± 1·88	—	—
24–48	4·77 ± 0·90	3·00 ± 2·28	13·9 ± 2·11	—
0–48	68·1 ± 13·1	14·1 ± 3·56	—	96·2 ± 7·99

*Means for two animals given 0·822 and 0·767 mg [^{14}C]DTB/kg body weight.
†Means for three animals each given 1 mg [^{14}C]DTB/kg body weight.

urine. Only unchanged surfactant was found in the faeces but several so far unidentified metabolites were detected in the urine after iv injection. No radioactivity could be found in the expired air.

The levels of radioactivity in the blood after iv injection showed a steep fall in less than 30 min and only very low levels of radioactivity (near the minimum detectable count, equivalent to 10 ng/g blood) could be detected 5 hr after the injection. Assuming no conversion to metabolites, the blood levels at 3, 9, 15, 30, 60, 120 and 300 min after the injection were 0·50, 0·10, 0·04, 0·03, 0·04, 0·02 and 0·009 μg/ml blood, respectively.

No significant difference was found in the pattern of excretion between iv and sc dosed rats. Thin-layer chromatography indicated the presence of several radiolabelled metabolites in the urine but only unchanged DTB was demonstrated in the faeces.

Tissue distribution of radioactivity after iv injection of [^{14}C]DTB

Tissue distribution of radioactivity was determined 15 min and 24 hr after injection of DTB (Table 2). At the earlier time, a high level of radioactivity was measured in the liver and kidneys, the organs concerned with the elimination of DTB. The high level of radioactivity in these organs explains the rapid fall in radioactivity in the blood. After 24 hr, only small amounts of radioactivity were found in the liver and kidneys. There was no sign of accumulation of the label in any organ.

Table 2. *Concentration of radioactivity in organs and tissues 15 min and 24 hr after a single iv dose of [^{14}C]DTB*

Organ or tissue	Concn of radioactivity	
	% of applied dose	μg equivs/g
	15 min	
Liver	24·8	7·01
Kidneys	5·54	10·4
Heart	0·48	2·09
Spleen	0·15	1·00
Lungs	0·83	3·01
Stomach	ND*	0·18
Intestinal tract	ND*	1·61
Blood†	1·47	0·13
	24 hr	
Liver	2·08	0·84
Kidneys	0·36	0·99
Heart	0·11	0·89
Spleen	0·029	0·34
Lungs	0·14	0·74
Stomach	0·18	0·16
Intestinal tract	1·41	0·58
Blood†	—‡	—‡

*Only specific activities were determined.
†Assuming a blood volume of 10 ml.
‡Below the minimum detectable count rate.

Cutaneous application of [^{14}C]DTB with rinsing of the skin

From Table 3, it can be seen that the percutaneous absorption of the surfactant was very low. The total absorption was 0·59% of the applied radioactivity. Most of the amount absorbed was excreted in the urine. Within the first 24 hr, 0·35% of the applied surfactant was excreted. Of the radioactivity applied, 13·2% remained on the skin after rinsing, demonstrating the relatively high affinity of the surfactant for skin.

Cutaneous application of [^{14}C]DTB in a hair-rinse preparation

Applying [^{14}C]DTB in a hair-rinse preparation (0·5% DTB) under conditions of normal use resulted in a marked decrease in the percutaneous absorption of the surfactant (Table 3). Only 0·016% of the amount applied was excreted in the first 24 hr and 4·11% of the surfactant remained at the application site. No significant radioactivity was detected in the blood of the treated animals during the experiment (detection limit 10 ng surfactant/g blood).

Cutaneous application of [^{14}C]DTB without rinsing

Application of a 3% solution of [^{14}C]DTB on the skin without any rinsing resulted in percutaneous resorption of 3·15% of the applied radioactivity (Table 3). In the experiments involving rinsing, the excretion of radioactivity was always lower on day 2 than on day 1, but in this experiment there was a marked increase in absorption on day 2. The possibility cannot be excluded that the relatively long contact of the skin with the surfactant caused a slight but invisible damage to the skin, resulting in higher absorption rates. In two of the rats, the blood level of the surfactant was below the detection limit of 10 ppb (10 ng/g) for the first 5 hr of cutaneous application of DTB (Table 4). Significantly higher levels of radioactivity

Table 3. *Effects of the vehicle and of rinsing on the distribution of radioactivity in rats after the cutaneous application of [^{14}C]DTB*

Materials analysed	Radioactivity (% of ^{14}C administered)		
	Aqueous DTB with skin rinsing*	DTB in hair-rinse with skin rinsing†	Aqueous DTB without skin rinsing‡
Urine: 0–24 hr	0·25 ± 0·11	0·011 ± 0·007	0·60 ± 0·71
24–48 hr	0·05 ± 0·003	0·008 ± 0·007	1·16 ± 0·56
48–72 hr	0·02 ± 0·006	—	—
Total...	0·32 ± 0·115	0·019 ± 0·013	1·76 ± 1·21
Faeces: 0–24 hr	0·10 ± 0·05	0·005 ± 0·006	0·02 ± 0·003
24–48 hr	0·04 ± 0·01	0·008 ± 0·01	0·26 ± 0·22
48–72 hr	0·04 ± 0·02	—	—
Total...	0·18 ± 0·08	0·013 ± 0·017	0·28 ± 0·22
Carcass + tissues	0·09 ± 0·06	0·061 ± 0·047	1·11 ± 0·75
Percutaneous absorption...	0·59 ± 0·13	0·093 ± 0·061	3·15 ± 1·65
Liver	0·004 ± 0·001	—	—
Kidneys	0·001 ± 0·0003	—	—
Rinsings	81·4 ± 1·77	80·4 ± 3·41	—
Application site	13·2 ± 2·96	4·11 ± 2·21	93·2 ± 5·76
Total recovered...	95·3 ± 2·41	92·6 ± 3·13	96·4 ± 7·09

*Means ± SD for five rats each given a dose of 1·92 mg [^{14}C]DTB in 1% aqueous solution on a 10-cm^2 application site, which was rinsed after 15 min with 100 ml warm water.

†Means ± SD for five rats each given a dose of 1·1–1·2 mg [^{14}C]DTB as an approximately 0·5% solution in a hair-rinse preparation applied to an 8-cm^2 site, which was rinsed after 5 min with 100 ml warm water.

‡Means ± SD for three rats each given a dose of 7·35 mg [^{14}C]DTB in a 3% aqueous solution on an 8-cm^2 application site, which was not rinsed afterwards.

could be determined in blood samples from rat 3. Within 30 min of the application, the blood contained measurable levels of radioactivity. This is in accordance with the significantly higher percutaneous absorption of DTB found in this rat.

DISCUSSION

After either iv or sc administration of the surfactant, more than 70% of the applied radioactivity was eliminated within the first 24 hr. Measurement of the blood concentration showed a rapid fall in the surfactant level, no radioactivity being detectable in the blood 5 hr after iv injection. The liver and the kidneys, organs involved in the elimination of the substance, showed the highest specific radioactivity among the organs and tissues of animals killed 15 min after treatment. The distribution of radioactivity in the different organs 24 hr after administration did not demonstrate any significant affinity of the surfactant for any organ or tissue. The radioactivity was excreted mainly in the urine. Whilst in the faeces only the unchanged surfactant was detectable by thin-layer radiochromatography, the urine contained several metabolites. A further study is planned to investigate the metabolism of this compound.

The absorption of the surfactant after cutaneous application of an aqueous solution of [^{14}C]DTB followed by rinsing of the skin was very low, only 0·59% of the applied dose being absorbed in 72 hr. The absorption gradually decreased with time. This low level of percutaneous absorption was further reduced by cutaneous application of [^{14}C]DTB in a hair-rinse preparation, the absorption in this case accounting for less than 0·1% of the dose in 48 hr. No radioactivity could be detected in the blood. Application of the surfactant in a hair-rinse preparation reduced the

Table 4. *Blood levels of surfactant in rats after cutaneous application of [^{14}C]DTB without subsequent rinsing of the skin*

Time after application (hr)	Blood concn of DTB (ng/g blood)* in rat no.		
	1	2	3
0·5	—†	33	—†
1	—	30	—
3	—	70	—
5	—	95	—
22	28	62	18
26	30	58	22
30	38	55	26
46	30	81	40
50	28	84	38

*Calculated from blood levels of radioactivity, assuming that no metabolic conversion had occurred.

†Below the minimum detectable count rate (10 ng/g blood).

affinity for the rat skin significantly compared with the application of an aqueous solution (4·11 and 13·2%, respectively, of the administered dose remaining on the skin after rinsing). Cutaneous application of the surfactant without rinsing resulted in a greater degree of percutaneous absorption, as expected. Under these conditions, blood levels in the ppb range could be detected.

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REPRODUCTION AND TERATOLOGY STUDIES OF ZINC PYRITHIONE ADMINISTERED ORALLY OR TOPICALLY TO RATS AND RABBITS

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Abstract—A 48% aqueous slurry of zinc pyrithione (ZPT) was administered topically to rats, without prevention of ingestion through grooming, from 8 wk before mating until day 15 of gestation at levels of 2·5, 7·5 or 15 mg ZPT/kg/day. Both sexes were treated but were mated with untreated rats. No adverse effects were seen on growth, pathology or conception in the parents or on viability, post-weaning growth or pathology in the neonates. Reproduction indices were similarly unaffected by the same doses applied topically on days 6–15 of gestation under conditions preventing any ingestion. The treated females were subsequently mated a second time and given the same treatment as before on days 6–15 of gestation. On the same gestation days, three additional groups were dosed orally with 7·5 or 15 mg ZPT/kg/day in corn oil or with corn oil alone. All the females were killed on day 20 for teratological evaluation. Those treated topically with 7·5 or 15 mg/kg, with no restriction on ingestion, gained significantly less weight than the controls during pregnancy, while two of ten dams given the 7·5-mg/kg doses and five of ten on 15 mg/kg developed hind-leg paralysis. The only effect on the foetuses was a significant reduction in weight at the 15-mg/kg dose level. No adverse effects were seen on either dams or foetuses when similar doses were administered topically and ingestion was controlled. Rats treated orally with 7·5 or 15 mg ZPT/kg gained less weight than controls and 50% of each group exhibited paralysis. Neither dose level had adverse effects on foetal soft-tissue development, but in the group on the higher level foetal weights were significantly lower than in the controls and the incidence of rib defects was significantly increased.

ZPT administered orally to groups of 15 pregnant rabbits from day 6 to day 18 of pregnancy was lethal to six at the 5-mg/kg level, ten at 10 mg/kg and 15 at 20 mg/kg. Survivors lost weight and had significantly higher incidences of embryonic resorption. Another group of rabbits dosed orally with 5 mg ZPT/kg/day lost weight significantly and showed a significantly high number of resorptions, but none of the dams died. The effects in dams dosed with 2·5 mg ZPT/kg were similar but were not statistically significant, while treatment with 1 mg/kg/day had no such effects. None of these ZPT doses affected foetal development adversely. No deleterious effects were seen in restrained rabbits treated topically with 25, 50 or 100 mg ZPT/kg/day on days 6–18, and no teratogenicity was observed.

INTRODUCTION

The salts of 2-pyridinethiol-1-oxide have been used as antifungal agents for many years. The zinc salt, known as zinc pyrithione (ZPT), has been used as an anti-dandruff agent in shampoos and has been subjected to extensive toxicological investigation (Collum & Winek, 1967; Opdyke, Burnett & Brauer, 1967; Snyder, Buehler & Winek, 1965; Wedig, Kennedy, Jenkins, Henderson & Keplinger, 1976; Winek & Buehler, 1966). In these studies, the primary toxic manifestations were ocular lesions in dogs and hind-limb paralysis in rats and rabbits, but they developed only when ingestion occurred.

These products are intended to be used only as topical agents and their safety when used in this fashion has been well documented by Snyder *et al.* (1965). Moreover, Howes & Black (1975), Klaassen (1976) and Okamoto, Hasegawa & Urakubo (1967) have shown that less than 1% of topically applied ZPT is absorbed in several species of rodent. Recently, we treated pregnant rabbits during organogenesis with a shampoo containing 2% ZPT, in topical doses equivalent to 20 or 50 mg ZPT/kg, and found no embryotoxic or teratogenic effects (Nolen, Patrick & Dierckman, 1975). We have extended these studies to compare the effects of oral and topical administration of ZPT on embryogenesis in the rat and the rabbit.

EXPERIMENTAL

For all of the studies reported here, a single lot of an aqueous slurry of ZPT, obtained from Olin Corp., New Haven, CT (lot no. 410-ZP-394) was used. The slurry contained 48% ZPT, with approximately 2% Darvan No. 1® (R. T. Vanderbilt Co., Inc., New York; lot no. C-10-5523-2) and 0·3% food-grade carboxymethylcellulose (CMC-7MF; Hercules, Inc., Wilmington, DE; lot no. 54703) as suspending agents, the remainder being water. Darvan No. 1 is the sodium salt of a polymerized alkylnaphthalenesulphonic acid. Analyses of the slurry indicated a zinc content of 10·11% (9·76%), a ZPT level of 48·0% (47·23%), a pH of 7·9 and a particle size below 5 μm. ZPT to be applied topically to either rats or rabbits was slurried with an additional 3% Darvan and 0·2% CMC to stop it running off the treated area. All vehicle solutions for the topical studies contained 3% Darvan and 0·2% CMC.

Rat studies

Pilot study. Before the reproduction and teratology studies were conducted in rats, a pilot study was done to establish the response from several topically applied doses of the ZPT slurry. No attempt was made to control ingestion resulting from the grooming habits of the rats, since the reproduction study was to be conducted under similar conditions. Adult Charles River Sprague-Dawley rats of both sexes (weighing about 200 g) were randomly distributed into six groups of five rats of each sex. A 3 × 5-cm area on the back of each animal was clipped with an Oster clipper to remove most of the hair and was re-clipped every 3 days during the treatment period. The animals were weighed at similar intervals, to monitor the effects of the material and to adjust the dosages.

A 2 × 4-cm area, within the clipped area, was marked and the ZPT solutions were applied to this area each day for 15 days. No attempt was made to remove any residues from previous applications. The slurry of ZPT was diluted with water so that each animal received 2·0 ml of solution/kg body weight giving one of the following doses: 2·5, 7·5, 15, 20 or 50 mg ZPT/kg body weight. A control group was treated with water. From the results of this pilot study, the doses were selected for the reproduction study.

Rat reproduction study. For this study, 80 weanling (21–23 days old) Charles River Sprague-Dawley rats of each sex were weighed and randomly assigned to eight groups of ten males and ten females after a 3-day acclimatization period in the animal laboratory. The rats were housed individually in stainless-steel cages with suspended wire floors. Purina Laboratory Chow and tap-water were available *ad lib.* throughout the study. The room temperature was maintained at 23 ± 1°C and the relative humidity at 50 ± 5%. Lighting was on a 12-hr light-dark cycle (8.00–20.00 hr).

At the beginning of the study, when the rats were small, a 2 × 4-cm area on the back of the appropriate animals was clipped with an Oster clipper but as the animals grew the area was enlarged to a 3 × 5-cm area. The area was re-clipped twice each week. To these areas, the ZPT or control solutions were applied daily on 5 days/wk for 8 wk. One group of males and females served as untreated controls and a second as vehicle controls, being clipped and treated with an aqueous solution containing only Darvan and CMC. In three groups the males only and in the remaining three groups the females only were treated with 2·5, 7·5 or 15 mg ZPT/kg body weight/day. These doses were applied to the clipped areas in a volume of 1·5 ml/kg. No attempt was made to prevent ingestion, nor was the residue from a previous treatment removed before applying another. The animals were weighed weekly during this phase.

At the end of wk 8, the rats were mated on a one-to-one basis within each group, so that untreated male rats were mated with treated females and *vice versa* (see Table 1). The day on which sperm and cornified epithelial cells were found in vaginal smears prepared daily during the mating phase was designated day 0 of pregnancy. The males received no further treatment, but the females were kept on their respective dosing regimens until day 15 of pregnancy.

Near the end of the 8-wk period, 80 additional adult Charles River rats (weighing *c.* 225 g) were obtained, and after a 3-day acclimatization period they were weighed and distributed into four groups of ten males and ten females and mated. The males received no treatment, but the females were treated on days 6–15 of pregnancy (determined as described above) with a Darvan–CMC–water solution or with 2·5, 7·5 or 15 mg ZPT slurry/kg/day. These females each had a small plastics container, adapted from those developed by Nixon & Reer (1975), glued to the clipped dorsal area with surgical adhesive (methyl 2-cyanoacrylate; Eastman 910®, Eastman Kodak, Rochester, NY) in order to prevent ingestion of the topically applied ZPT. Body weights were recorded on days 0, 6, 11 and 15 of pregnancy.

The females were transferred to nesting cages shortly before parturition and were given shredded paper to make nests. At birth, the live and stillborn pups were counted, but the litters were not disturbed until 4 days after birth, when they were weighed and sexed. At this time, litters containing more than eight pups were randomly reduced to that number, divided as evenly as possible between the sexes. On day 21 the pups were weighed and discarded.

Five parent males were selected randomly from each of the control groups and from the three groups in which they had been treated topically with ZPT from weaning, and were killed with ether and autopsied. The liver, kidneys and heart were weighed. These and the following organs or tissues were removed, fixed in 10% neutral buffered formalin, blocked in paraffin, stained with haematoxylin and eosin and sectioned for histopathological evaluation: skin from test site, lung, trachea, thyroid, oesophagus, stomach, small intestine, large intestine, spleen, pancreas, adrenal, urinary bladder, testes, brain and spinal cord. No females were killed since they were to be used for breeding again for the teratology study.

Rat teratology studies. The reproduction study just described was extended to include teratology, but there were some changes in the grouping and treatment of the animals. Additional adult Charles River male rats were obtained to replace those killed for histopathology, since no males were to be treated during this study. The females previously treated topically with ZPT in the reproduction study were treated similarly during the teratology study. The untreated females that had been mated with treated males were reassigned to oral treatment, duplicating the treatment used by Wedig & Henderson (1974). All females were treated daily from day 6 to day 15 of pregnancy, after being mated as described above. Corn oil was used as the vehicle for the oral dosing of ZPT, the rats being given 2 ml of vehicle or of ZPT-vehicle solution/kg body weight and ZPT doses of 7·5 or 15 mg/kg.

On day 20 of gestation, the dams were killed by CO_2 inhalation, the uterine contents were evaluated, and corpora lutea of pregnancy were counted. The foetuses were removed, blotted dry of amniotic fluid, weighed, sexed and inspected for gross abnormalities. Half of each litter was cleared and stained with alizarin according to the method of Staples & Schnell

(1964) and examined for skeletal defects and variations. The other half was fixed whole in Bouin's fixative, razor-blade sectioned and examined for soft-tissue defects (Wilson, 1965).

Rabbit teratology studies

An initial pilot study was conducted with five groups of four non-pregnant does. The ZPT slurry incorporated into Purina Rabbit Chow at levels equivalent to a daily intake of 2·5, 5, 10, 20 and 40 mg/kg body weight was fed for 10 days. During this period the feed consumed and the body weights were recorded daily. The rabbits fed ZPT at any level in the diet ate very little after the first day, yet developed the characteristic hind-leg paralysis. A second pilot study was carried out using similar levels of ZPT administered by gavage to pairs of non-pregnant does for 7 days. In this case, loss of body weight and deaths occurred with doses of 20 and 40 mg/kg, but there was no paralysis.

The following general procedures apply to two studies involving oral dosing and one study in which ZPT was applied topically. Sexually mature (5-6 months old) virgin New Zealand does obtained from Pel-Freez Bio-Animals, Rogers, AR, were housed individually in stainless-steel cages with raised-wire floors. The environmental conditions were as described for the rat. Purina Rabbit Chow and tapwater were available *ad lib.* The does were maintained in the laboratory for 18–20 days before the teratology studies started. Then they were weighed and distributed into groups of 15 or 20 (except for one group of ten used as a positive control) so that the mean weights of the groups were similar and the litters were evenly distributed among the groups.

The does were artificially inseminated via an artificial vagina with 0·25 ml of undiluted semen obtained from proven bucks. Immediately prior to insemination, each doe received a 1-mg/kg dose of pituitary leuteinizing hormone to facilitate ovulation. This was designated day 0 of pregnancy. Two or three does from each group of 20 were inseminated daily, until all of the does for a particular experiment were inseminated.

All of the rabbits were treated with ZPT from day 6 to day 18 of pregnancy. In the first study, groups of 15 rabbits were gavaged daily with the 48% ZPT slurry at doses of 5, 10 and 20 mg ZPT/kg body weight. Untreated and vehicle-dosed control groups were included. In the second experiment, the slurry was given by gavage to groups of 20 rabbits at doses of 1·0, 2·5 and 5 mg ZPT/kg. A vehicle control group (20 rabbits) and a positive control group of ten rabbits given 50 mg thalidomide/kg were included. In the second oral study, the feed consumed during pregnancy was measured. All doses were given in a volume of 1 ml/kg body weight.

In the third rabbit experiment, the ZPT slurry was applied topically at doses of 25, 50 and 100 mg ZPT/kg body weight/day to groups of 20 rabbits. Starting on day 6 of gestation, a 14 × 24 cm area on the back was clipped with an Oster clipper (00 blades) every 3 days during the treatment period. The ZPT slurry in a volume of 1 ml/kg was applied to a 10 × 20 cm rectangle within this area with a perforated 13-ga needle fitted to a 10-ml syringe. The rabbits were then fitted with the Newmann (1963) harness to prevent ingestion of the slurry. After 2 hr, the harnesses were removed, the residue of slurry was washed off with a spray of 37°C water and the area was blotted dry with soft towels. Before the doe was put back into her cage, the interior of the cage was wiped with a clean damp cloth to remove any slurry that may have been deposited.

The does in all three experiments were weighed every 3 days so that dosing solutions could be adjusted. They were killed on day 29 of gestation by an overdose of pentobarbital and the uterine horns were exteriorized. The numbers of corpora lutea of pregnancy, live and dead foetuses, and resorption sites, as well as their positions in the horns, were recorded. The foetuses were removed, dried, weighed and inspected for external anomalies. One third of each litter was examined for skeletal defects and two thirds for soft-tissue defects, using the procedures described previously for the rat. The rabbit foetuses were sexed during the visceral examinations.

The does in the topical and the first oral dosing study were examined for histopathology after being killed. The following tissues were fixed in 10% neutral buffered formalin, blocked in paraffin and stained with haematoxylin and eosin before sectioning: heart, liver, lungs, kidneys, skin from test sites, trachea, thyroid, oesophagus, stomach, small and large intestines, spleen, pancreas, adrenal, urinary bladder, ovary, uterus, brain, and spinal cord. No organs were weighed.

Statistical analyses

The continuous data from all of the studies were analysed by the analysis of variance (Snedecor, 1946). When the *F*-test was significant, Tukey's minimum significant difference (MSD) as described by Scheffe (1952) was calculated and used to determine which treated groups differed significantly from the controls. When the *F*-test was significant but no significance was indicated by the MSD technique, the two groups with the highest and lowest means were considered to differ significantly. The discrete data were analysed by the chi-square method (Snedecor, 1946).

RESULTS

Rat studies

Pilot range-finding study. In the pilot study, the application of the 48% ZPT slurry to the rats' dorsal surface without restraints to prevent ingestion resulted in paralysis and deaths in both sexes treated with 50 mg ZPT/kg and paralysis in four of five females given 20 mg/kg. There was evidence of toxicity at all levels, as indicated by either a lower gain in body weight or an actual loss of weight. In addition, three of the males treated with 50 mg ZPT/kg had prolapsed penes.

Reproduction studies. Rats treated from weaning with topical applications of the ZPT slurry and not restrained from grooming the treated area did not differ significantly from the controls in either growth or reproductive characteristics, except that the lactation index of the females treated with 15 mg ZPT/kg

Table 1. *Growth, reproductive performance and neonatal viability in Charles River rats treated topically with a 48% zinc pyrithione (ZPT) slurry either for 8 wk before mating and on days 1–15 of gestation (without restriction on ingestion) or only on days 6–15 of gestation with ingestion prevented*

Treatment†		Body-weight gain (g)			PR	Mean no. of live births/	Mean no. of pups/litter	Mean no. of pups weaned/	LI	Mean pup weight at	Mean weight at weaning (g)	
		In 8-wk treatment period		Days 0–15 of			at day 4‡	litter‡		day 4 (g)		
Males	Females	Males	Females	gestation	(%)	litter					Males	Females
From weaning and during organogenesis§												
None	None	323·9 ± 26·1	168·0 ± 14·4	64·3 ± 8·2	90	8·9 ± 4·8	9·6 ± 3·9	7·0 ± 1·9	100	8·5 ± 1·2	42·8 ± 5·7	40·0 ± 3·4
Vehicle	Vehicle	316·7 ± 31·6	167·0 ± 17·1	69·0 ± 8·9	100	13·4 ± 3·2	12·4 ± 2·3	8·0 ± 0·0	100	8·5 ± 1·7	44·7 ± 4·4	40·8 ± 5·1
2·5 ZPT	None	294·5 ± 15·7	—	—	100	11·5 ± 4·0	10·8 ± 3·5	7·4 ± 1·6	98	8·8 ± 1·3	41·9 ± 3·5	39·9 ± 2·0
7·5 ZPT	None	300·4 ± 36·1	—	—	90	10·7 ± 4·7	10·5 ± 4·6	7·0 ± 2·1	100	8·5 ± 1·7	41·5 ± 4·0	39·5 ± 3·1
15·0 ZPT	None	304·0 ± 30·2	—	—	90	11·3 ± 2·8	11·1 ± 2·7	7·8 ± 0·7	100	9·1 ± 0·6	41·6 ± 4·9	41·8 ± 4·1
None	2·5 ZPT	—	157·5 ± 14·3	73·3 ± 10·9	100	12·7 ± 1·3	12·0 ± 1·6	7·7 ± 0·5	96	8·6 ± 0·8	42·5 ± 2·9	40·9 ± 2·7
None	7·5 ZPT	—	153·3 ± 20·0	69·8 ± 12·5	90	10·6 ± 2·1	8·8 ± 2·6	7·1 ± 1·3	96	9·1 ± 1·9	41·6 ± 3·4	38·8 ± 6·8
None	15·0 ZPT	—	154·0 ± 24·8	66·1 ± 9·5	90	9·7 ± 4·6	10·6 ± 3·5	7·0 ± 1·2	94*	9·6 ± 1·1	46·3 ± 4·2	42·0 ± 4·6
During organogenesis only‖												
—	Vehicle	—	—	96·3 ± 18·0	80	11·9 ± 4·1	11·9 ± 4·1	7·5 ± 0·9	100	9·6 ± 2·1	53·8 ± 5·0	50·1 ± 4·3
—	2·5 ZPT	—	—	90·6 ± 20·8	80	12·5 ± 3·4	12·1 ± 3·3	7·9 ± 0·4	99	9·1 ± 1·4	48·1 ± 7·1	42·8 ± 17·1
—	7·5 ZPT	—	—	81·1 ± 18·6	90	12·8 ± 2·1	12·7 ± 2·1	7·8 ± 0·4	97	9·2 ± 0·8	51·1 ± 3·9	47·2 ± 3·7
—	15·0 ZPT	—	—	90·6 ± 13·6	80	14·0 ± 1·5	13·8 ± 1·6	8·0 ± 0·0	100	9·3 ± 0·5	49·9 ± 5·4	48·1 ± 4·3

PR = Pregnancy rate (no. of females pregnant × 100/no. mated) LI = Lactation index (no. of pups weaned × 100/no. alive at day 4 litter reduction)

†Vehicle: 3% Darvan + 0·2% carboxymethylcellulose in water; 2·5, 7·5 and 15·0 ZPT represents the dose (in mg/kg) of zinc pyrithione applied as a 48% aqueous slurry. Each test and control group consisted of ten males and ten females.

‡Litters of more than eight pups were reduced to that number on day 4.

§Animals were treated on 5 days/wk between weaning and mating (8 wk) and treatment of females was continued on days 0–15 of pregnancy.

‖Ingestion of test material during grooming was prevented by a plastics device glued over the site of application.

Values are means ± 1 SD and that marked with an asterisk is significantly lower ($P \leq 0{\cdot}05$) than the control value (see Experimental, p. 641, for details of the methods of statistical analysis used).

was lower than that of the controls to a slight but statistically significant degree (Table 1). In addition, no toxic signs, such as paralysis, were noted and no test-related histopathology was seen in the males. Similarly, neither reproduction nor neonatal viability was affected by the topical application of the ZPT slurry to females on days 6–15 of pregnancy, when ingestion was prevented by plastics containers cemented to the dorsal skin (Table 1).

Teratology studies. Rats that were treated topically with 7·5 or 15 mg ZPT/kg during the organogenesis period of their second pregnancy and were allowed to groom the treated area lost a significant amount of body weight, while the weights of rats treated with the lower level (2·5 mg/kg) were not significantly different from those of the controls (Table 2). In addition, the foetuses from the dams treated with the high level were smaller than the control foetuses, although they were statistically different only from those in the group treated with 2·5 mg ZPT/kg. Five dams treated with the 15 mg ZPT/kg and two dams treated with the 7·5 mg/kg exhibited hind-leg paralysis, but there were no significant differences in the numbers of implantations, resorptions, or live foetuses among the groups. No dead foetuses were found in any of the dams.

Use of a plastics device to prevent the ingestion of the topically applied ZPT slurry reduced the toxicity of the ZPT, in that the treated animals gained weight during pregnancy, although they gained much less weight than the controls (Table 2). No signs of paralysis were noted in any of the animals treated topically when ingestion was prevented. No significant differences were observed in any of the reproductive or foetal viability characteristics, including foetal weights. The two dead foetuses in the group treated with 15 mg/kg were probably due to causes other than the treatment with ZPT, since it is not uncommon to have this number of deaths in control groups.

The dams treated with 15 mg ZPT/kg by gavage gained significantly less weight during the first 15 days of pregnancy than did either of the control groups, while the group treated with 7·5 mg/kg gained significantly less weight than the untreated controls (Table 2). In addition, five (50%) of the dams in each group exhibited hind-leg paralysis. There was one death in the group given 7·5 mg ZPT/kg and one in the group given only the corn-oil vehicle.

As in the previous rat studies, there were no significant differences in the numbers of implantations, resorptions or live foetuses. Two dead foetuses were seen in dams treated with 15 mg ZPT/kg, and the foetal weights of this group were significantly lower than the weights of the foetuses from dams treated only with the corn oil vehicle.

In all of the rat studies, rats that were becoming paralysed ate less feed, although the feed consumption was not measured. In addition, as soon as the ZPT treatments were stopped, the hind-leg paralysis began to remit and the quantity of feed consumed increased. However, none of the rats exhibiting paralysis during treatment had fully recovered by the end of pregnancy, when they were killed. At autopsy no gross internal lesions were evident in any of the rats, including those that were paralysed. In addition, histological studies of males treated topically with ZPT from weaning through sexual maturity revealed no treatment-related changes in the gonads or other tissues.

Of the foetuses examined for soft-tissue abnormalities, only seven had a major anomaly. All seven had hydrocephalus and were from a single litter in the group treated topically with 7·5 mg ZPT/kg. Therefore the abnormality was not considered to be treatment-related. Otherwise, the normal variations, such as hydronephrosis, were observed and were not statistically increased among the groups receiving the different ZPT treatments (Table 3).

The results of skeletal examinations are shown in Table 3. There was a significant increase in the number of skeletal defects in the foetuses of dams given 15 mg ZPT/kg by gavage, but as in the case of most studies, many of these were due to alterations in the sternebrae. However, there was a significant increase in the numbers of rib anomalies, such as split and fused ribs, in foetuses from dams given 15 mg ZPT/kg orally. These results are similar to the ones described by Wedig & Henderson (1974), who reported rib defects in rats treated orally with either 7·5 or 15 mg ZPT/kg. These defects were not seen in any foetuses from dams treated topically with the ZPT slurry, even where some ingestion occurred through grooming. There was a significant increase compared to the untreated control group in the number of foetuses with extra (14th) ribs in the groups treated orally with ZPT and also in the groups treated topically, but only when some ingestion occurred from grooming. The number of foetuses with extra ribs is within the incidence range for this defect in Charles River rats, and since there were no significant differences from the vehicle controls, this defect or variation was probably not linked to the other rib defects in ZPT-treated animals.

Rabbit studies

Pilot range-finding studies. Although it was intended to conduct the oral teratology studies by including the ZPT in the feed, the rabbits became anorectic after 24 hr on the ZPT-containing diet, and consequently there was very little inter-group difference in the actual intake of ZPT. In addition, nearly all of the animals developed characteristic hind-leg paralysis and two animals died. When the ZPT was given by gavage, two deaths occurred at the 40-mg/kg level and one at the 20-mg/kg and there was extensive loss in body weight, but no paralysis. Therefore, dose levels of 5, 10 and 20 mg ZPT/kg were chosen for the first rabbit teratology study.

Rabbit teratology—oral administration. The ZPT administered as an aqueous slurry by gavage was toxic to the dams in a dose-related fashion (Table 4). All of the dams given 20 mg/kg died, as did two thirds of those given 10 mg/kg and one third of those given 5 mg/kg. Regardless of the dose level, all of these dams died after 3–5 daily treatments. No histological lesions could be related to the ZPT treatment in animals that were examined histologically.

Survivors receiving either 5 or 10 mg ZPT/kg lost a significant amount of body weight, in proportion to the dose. In the living dams, there was a significant increase in the resorption of embryos, resulting in no foetuses in dams fed 10 mg ZPT/kg and only nine foetuses in five dams given 5 mg/kg. In both

Table 2. *Embryo viability and teratogenesis in Charles River rats treated topically (with or without restriction on ingestion) or orally with a 48% zinc pyrithione (ZPT) slurry on days 6–15 of gestation*

Treatment	No. of rats/group§	Pregnancy rate (%)	Weight gain: days 0–15 (g)	Mean no. (per litter) of: Corpora lutea	Implantations	Resorptions	Live foetuses	Total no. of dead foetuses/group	Mean foetal weight (g)
Topical administration with possible ingestion									
None	10	80	63·5 ± 7·2	15·5 ± 1·9	15·3 ± 2·6	1·5 ± 3·1	13·8 ± 2·2	0	5·23 ± 0·43
D/CMC vehicle (1·5 ml/kg)	10	90	52·3 ± 14·6	11·7 ± 4·7	10·9 ± 6·1	1·0 ± 1·1	9·9 ± 6·3	0	5·27 ± 0·66
ZPT (mg/kg): 2·5	10	100	50·6 ± 16·0	13·8 ± 2·8	12·5 ± 3·9	0·7 ± 0·8	11·8 ± 4·2	0	5·41 ± 0·37
7·5	9	100	38·8 ± 23·2	13·5 ± 4·5	13·1 ± 5·0	0·6 ± 0·7	12·5 ± 4·8	0	5·11 ± 0·73
15·0	10	80	−5·5 ± 21·4*†	14·4 ± 2·2	13·6 ± 3·6	0·5 ± 0·5	13·0 ± 3·3	0	4·18 ± 0·15‡
Topical administration without ingestion‖									
D/CMC vehicle (1·5 ml/kg)	10	100	54·6 ± 19·1	16·3 ± 2·5	13·3 ± 5·2	1·2 ± 1·6	12·1 ± 5·6	0	5·53 ± 0·32
ZPT (mg/kg): 2·5	10	80	57·6 ± 64·5	15·6 ± 2·7	14·5 ± 2·3	1·4 ± 2·8	13·1 ± 4·8	0	5·19 ± 1·25
7·5	10	60	41·2 ± 27·4	14·5 ± 6·3	14·5 ± 6·4	2·0 ± 2·1	12·5 ± 6·4	0	4·44 ± 1·18
15·0	10	60	18·5 ± 33·7	14·2 ± 6·7	13·3 ± 6·1	1·7 ± 2·0	11·3 ± 6·4	2	5·32 ± 0·74
Oral administration									
None	10	80	63·5 ± 7·2	15·5 ± 1·9	15·3 ± 2·6	1·5 ± 3·1	13·8 ± 2·2	0	5·23 ± 0·43
CO vehicle (2 ml/kg)	10	70	47·3 ± 16·2	14·6 ± 3·4	12·6 ± 3·7	0·9 ± 1·1	11·9 ± 4·3	0	5·50 ± 0·63
ZPT (mg/kg): 7·5	10	70	18·3 ± 30·1*	14·4 ± 1·7	13·7 ± 2·1	0·6 ± 1·1	13·1 ± 2·7	0	4·85 ± 0·65
15·0	10	70	10·7 ± 21·2*†	16·1 ± 2·1	14·7 ± 3·3	0·3 ± 0·5	14·1 ± 3·8	2	4·23 ± 0·97†

D/CMC = 3% Darvan + 0·2% carboxymethylcellulose in water CO = Corn oil

§Paralysis was evident in two dams treated with 7·5 mg ZPT/kg topically with no restriction on ingestion, in five of those treated similarly with 15 mg/kg and in five dams from each of the groups treated orally with ZPT. One dam died in the group treated orally with 7·5 mg ZPT/kg and in that given the corn oil vehicle.

‖Ingestion of test material during grooming was prevented by a plastics device glued over the site of application.

Values are means ± 1 SD; superscripts indicate those differing significantly ($P \leq 0{\cdot}05$ by analysis of variance and Tukey's minimum significant difference technique) from the untreated controls (*), the vehicle controls (†) or the group treated with 2·5 mg ZPT/kg (‡).

Table 3. *Incidence of soft-tissue and skeletal abnormalities in Charles River rats treated topically (with or without restriction on ingestion) or orally with a 48% zinc pyrithione (ZPT) slurry on days 6–15 of gestation*

	No. of foetuses affected (expressed in parentheses as % of no. examined)											
Treatment....	None	Oral			Topical with possible ingestion				Topical without ingestion‡			
Observations Dose§...	—	CO	7·5 ZPT	15·0 ZPT	D/CMC	2·5 ZPT	7·5 ZPT	15·0 ZPT	D/CMC	2·5 ZPT	7·5 ZPT	15·0 ZPT
Soft-tissue investigation												
No. of foetuses examined	59	43	48	52	49	61	61	63	63	53	42	35
All defects	11 (19)	7 (16)	4 (8)	1 (2)*	5 (10)	8 (13)	1 (2)*	7 (11)	11 (17)	4 (8)	11 (26)	0*
Specific abnormalities												
Hydronephrosis	2 (3)	3 (7)	1 (2)		3 (6)	4 (7)			5 (8)			
Enlarged bladder	8 (14)	6 (14)	3 (6)	1 (2)		5 (8)	1 (2)*	7 (11)	7 (11)	3 (6)	3 (7)	
Cryptorchism	1 (2)				1 (2)					1 (2)	1 (2)	
Monorchism					1 (2)							
Hydrocephalus											7‖ (17)*	
Skeletal investigation												
No. of foetuses examined	51	40	38	45	40	44	56	42	56	46	33	33
All defects	30 (59)	18 (45)	21 (55)	37 (82)*	19 (48)	22 (50)	27 (48)	26 (62)	28 (50)	24 (52)	12 (36)	19 (58)
Specific abnormalities												
Sternebrae –hypoplastic	29 (57)	14 (35)	14 (37)	20 (44)	16 (40)	21 (48)	21 (38)	23 (55)	21 (38)	16 (35)	9 (27)	19 (58)
–missing				2 (4)	2 (5)	1 (2)						3 (9)
–bipartite				2 (4)	1 (3)							
Vertebrae –bipartite			1 (3)	2 (4)		1 (2)			1 (2)			1 (3)
–cleft					2 (5)		3 (5)		4 (7)		2 (6)	
Rib –14th		3 (8)	4 (11)	5 (11)†			6 (11)*	6 (14)*	7 (13)†	11 (24)†	4 (12)†	2 (6)
–short	2 (4)	1 (3)	1 (3)	3 (7)				1 (2)				
–fused			4 (11)	5 (11)*								
–forked			2 (5)	8 (18)*								
–missing			2 (5)	13 (29)*								
–floating				1 (2)								
–15th				1 (2)								

CO = Corn oil D/CMC = 3% Darvan + 0·2% carboxymethylcellulose in water

‡Ingestion of test material during grooming was prevented by a plastics device glued over the site of application.

§For corn oil, 2 ml/kg and for ZPT 7·5 or 15·0 mg/kg as indicated.

‖All from one litter.

Values marked with an asterisk differ significantly (*$P \leq 0{\cdot}05$ by chi-square analysis) from both no-treatment and vehicle controls, while those marked with a dagger differ significantly (†$P \leq 0{\cdot}05$ by chi-square analysis) only from the no-treatment control.

instances, there were no dead foetuses, indicating that the toxicity of ZPT was directed at the early embryo.

Although there were few foetuses from ZPT-treated dams to evaluate, the limited data are shown in Table 4. The abnormalities or variations were those usually seen in rabbit foetuses.

In the second rabbit study, in which ZPT was given by gavage at lower levels, no deaths occurred even at the 5-mg/kg level, although the rabbits given this dose lost considerable weight during the treatment period and consumed significantly less feed than the controls (Table 5). There was one death in the thalidomide-treated dams. As in the rat studies, ZPT had no effect on implantation, but was embryotoxic at both the 2·5- and 5-mg/kg levels, although only at the latter level was the finding statistically significant. Although the number of resorptions (4·0) in the dams treated with 2·5 mg/kg was not statistically significant, it was higher than normal and therefore was probably biologically significant. There were no significant differences in the foetal weights of the groups.

Table 5 also lists the soft-tissue and skeletal anomalies seen in the rabbit foetuses. Because of the embryotoxicity at the 2·5- and 5·0-mg/kg levels of ZPT, the numbers of foetuses were few in these two groups. Nevertheless, it is readily apparent not only that there were no significant increases in either type of anomaly but that they were mostly variations rather than frank defects.

Rabbit teratology—topical administration. Topical exposure of rabbits, restrained from grooming, to levels of 25, 50 or 100 mg ZPT/kg during organogenesis (days 6–18) produced no paralysis or other maternal toxicity and no mortality (Table 6). Although five dams died during the study, the apparent cause of the deaths was respiratory disease. All of the remaining dams, except one in the 50-mg/kg group, were examined histopathologically and no test-related pathology was seen.

No statistically significant differences were noted in the average numbers of corpora lutea, implantations, resorptions or live or dead foetuses. There were significant increases in the foetal weights from dams treated with 100 mg ZPT/kg, probably because of the slightly smaller litters in this group. There were no gross abnormalities in these foetuses and the anom-

Table 4. *Reproductive performance, embryo viability and foetal development in New Zealand rabbits given a 48% zinc pyrithione (ZPT) slurry by gavage in doses of 5–20 mg ZPT/kg on days 6–18 of gestation*

Observations	Values† for control groups		Values† for groups treated with ZPT‡ (mg/kg)	
	No treatment	Distilled H_2O (1 ml/kg)	5·0	10·0
No. pregnant	14 (93)	13 (87)	14 (93)§	15 (100)
No. of dams dying during gestation	1	0	6	9*
Mean maternal weight gain/loss (g) between days 6 and 18	72 ± 158	190 ± 150	−126 ± 154	−292 ± 185*
Mean no. of corpora lutea	10·3 ± 2·5	8·6 ± 2·1	8·1 ± 2·2	8·2 ± 1·9
implantations	9·9 ± 2·1	8·2 ± 3·1	8·1 ± 2·6	8·0 ± 2·3
resorptions	0·4 ± 0·7	1·3 ± 1·9	6·4 ± 3·7*	8·0 ± 2·3*
live foetuses	8·6 ± 2·7	6·8 ± 3·0	1·8 ± 2·4*	0·0 ± 0·0*
dead foetuses	0·8 ± 2·2	0·1 ± 0·3	0 ± 0	0 ± 0
Mean foetal weight (g)	34·4 ± 5·8	41·9 ± 5·3	45·4 ± 8·4*	—
Soft-tissue anomalies				
No. of foetuses examined	74	54	9	0
No. abnormal	1 (1·4)	0	1 (11)	0
Specific abnormalities				
Acephaly	1 (1·4)			
Bloodless atrium	1 (1·4)		1 (11)	
Umbilical hernia			1 (11)	
Acaudia			1 (11)	
Atrophied lung			1 (11)	
Hydronephrosis			1 (11)	
Skeletal anomalies				
No. of foetuses examined	40	28	5	
No. abnormal	27 (67·5)	22 (78·6)	5 (100)	
Specific abnormalities				
Sternebrae –hypoplastic	11 (27·5)	5 (17·9)	2 (40·0)	
–missing	4 (10·0)	5 (17·9)	1 (20·0)	
–bipartite	1 (2·5)			
Rib –13th	19 (48·0)	16 (57·1)	4 (80·0)	
Fused caudal vertebrae			1 (20·0)	

†Where appropriate, numbers are expressed (in parentheses) as a percentage of the total (i.e. of the total number of females (15) inseminated or the total number of foetuses examined). Since a foetus may have had more than one defect, the numbers or percentages of individual abnormalities may not equate with the total incidence of abnormal foetuses. Other values are means ± 1 SD.

‡All of the 15 pregnant females given doses of 20 mg ZPT/kg died during gestation. Three of the females given 5 mg ZPT/kg resorbed their entire litters.

Values differing significantly from the controls are marked with an asterisk: *$P \leq 0{\cdot}05$ by chi-square analysis for maternal deaths and total or individual foetal abnormalities and *$P < 0{\cdot}05$ by analysis of variance and Tukey's minimum significant difference technique for other parameters.

alies seen in either the soft tissues or the skeleton were ones normally seen in the rabbit (Table 6). The incidences of both kinds of anomaly were not statistically different among the control and treated groups.

DISCUSSION

Although ZPT is toxic to laboratory animals when given by the oral route, these studies have demonstrated again that ZPT applied topically at levels up to 100 mg/kg body weight is not toxic. These studies further show that when ZPT is applied to the skin without any control of ingestion, the animal may obtain a toxic amount through grooming. This appears to be true for both rats and rabbits.

In the rat, topical treatments with ZPT, with no control of ingestion, produced death and paralysis at the 50-mg/kg dose level, while at 20 mg/kg paralysis

Table 5. *Reproductive performance, embryo viability and foetal development in New Zealand rabbits given a 48% zinc pyrithione (ZPT) slurry by gavage in doses of 1–5 mg/kg on days 6–18 of gestation*

Observations	Values† for distilled water (1 ml/kg) controls	Values† for groups treated with ZPT (mg/kg)			Values† for positive controls (50 mg thalidomide/kg)
		1·0	2·5	5·0	
No. inseminated	20	20	20	20	10
No. pregnant	16 (80)	19 (95)	15 (75)	17 (85)	9 (90)
No. of dams dying during gestation	0	0	0	0	1
Mean maternal weight gain/loss (g) between days 6 and 18	175 ± 79	125 ± 135	50 ± 114	−136 ± 142*	−30 ± 112*
Feed consumed during gestation (g)	3919 ± 517	3341 ± 562	3483 ± 793	3278 ± 785*	—
Mean no. of corpora lutea	8·3 ± 2·5	10·9 ± 2·2	9·9 ± 3·3	8·9 ± 2·1	11·8 ± 2·1*
implantations	6·6 ± 3·2	9·8 ± 3·1*	8·5 ± 3·1	8·2 ± 1·9	9·8 ± 3·9*
resorptions	0·8 ± 0·8	2·3 ± 3·0	4·0 ± 3·4	6·8 ± 2·6*	4·3 ± 3·2*
live foetuses	5·8 ± 3·0	7·4 ± 3·1	4·1 ± 3·5	1·4 ± 2·2*	5·3 ± 3·2
dead foetuses	1	2	0	0	1
Mean foetal weight (g)	45·9 ± 7·9	41·2 ± 5·1	43·3 ± 7·0	43·6 ± 7·4	39·6 ± 4·1
Soft-tissue anomalies					
No. of foetuses examined	63	91	41	17	33
No. abnormal	0	0	2 (4·9)	1 (5·9)	8 (24)*
Specific abnormalities					
Cleft palate			1 (2·5)		
Undescended testes			1 (2·5)		
Acephaly			2 (4·9)		
Microbrain			1 (2·5)		
Club feet			1 (2·5)		
Septal defect				1 (5·9)	1 (3·0)
Microprobiscus					1 (3·0)
Bidactyly				1 (5·9)	
Bladder –enlarged					1 (3·0)
–missing				1 (5·9)	
Anal atresia					1 (3·0)
Hydrocephalus					5 (15·0)*
Hydronephrosis					1 (3·0)
Short tail					1 (3·0)
Skeletal anomalies					
No. of foetuses examined	30	42	17	7	15
No. abnormal	21 (70)	28 (67)	15 (88)	4 (57)	13 (87)
Specific abnormalities					
Sternebrae –hypoplastic	9 (30)	19 (45)	8 (47)		6 (40)
–missing	3 (10)	2 (5)	1 (6)	2 (29)	3 (20)
–bipartite	1 (3)	0			
Ribs –fused		1 (2)			1 (7)
–13th variation	14 (47)	16 (38)	9 (53)	2 (29)	8 (53)
Incomplete parietal ossification		1 (2)			
Spina bifida			1 (6)		

†Where appropriate, values are expressed (in parentheses) as a percentage of the total (i.e. of the total number of females inseminated or the total number of foetuses examined). Since a foetus may have had more than one defect, the numbers or percentages of individual abnormalities may not equate with the total incidence of abnormal foetuses. Other values are means ± 1 SD.

Values differing significantly from the distilled-water controls are marked with an asterisk: *$P \leq 0{\cdot}05$ by chi-square analysis for total and individual foetal abnormalities and *$P < 0{\cdot}05$ by analysis of variance and Tukey's minimum significant difference technique for other parameters.

Table 6. *Reproductive performance, embryo viability and foetal development in New Zealand rabbits treated topically with a 48% zinc pyrithione (ZPT) slurry in doses of 25–100 mg ZPT/kg on days 6–18 of gestation*

Observations	Values† for control groups		Values† for groups treated with ZPT (mg/kg)		
	No treatment	D/CMC‡ (1 ml/kg)	25	50	100
No. pregnant	18 (90)	16 (80)	19 (95)	18 (90)	20 (100)
No. of dams dying during gestation	0	2	2	1	0
Mean maternal weight gain (g) between days 6 and 18	201 ± 84	146 ± 92	80 ± 228	111 ± 134	82 ± 164
Mean no. of corpora lutea	9·5 ± 1·9	10·3 ± 1·6	10·5 ± 2·0	10·5 ± 1·9	10·2 ± 2·6
implantations	9·4 ± 2·0	9·9 ± 1·8	9·8 ± 2·9	10·1 ± 1·8	9·4 ± 2·4
resorptions	0·8 ± 1·9	0·8 ± 2·0	0·4 ± 0·8	0·8 ± 0·9	1·8 ± 2·4
live foetuses	8·3 ± 3·3	9·5 ± 1·7	9·9 ± 1·9	9·2 ± 2·1	7·4 ± 3·6
dead foetuses	0·4 ± 1·6	0·2 ± 0·5	0·1 ± 0·3	0·1 ± 0·3	0·2 ± 0·7
Mean foetal weight (g)	39·2 ± 4·9	40·8 ± 4·1	40·5 ± 3·8	39·7 ± 4·9	44·3 ± 6·6*
Soft-tissue anomalies					
No.of foetuses examined	110	94	117	112	102
No. abnormal	1 (0·9)	0	0	1 (0·9)	1 (1·0)
Specific abnormalities					
Undescended testes					1 (1·0)
Heart defects	1 (0·9)			1 (0·9)	
Skeletal anomalies					
No. of foetuses examined	55	48	52	52	46
No. abnormal	36 (65)	34 (70)	34 (65)	30 (58)	27 (59)
Specific abnormalities					
Sternebrae -hypoplastic	21 (38)	21 (43)	13 (25)	12 (23)	12 (26)
-missing	7 (13)	9 (19)	11 (19)	6 (11)	5 (11)
-bipartite		5 (10)	1 (1·8)	2 (3·8)	2 (4·4)
-fused		2 (4			
extra			1 (1·8)		
Ribs -13th variation	17 (31)	13 (27)	16 (31)	13 (25)	15 (33)
-floating	1 (2)	1 (2)		1 (1·9)	

†Where appropriate, values are expressed (in parentheses) as a percentage of the total (i.e. of the total number of females (20) inseminated or the total number of foetuses examined). Since a foetus may have had more than one defect, the numbers or percentages of individual abnormalities may not equate with the total incidence of abnormal foetuses. Other values are means ± 1 SD.

‡Vehicle controls treated with 3% Darvan + 0·2% CMC in water.

The value marked with an asterisk differs significantly from that of the untreated control: $*P \leq 0.05$ by analysis of variance and Tukey's minimum significant difference technique. There were no significant differences from the controls ($P \leq 0.5$ by chi-square analysis) in the incidence of any foetal abnormalities.

was produced, and the toxicity of lower doses was reflected in the body weight. Yet similar levels applied topically to young animals for longer periods (8 wk) produced no toxic signs. Intubation of 7·5 or 15 mg ZPT/kg in the teratology study produced paralysis in adult animals, although it had been reported elsewhere that paralysis did not occur with this method of administration (Winek & Buehler, 1966).

The rabbit appears to be much more sensitive to ZPT than the rat, since in these studies intubation of rabbits at levels similar to those given to the rats produced a much higher incidence of death. However, no paralysis was seen in the rabbit except in the pilot study. ZPT administered in feed was much more toxic than ZPT given orally by gavage. As with the rat, and as we reported previously (Nolen *et al.* 1975) for a shampoo containing ZPT, no toxic effects were seen when ZPT was applied topically to rabbits and grooming of the treated area was controlled.

During pregnancy, rat embryos appear to be less susceptible to ZPT-induced lethality than their mothers, since no increase in the incidence of resorption was observed even in dams showing significant evidence of toxicity. This was not the case for rabbits, as there was a significant increase in resorptions in the living dams in groups in which maternal deaths occurred. Again, these data indicate that the rabbit is more sensitive than the rat to the toxic effects of ZPT.

The only teratogenic effects seen in these studies were the increased incidences of rib defects in the foetuses of rats given ZPT orally at a level of 15 mg/kg body weight. This finding confirms the results of studies reported by Wedig & Henderson (1974).

Although there is some variation from study to study in this series, the no-effect level for toxicity among rats treated orally with ZPT is 2·5 mg/kg. From a teratogenic/embryotoxic viewpoint, the no-effect level is 7·5 mg ZPT/kg. Among the rabbits, which were more sensitive to ZPT, oral doses down to 2·5 mg/kg were embryotoxic, but no teratogenicity was seen at any of the levels tested. Thus, a considerable amount of ZPT must be ingested, either through oral administration or grooming, in order to be toxic to the dam or embryo.

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NEUROLOGICAL, MICROSCOPIC AND ENZYME-HISTOCHEMICAL ASSESSMENT OF ZINC PYRITHIONE TOXICITY

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Abstract—Male rats were given an *ad lib.* diet containing 250 ppm zinc pyrithione (ZPT) for 9 or 14 days and were then given untreated food during recovery. Each animal was matched to a pair-fed control. Clinical signs observed in all treated rats included progressive hind-limb weakness, motor incoordination, spinal kyphosis with muscle atrophy, and penile prolapse. Electrophysiological recording *in vivo* from purely sensory sural nerves showed a significant decrease in peak-to-peak potential amplitudes but no reduction in conduction velocity. The sensory nerve-evoked potential amplitudes remained significantly reduced after 4-wk recovery although no clinical signs of toxicity persisted. Similarly, recording *in vitro* from sections of the mixed sensory and motor sciatic nerves also showed significantly lower potential amplitudes with no reduction in nerve conduction velocity. Histological and enzyme-histochemical studies failed to reveal any abnormal muscle morphology. Light- and electron-microscopic examination of spinal roots, sciatic and sural nerves failed to reveal any pathological effects in the acutely affected rats. However, accumulations of dense granular axoplasmic deposits and accumulations of tubulo-vesicular profiles were noted in the axons of sural nerves and intramuscular lumbrical nerves. These studies and others recently completed (Sahenk & Mendell, *J. Neuropath. exp. Neurol.* 1979, in press) suggest an intracellular mechanism of ZPT toxicity involving axonal terminal structures. The distal concentration of membrane-associated proteins in treated animals is indicative of a fast axoplasmic transport neuropathy leading to defective maintenance of axon terminal structures and loss of function. Whether this is due to a specific enzyme deficiency, altered vitamin metabolism or some other biochemical process remains to be determined.

INTRODUCTION

The pyrithiones and their salts have many practical applications, the more important of which include uses in shampoos, cosmetics and industrial cutting fluids which take advantage of their broad-spectrum antibacterial and antifungal properties. The zinc salt of 1-hydroxy-2(1*H*)-pyridinethione (zinc bis-(1-hydroxy-2(1*H*)-pyridinethionate); ZPT), the most widely used salt, has undergone numerous safety evaluation studies in various animal species (Adams, Wedig, Jordan, Smith, Henderson & Borzelleca, 1975; Delahunt, Stebbins, Anderson & Bailey, 1962; Klaassen, 1976; Moe, Kirpan & Linegar, 1960; Snyder, Buehler & Winek, 1965; Snyder, Gralla & Coleman, 1977; Wedig, Goldhamer & Henderson, 1974; Wedig, Kennedy, Jenkins & Keplinger, 1977; Wedig, Wentworth, Gallo, Babish & Herrion, 1978; Winek & Buehler, 1966; Ziller, 1977). Pharmacotoxicity in the rat when ZPT was incorporated in the diet was first noted in 1958 by P. S. Larsen (unpublished data) and was characterized by skeletal-muscle wasting and weakness, initially affecting the hindquarters. Extensive histological examination of the rats in Larson's study, including examination of the peripheral and

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central nervous systems by light microscopy, did not reveal any lesions to explain the muscular weakness. Snyder *et al.* (1965) found this effect was reversible in rats given ZPT-treated and then untreated feed for three cycles.

Neurophysiological studies in rats fed 250 ppm ZPT in the diet indicated no decrease in the conduction velocities of the sciatic nerve (Snyder *et al.* 1977). However, subsequent electrophysiological studies of the sural nerve in treated rats demonstrated that axonal disturbances were present (de Jesus, Towfighi & Snyder, 1978). In ZPT-treated rats, Wallerian degeneration of the lateral plantar nerve and a decrease in fast axoplasmic transport (FAT) in the dorsal root ganglion after injection of [^{3}H]leucine have also been described (Sahenk & Mendell, 1977). A correlation was noted between the rate of FAT and a change in nerve morphology which suggested that a reduction in FAT preceded Wallerian degeneration.

The studies reported here were designed to investigate a more precise site and mechanism of action for the muscular weakness by studying nerve-fibre potentials, by using special muscle- and nerve-histochemical stains for enzymes, and by a detailed morphological evaluation of selected neural tissues using light and electron microscopy.

EXPERIMENTAL

Animals and treatment. Male CD strain rats (Charles River Breeding Laboratories, Wilmington, MA), weighing 150–250 g, were divided into 63 pairs, matched by weight, and housed in individual plastic boxes containing hardwood-chip bedding. Water was supplied *ad lib.* from individual bottles. Technical grade ZPT (97·0% pure) was incorporated weekly in a homogeneous mixure of ground Purina rat chow (Nutritional International, New Brunswick, NJ) at a concentration of 250 ppm. Each batch was assayed by polarography before use (Krivis, Gazda, Supp & Robinson, 1963). These assays were performed to check the stability of each batch over several weeks. The dose (250 ppm ZPT) was based on our earlier determinations of the dose-latency relationship between dietary concentrations of ZPT and the appearance of profound hind-limb pareses after 9–14 days of treatment (Snyder *et al.* 1977).

All rats were acclimatized to the laboratory environment for 1 wk during which time daily records of weight and food consumption were kept. During the treatment periods, the pair-fed controls were given an amount of untreated ground chow equal in weight to that eaten by the treated partner during the previous 24 hr. During the course of the experiment, the water bottles of affected animals were laid inside the cage next to the food jars (which were fitted with wide-mesh screen inserts) to permit each prostrate animal access to food and water. All pair-fed animals, whether used for electrophysiological, enzyme histochemical or ultrastructural studies, were treated identically.

Histology and enzyme histochemistry. The gastrocnemius and soleus muscles from five treated and five pair-fed control rats were examined. The animals were killed with carbon dioxide and the muscles were removed immediately from the right legs. Transverse 2-mm thick sections were taken from the midportion of each muscle, placed on metal chucks and flash-frozen in isopentane at -170°C. The frozen specimens were transferred to a cryostat and allowed to warm to -20°C over 2 hr. Sections (6 μm) of the specimens were taken and stained either with haematoxylin and eosin or for adenosine triphosphatase at pH 9·4 by the calcium method (Padykula & Herman, 1955).

The sections were evaluated by light microscopy. The ratios of type I (light) to type II (dark) myofibres were determined by counting 200 fibres per section from randomly selected bundles. All the fibres in each high power ($\times$ 400) field were counted.

Light and electron microscopy. Eighteen treated and eighteen pair-fed control rats were used for ultrastructural evaluation. Ten of the treated rats were at the height of induced toxicity (250 ppm ZPT in the diet for 14 days). The remaining eight treated animals were allowed to recover for 14–28 days on an untreated diet before being studied.

Half of the treated animals in each group together with their pair-fed controls were perfused intracardially with 5% phosphate-buffered glutaraldehyde under halothane anaesthesia. The sural and sciatic nerves of the remaining animals were fixed *in situ* with the same solution. Segments of sural and sciatic nerves from all animals plus anterior and posterior lumbosacral roots from the perfused animals were fixed in 1% osmium tetroxide in 0·1 M-phosphate buffer, embedded in Epon 812 (Polysciences, Inc. Warrington, PA), and sectioned for light- and electron-microscopic (EM) evaluation. Pacinian corpuscles, muscle spindles, intramuscular and subcutaneous nerves from hind-limb toe pads, and lumbrical muscles were examined ultrastructurally in rats that were perfused after 2- or 4-wk recovery.

Electrophysiology. (a) Recording *in vivo.* Sural nerve conduction velocities and potential amplitudes to orthodromic stimulation were recorded in a total of 74 treated and pair-fed control rats using the technique described recently by de Jesus *et al.* (1978) for the study of electrophysiological correlates of experimental neuropathy in a sensory nerve. The purely sensory sural nerve was chosen because of its increased sensitivity, reliability and accuracy as a pathophysiological indicator compared with recording from mixed- or motor-nerve fibres (Buchthal, 1973; Buchthal & Rosenfalck, 1971).

The rats were anaesthetized with sodium pentobarbital and the sural nerve was surgically exposed with the aid of a dissecting microscope. A short segment of the nerve in the popliteal fossa was dissected loose for the recording electrode and a similar section proximal to the hock was freed for the stimulating electrodes. The nerve was bathed in Ringer's solution at both sites to prevent drying and the tibial and peroneal nerves and the nerves to the thigh muscles were sectioned to prevent twitch artefacts.

Recording and stimulation was done with fine Teflon-coated stainless-steel electrodes with the sural-nerve ends laid at the tips. Indifferent electrodes were inserted into tissue 3–5 mm from the recording electrodes and an earth clip was attached to the muscle between the stimulating electrodes which were placed as close together as possible. The tissue temperature was maintained at 35° $\pm$ 0·3°C. In order to achieve

maximum comparability with later experiments *in vitro*, the recording electrode was placed above the surface of the Ringer's solution at high relative humidity.

Measurements were taken from paper prints of the potentials with a constant pulse duration of 0·05 msec at 20 μV/cm amplification after averaging over 1000 supramaximal responses. Threshold measurements were made after averaging 32–64 responses at 5 μV/cm amplification. The neural parameters that were quantified included the sensory nerve conduction velocity (SNCV), peak-to-peak sensory nerve potential amplitude, total sensory nerve potential duration, duration of negative spike potential, supramaximal current and current at sensory potential threshold. (A more detailed description of the surgical, electrophysiological and quantification procedures has been given by de Jesus *et al.* 1978.)

(b) Recording *in vitro*. The sciatic nerves of ZPT-treated and pair-fed rats were dissected free under sodium pentobarbital anaesthesia. The cut sections were ligated and fixed at both ends with miniature adjustable clamps. The fixture holding the nerve section was then placed *en bloc* in a recording chamber filled with heated, circulating Ringer's solution. As in the experiments on sural nerves *in vivo*, the recording electrode with the sciatic nerve lying over it was lifted just above the surface of the solution. The same high humidity/35°C ambient environment was maintained. The sciatic nerve was stimulated distally; the recording electrodes were placed proximally.

RESULTS

Histology and enzyme histochemistry

The histological morphology of all muscles was normal. The ratios of type I to type II muscle fibres in treated rats did not differ significantly from those of pair-fed controls (Table 1). There was, however, some intragroup variation in the fibre-type ratios of different sections of gastrocnemius muscle.

Light and electron microscopy

Light-microscopic examinations of 1 μm-thick sections and electron microscopy showed that sciatic-, sural- and spinal-nerve roots were not altered in the acutely-affected rats studied after 14-days treatment. Similarly, the spinal roots and sciatic nerves showed neither light-microscopic nor ultrastructural changes after 14–28 days recovery.

The myelinated sural-nerve axons in the recovered rats, however, showed occasional accumulation of dense granular axoplasmic deposits (Fig. 1). The most prominent changes in the treated rats were seen in the intramuscular lumbrical fibres. Many myelinated and unmyelinated axons were enlarged and contained abundant mitochondria and electron-dense granules (Fig. 2). Alterations in Schwann cells or myelin sheaths were not seen. Similar but less extensive changes were present in subcutaneous-nerve fibres. Only slight effects were observed in the sensory endings. These included an increased number of dense bodies in some intrafusal fibres (Fig. 3a) and Pacinian-corpuscle axonal terminals. Occasional intrafusal fibres had accumulated tubulo-vesicular profiles (Fig. 3b).

Electrophysiology

(a) Recording *in vivo*. SNCVs did not differ significantly between the ZPT-treated rats and the pair-fed controls (Table 2). However, the peak-to-peak sensory potential amplitudes were significantly different after 9-days ingestion of 250 ppm ZPT ($P < 0·01$) when only early, mild signs of neural involvement were apparent. After 14-days treatment, when the rats showed profound hind-limb weakness, spinal kyphosis, penile prolapse and locomotor dysfunction, the differences in sensory potential amplitude were significantly increased ($P < 0·001$). The apparent increase in the absolute amplitude values between 9- and 14-days treatment was due to the raising of the recording electrode above the Ringer's solution pool during the later experiments. This was done to avoid

Table 1. *Enzyme histochemistry and histopathology of rats fed 250 ppm ZPT in the diet for 14 days and of their pair-fed controls*

		Appearance of fibres in			
		Gastrocnemius muscle		Soleus muscle	
Treatment	Rat number	Type I/type II* fibre ratio	HE†	Type I/type II* fibre ratio	HE†
ZPT	1	84/116	N	NE	N
	2	68/132	N	124/76	N
	3	84/116	N	154/46	N
	4	71/129	N	138/62	N
	5	NE	N	156/44	N
Pair-fed controls	1	25/175	N	168/32	N
	2	79/121	N	146/54	N
	3	26/174	N	128/72	N
	4	78/122	N	154/46	N
	5	NE	N	145/55	N

N = Normal histology NE = Poor section, not examined

*Stained for adenosine triphosphatase at pH 9·4 by the calcium method (Padykula & Herman, 1955).

†Stained with haematoxylin and eosin.

unnecessary current spread and to take full advantage of the differential amplifier, thereby avoiding amplitude changes due to variations in inter-electrode distance. The SNCVs were unaffected.

Additional studies were performed 2 or 4 wk after 14 days treatment. All clinical signs of ZPT toxicity were absent 7–10 days after removal of the treated food. The SNCVs were not affected during recovery. The sensory nerve-evoked potential amplitudes between treated and pair-fed control rats were still significantly different after 2 wk of recovery ($P < 0.005$) and the difference tended to decrease only at the end of the 4-wk recovery ($P < 0.025$). Thus, 4 wk after cessation of ZPT ingestion, sensory nerve-evoked potential amplitudes remained significantly disturbed despite the rapid (within 1 wk) disappearance of muscular and locomotor signs of toxicity and the apparently normal gross behavioural appearance of the treated rats. As in an earlier study (Snyder *et al.* 1977) the treated animals' individual and group-mean weights remained significantly lower than the control animals' weights ($P < 0.025$) 4 wk after the end of treatment.

(b) Recording *in vitro*. As shown in Table 3, the mean conduction velocity of the mixed sensory and motor sciatic nerve in the ZPT-treated group of rats was not significantly different from that in the pair-fed controls and was similar to our previously reported values for motor nerve conduction velocities for rats of the same size and age (Snyder *et al.* 1977). As they were in the studies *in vivo*, the sciatic nerve-evoked potential amplitudes were significantly lower in the ZPT-treated rats ($P < 0.001$). However, unlike the sural SNCV, the severity of clinical signs of disease did not correlate with the degree of potential amplitude diminution.

DISCUSSION

Blood levels

In order to relate blood levels of ZPT and metabolites to the initial pharmacotoxic sign of skeletal-muscle weakness that occurred in this study between days 9 and 14, pharmacokinetic analyses of ^{14}C blood levels in male rats given a single oral dose of 12·5 mg ZPT/kg/day (15 μCi [2,6-^{14}C]ZPT plus technical-grade material) were conducted (Wedig *et al.* 1978). This dose is equivalent to dietary levels of 250 ppm ZPT. Analysis predicted blood levels of 15·89 μg ZPT/ml on day 9 and 18·31 μg ZPT/ml on day 14, assuming that the required dose was ingested (Wagner, 1975).

Enzyme histochemistry

The intragroup variation in fibre-type ratios in gastrocnemius muscle may be accounted for by the expected variability in samples of a muscle with multiple heads, each with different ratios of slow to fast muscle fibres. There were no detectable changes in muscle morphology with respect to fibre type after 14-days ZPT treatment. This was probably due to the fact that ZPT is not directly toxic to muscle and that neurological changes had not persisted long enough to be reflected in altered muscle morphology. Even after crush injury to nerves to the legs of rats, atrophy of muscles such as the soleus takes place over several weeks and fibre diameters are reduced by about a third. Also, the number of type II fibres in soleus muscle does not change appreciably for up to 3 wk after crush injury to the sciatic nerve (Jaweed, Herbison & Ditunno, 1975).

Neuropathology

The principle electrophysiological findings were significant decreases in evoked potential amplitudes recorded from sural nerve *in vivo* and sciatic nerve *in vitro*. However, marked involvement of intramuscular nerve fibres with somewhat less severe involvement of sensory endings in recovering rats suggests but does not necessarily indicate that ZPT exerts a primary toxic effect on motor fibres.

Recent studies by Sahenk & Mendell (1979; personal communication, 1978) have demonstrated ZPT-induced Wallerian-type, dying-back neuropathy characterized by collections of tubulo-vesicular profiles composed of membrane-associated proteins in the

Table 2. *Electrophysiology* in vivo *of rats given 250 ppm ZPT in the diet and of pair-fed controls*

Treatment	No. of rats	Purely sensory (sural) nerve	
		Mean conduction velocity (m/sec)	Mean evoked potential amplitude (μV)
250 ppm ZPT, 9 days	7	28·6 ± 1·2 (3·1)	18·9 ± 3·8 (10·0)**
Pair-fed controls	7	29·6 ± 0·7 (1·9)	42·9 ± 5·9 (15·7)
250 ppm ZPT, 14 days	10	28·8 ± 1·0 (3·1)	1048 ± 152 (482)****†
Pair-fed controls	10	29·8 ± 0·8 (2·7)	2315 ± 276 (873)
250 ppm ZPT, 14 days + 2-wk recovery	10	33·8 ± 0·44 (1·4)	1079 ± 164 (520)***
Pair-fed controls	10	33·1 ± 0·57 (1·8)	2030 ± 190 (602)
250 ppm ZPT, 14 days + 4-wk recovery	10	33·9 ± 0·7 (2·2)	1465 ± 167 (529)*
Pair-fed controls	10	33·3 ± 0·5 (1·7)	2335 ± 284 (898)

†The apparent increase in the evoked potential amplitude between 9 and 14 days was due to a modified recording technique—the raising of the recording electrode above the Ringer's solution—to reduce the uncontrolled spread of current and to take full advantage of the differential amplifier.

Values are means ± SEM and. in parenthesis, ISD. Those marked with asterisks differ significantly by Student's t test from the corresponding pair-fed control: *$P < 0.025$; **$P < 0.01$; ***$P < 0.005$; ****$P < 0.001$. Unmarked values do not differ significantly from the pair-fed control: $P \geqslant 0.5$.

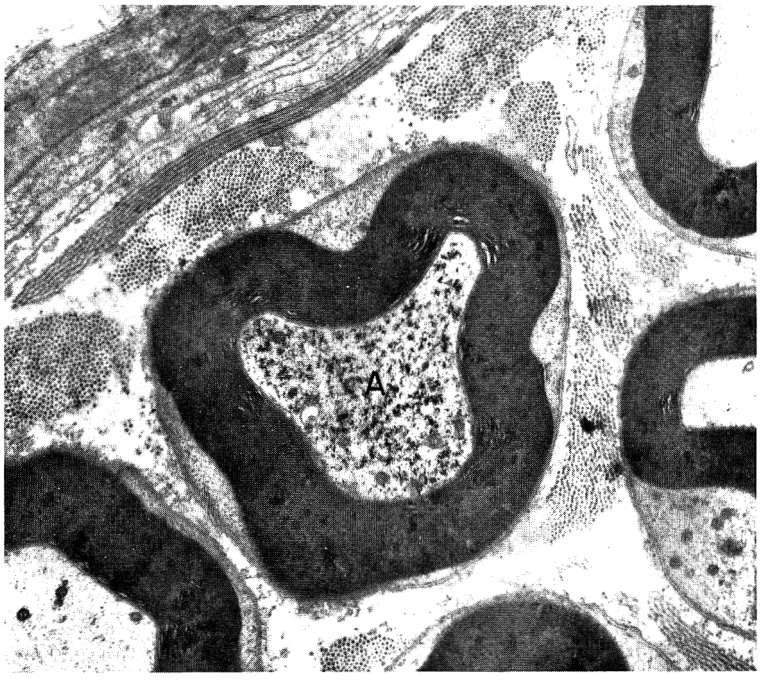

Fig. 1. Representative electron micrograph of the sural nerve of a rat killed during wk 3 of recovery from ZPT treatment (250 ppm in the diet for 14 days). Axon (A) shows accumulation of electron-dense granular deposits. × 12.000.

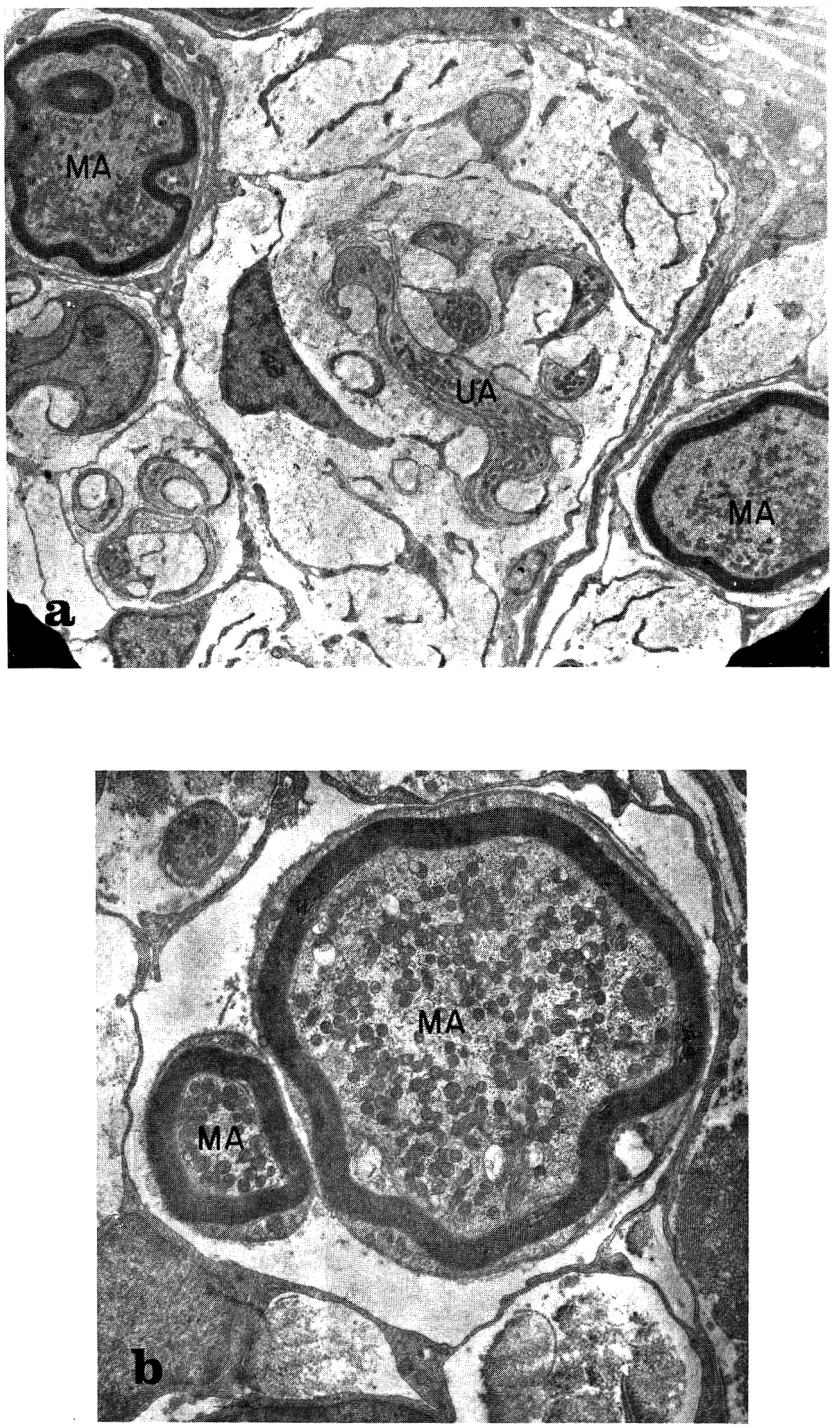

Fig. 2. Representative electron micrographs of intramuscular, lumbrical nerves of a rat killed during wk 3 of recovery from ZPT treatment (250 ppm in the diet for 14 days). (a) Accumulation of mitochondria in myelinated (MA) and unmyelinated (UA) axons. × 4000. (b) Myelinated axons (MA) with accumulation of mitochondria and lightly electron-dense granular deposits. × 12,000.

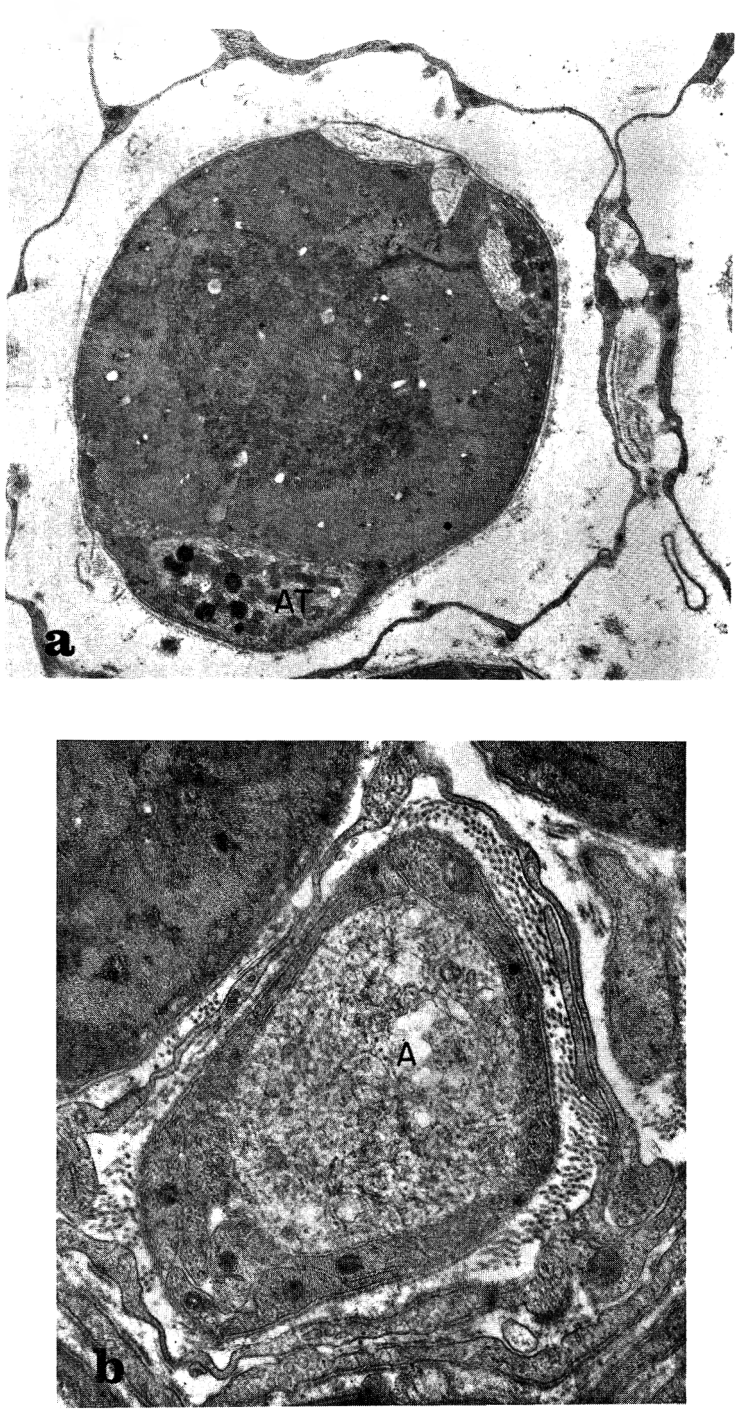

Fig. 3. Representative electron micrographs of intrafusal nerves of a rat killed during wk 3 of recovery from ZPT treatment (250 ppm in the diet for 14 days). (a) Axon terminal (AT) with accumulation of electron-dense bodies. × 10,000. (b) Axon (A) with accumulation of tubulo-vesicular profiles. × 20,000.

Table 3. *Electrophysiology* in vitro *of rats given 250 ppm ZPT in the diet and of pair-fed controls*

Treatment	No. of rats	Mixed sensory and motor sciatic nerve: Mean conduction velocity (m/sec)	Mixed sensory and motor sciatic nerve: Mean evoked potential amplitude (μV)
250 ppm ZnPt, 14 days	10	63·7 ± 1·2 (4·0)	5·58 ± 0·5 (1·59)****
Pair-fed controls	10	64·3 ± 0·7 (2·3)	9·12 ± 0·45 (1·43)

Values are means ± SEM and, in parenthesis, ISD. That marked with asterisks differs significantly by Student's t test from the pair-fed control value: ****$P < 0.001$. The unmarked value is not significantly different from that of the pair-fed control.

terminal endings of intramuscular nerves and sciatic motor nerves. Progressive exposure to ZPT produced similar changes in the tibial-, peroneal- and fine-nerve branches innervating separate muscle groups. Compared to motor units, the sensory endings at the muscle spindles were relatively unaffected. These authors also reported similar but fewer alterations in CNS motor-tract axons in the spinal cord and in axons in the cerebellar vermis after prolonged exposure to ZPT.

Sahenk & Mendell (1979) speculated on the pathogenesis of the axonal loss on inactivation. The accumulations of tubulo-vesicular profiles derived from endoplasmic reticulum appear to be associated with a dysfunction in axoplasmic transport produced by ZPT. From their studies using EM autoradiography they concluded that ZPT caused a breakdown in retrograde axoplasmic transport returning cellular material towards the soma. At present, the relationship between defective axoplasmic transport and axonal dying-back remains unclear. Similarly, it is not known whether chronic defective transport of these membrane-associated proteins leads to long-term ultrastructural membrane changes in the neuron.

Electrophysiology

The absolute difference between human and rodent SNCVs (rodents showing 54–65% of human values) has been explained morphometrically (de Jesus *et al.* 1978). Normal human sural nerves contain faster-conducting fibres of larger diameter (up to 13 μm). Sural nerves from our CD strain rats contained no fibres greater than 8 μm in diameter and therefore had a lower theoretical limit to normal SNCV.

The electrophysiological indication of axonal disease is a decrease in evoked potential amplitude, like that reported here, in conjunction with conduction velocities that are within normal limits. Only a few axons are necessary to maintain the normal conduction velocity, but a graded response such as the evoked potential must decrease as progressively more axons are lost. Our results are consistent with a process of random axonal loss by Wallerian degeneration or inactivation. A possible explanation for the lack of correlation between the severity of clinical signs and the level of reduction in potential amplitude might be the random nature of axonal loss. Functional blocking of axons innervating major muscle groups would be expected to produce more disabling signs than loss of input to minor muscles or those with redundant innervation. Preliminary data indicate reversibility of evoked potential amplitude diminution after long-term recovery.

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Review Section

TOXICOLOGY STUDIES. II. THE LABORATORY ANIMAL

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Abstract—The laboratory animal can be thought of as the toxicologist's major instrument for use in a safety evaluation programme. Consequently, to obtain reliable results from such an 'instrument', the toxicologist must obtain animals of the highest quality available, and maintain as well as monitor them in such a manner that intercurrent infections or latent diseases do not produce effects leading to erroneous conclusions. The procedures described will enhance the reliability of the results obtained in toxicological studies.

Introduction

The first paper in this series considered the training, education and experience required for various personnel in a toxicological testing programme. Particular attention was given to the technical staff working with the animals (Arnold, Fox, Thibert & Grice, 1978). The present paper will be concerned with the laboratory animal, emphasizing procedures that will help to ensure a healthy animal for toxicological testing.

The acquisition of animals conforming to a specified health standard is the initial step towards obtaining accurate and reproducible experimental results with animal models. The principal investigator may devise an excellent experimental design and have access to a modern research facility containing the best laboratory equipment and safety devices, but animals of inferior quality will place the experiment in jeopardy even before it commences.

Reliable commercial breeding laboratories should be able to substantiate claims concerning the health and genetic integrity of their animals. Although animals of good quality may cost more, the reduced risk of premature termination or the invalidation of a study due to intercurrent disease justifies such expenditures. The cost of the animals in a toxicological testing programme usually represents less than 5% of the total cost for the study (Box, 1974).

A disease-surveillance programme for rodents similar to the one detailed here is probably not undertaken routinely by most laboratories. However, a basic disease-surveillance programme should be followed by laboratories conducting subchronic or chronic studies with rodents. The objectives of such a programme are: (i) to ascertain the breeder's ability to supply animals of acceptable quality; (ii) to minimize contamination of the animal quarters with unwanted pathogenic organisms; (iii) to facilitate early detection of latent disease(s) that may adversely affect the experimental model; (iv) to minimize the occurrence of communicable diseases that could invalidate a toxicological study; (v) to minimize the occurrence of zoonotic diseases (Wallbank, 1978).

In view of the more sophisticated experimental designs currently being used in toxicological studies, alternative methods for allocating the available test animals among treatment groups are outlined. Additional protocol considerations relating to the animal's macro- and microenvironment are also discussed.

The proposals and concepts presented here are based primarily on programmes that have evolved in our laboratories over a number of years, and should be applicable to a wide variety of toxicological research or safety-testing programmes.

Obtaining a quality animal

Once a particular animal model has been selected for a toxicological study, every effort should be made to ensure that animals of acceptable quality are obtained. Potential animal vendors must be evaluated using specific criteria based on the requirements for

the pending experiment. These criteria should be determined in consultation with a specialist in laboratory animal medicine or the veterinarian for the in-house animal colony. Consideration should be given to five major aspects when selecting a vendor.

The animal model

Theoretically, any animal species could be selected for a toxicity testing programme. Ideally, the absorption, tissue distribution, metabolism and excretion of the test chemical in the animal model should be comparable to that of man (National Academy of Sciences, 1971), but this information is seldom available. In fact, practical limitations of space, time and economics, familiarity, and the requirements of various regulatory bodies have produced a situation where small rodents (rat, mouse and hamster) are the primary species used (Arnold & Grice, 1978; Page, 1977). Many international groups and agencies have expressed the need for a 'new' test species that overcomes the concerns often expressed about small rodents (Arnold & Grice, 1978); however, very little progress has been made in this regard.

The choice between inbred and outbred strains for toxicological testing has been the subject of some controversy (Festing, 1975; Sontag, 1977). While outbred strains may be more analogous to human populations, the genetic heterogeneity may not be as extensive as anticipated because of closed-colony breeding techniques and the breeder's culling of the less desirable animals. Since inbred animals may be expected to exhibit more uniform sensitivity because of their isogenicity, experimental results with inbred animals should be more uniform than those obtained using an outbred stock. Although the results obtained with inbred animals may thus be more reproducible, it is possible that the particular strain selected for a study could be resistant to the toxicological effects of the test compound.

The animal

A supplier should be able to provide data concerning a strain's age and weight range, lifespan and behavioural peculiarities such as docility or intractability, and the availability of the species being considered for an experiment. Depending upon the duration and type of toxicological study, other data may be desirable and should also be available from the supplier. These data would include a growth chart for use in detecting abnormal growth patterns and possibly subtle subclinical abnormalities, historical data concerning the incidence of spontaneous tumours and chronic disease problems such as enteritis, nephrosis, cardiomyopathy and hydronephrosis, and information on biochemical peculiarities, such as elevated serum lactic dehydrogenase (LDH) levels which may suggest the presence of the lactic dehydrogenase or Riley virus. Additionally, the genetic background of the strain should be known and the integrity of the strain confirmed (Festing, 1974). The number, type and frequency of visible genetic mutations should also be known by the supplier.

Sources of historical information other than that provided by the supplier include published reports and information from other toxicological testing laboratories with experience involving the strain in question. However, data reported from other laboratories may be expected to diverge somewhat from those obtained in one's own laboratory (Clayson, 1962; Toxicology Forum, 1977).

The facilities

A visit to a supplier's animal facilities to evaluate the quality control programme, husbandry techniques, health monitoring procedures, type of staff employed, and the general appearance of the facilities may be desirable. Specific items of concern include the environmental control, the watering system used, dietary factors and housing and bedding.

Environmental control. To provide some degree of continuity and to minimize the stress on the animals when they are first received from the supplier, the environmental conditions (temperature, humidity, light cycle and number of air changes/hour) and such husbandry procedures as the feeding schedules used by the supplier should be known by the purchaser. However, during the in-house quarantine period, several of these environmental factors will be gradually altered to coincide with the requirements for the experimental protocol.

Watering system. The type of water surveillance and control programme in use at the supplier's facilities to monitor bacterial content (e.g. *Pseudomonas aeruginosa* and coliforms), possible chemical (e.g. chloroform) or pesticide contamination, as well as the level of $CaCO_3$, should be ascertained.

Dietary factors. The supplier should provide information concerning the type of diet used (i.e. chow, open or closed formula, cubes or ground), any changes in the source or type of diet, the feeding method (i.e. restricted intake or *ad lib.*, and whether the diet is monitored for bacteria, heavy metals, mycotoxins, oestrogenic activity or chemicals such as halogenated hydrocarbons that persist in the environment. The supplier should also advise the purchaser if rodenticides or insecticides are used within the breeding facility, since the animals might inadvertently ingest or inhale these agents as a result of contamination of stored feed or aerosolization.

Housing and bedding. Information concerning the type of housing (single or group) and bedding (type and particle size) used by the supplier will be more relevant to some research programmes than others, since bedding may be treated with or may naturally contain enzyme inducers.

Transportation logistics

Shipping procedures must be directed towards protecting the research animal from exposure to unwanted pathogens (e.g. sendai virus and Mycoplasma spp.) and minimizing shipment stress. Vendors will provide, in some instances at extra cost, shipping containers of varied construction with polyester-fibre filters designed to withstand the rigours of travel and to minimize the exposure of research animals to pathogenic organisms. Steps must be taken to reduce dehydration of research animals during transit (Weisbroth, Paganelli & Salvia, 1977).

Some institutions and many suppliers have vans specifically equipped to transport the animals in a fully controlled environment. It is important that such vehicles are easy to decontaminate and disinfect. The

responsibility of the driver should not be limited solely to transporting animals; in fact, the driver should be a staff member who is trained in all aspects of animal care and handling, using either an internal instructional programme or the auspices of CALAS (Canadian Association for Laboratory Animal Science) or AALAS (American Association for Laboratory Animal Science).

Ordering animals from a more distant supplier requires that the animal be shipped via a public carrier, usually by aeroplane. The supplier should provide the flight number, time of departure and arrival to speed the collection of the animals upon their arrival at the airport, since most airports do not have the facilities to house laboratory animals properly.

When laboratory animals are shipped by public carrier, their husbandry during transit is beyond the control of the supplier and the investigator, and is a continuing area of concern for all parties involved in the field of laboratory animal science. There have been many instances in which the animals were left without proper protection during inclement weather or the containers were crushed and the animals asphyxiated. While some of these problems no longer exist in the US because of recent legislation requiring strict control of animals during transportation (USDA, 1977), research animals in many countries, including Canada, are still handled as 'freight' and the carrier is not required to exercise any special precautions.

In any case, the following procedures will help to alleviate the problem of inappropriate handling of laboratory animals by airport personnel: (i) maintain good rapport with the local airport authorities and custom agents so that they are aware of the husbandry requirements for laboratory animals, (ii) offer an educational programme for airport personnel, and (iii) assign the same animal laboratory personnel to pick up the animals at the airport to establish good relations with airport personnel and possibly to fulfill the requirements of the educational programme on an informal basis.

In-house quality check programme

Having evaluated the available suppliers and possibly selected one or two as having the quality of animal desired, it is necessary to order a few animals of different ages from the supplier and subject them to an intensive health screening programme. This will serve as an aid for determining the effectiveness and reliability of the supplier's quality control programme.

Reception of the animals

A receiving area for the exclusive use of incoming animals helps to minimize animal health care concerns. It should have sufficient floor space to accommodate each animal shipment, as well as the bench space, storage facilities and equipment necessary for an adequate assessment of the health of all incoming animals. The design of the receiving area should also facilitate cleaning and sanitizing operations. Appropriate ventilation and the use of sanitizing agents which will not jeopardize the integrity of the animal for research purposes are also important.

The animal receiving area or room should be used primarily as a place to screen animals initially and reject those that are undesirable. The examining team might consist of the driver who received the animals at the airport, an animal technician and the veterinarian or the veterinary technician. A more detailed examination will be conducted during the quarantine period.

Standard procedures used in the receiving area include counting, sexing, weighing and examining the animals thoroughly for ectoparasites. The shipping containers should be examined for defects or damage and the contents should also be examined for signs that may be indicative of diarrhoea. Each animal should be examined for any other signs of disease. If the animals are clinically asymptomatic, they are transferred to a quarantine room.

The receiving area can then be thoroughly cleaned or sanitized. Disposable materials should be bagged, removed from the area and stored or incinerated as dictated by standard operating procedures and municipal requirements.

Quarantine

Ideally, the receiving area and quarantine rooms are in an area or building physically separated from the main animal housing facility. Traditionally, however, rodents are quarantined in one of several rooms predesignated for such purposes. The quarantining of newly received animals is undertaken to provide a period in which the veterinarian can make a more extensive evaluation of the animals' health and identify disease processes and aetiological agents while minimizing their possible introduction into the colony, and in which the animal adjusts to the new environment while recovering from the stress of shipment (Grant, Hopkinson, Jennings & Jenner, 1971; *ILAR News*, 1977; Sontag, Page & Saffiotti, 1976; Weihe, 1965). The veterinarian may use clinical laboratory tests and the pathologist's findings to assist in this endeavour. Selected use of prophylactic or therapeutic regimens should be undertaken during the quarantine period, since such treatments are generally not compatible with many experimental protocols using rodents.

Usually, animals spend less than one day in transit between the supplier and their destination. Many commercial breeding laboratories maintain excellent facilities and consistently supply animals of high quality. These animals are often shipped in boxes with polyester-fibre filters and transported in environmentally-controlled vehicles, which eliminate a major source of stress to the animals. In such circumstances one may be sufficiently confident of the animals' quality to permit newly arrived animals to be quarantined in the 'test' room. This is one of the major benefits of seeking out a supplier who can consistently provide animals of acceptable quality. This also eliminates many logistical problems, especially if these animals are to be used in a chronic or life-time experiment. However, this latter procedure requires adherence to the following standard operating procedures

and the presence of certain physical plant capabilities which would minimize the spread of disease:

(i) House only one species in each room.
(ii) House similar species from different suppliers, or similar species from the same supplier shipped at different times, in different rooms.
(iii) Thoroughly sanitize each room whenever a group of animals is removed.
(iv) Strictly follow procedures concerning the flow of personnel, supplies and equipment.
(v) Have 100% exhausted air with individual temperature and humidity controls monitored daily in each room. The problems associated with recirculated air (Sansone & Losikoff, 1978; Sansone, Losikoff & Pendleton, 1977) and faulty environmental controls (Fox, 1978) are well recognized.
(vi) Use monitoring devices on the washing equipment, to ensure that the water temperature is appropriate (i.e. >83°C/180°F) for sanitation, and test cages, washers, autoclaves and water bottles for the presence of microbiological organisms.
(vii) Ensure that used water bottles are never refilled; they should be washed and sanitized prior to reuse. Automatic watering systems must be checked daily to confirm their proper functioning. Water samples must be tested periodically for microbiological organisms.
(viii) Sponge walls weekly and wet-mop floors daily, preferably with a disinfectant, although some disinfectants may be contra-indicated in certain situations. Walls and floors should also be monitored for the presence of microbiological organisms.
(ix) Prohibit eating, drinking, smoking or the application of cosmetics by staff in the animal rooms.
(x) Have locker room facilities available to permit removal and storage of personal clothing, including shoes. Only suitable garments provided by the research institutions should be worn in the animal rooms. Footwear should consist of safety shoes with steel toes and skid resistant soles and heels. The prescribed footwear and outer garments should not be taken or worn outside the locker room and animal quarters, except when the garments are sent for laundering. While the use of a commercial laundry is acceptable for many experimental situations, in-house laundering of clothing contaminated with potentially toxic or carcino genic agents is essential. Alternatively, the use of disposable coveralls, gloves, head cover, mask or respirator and shoe covers, may be more appropriate in these experimental situations.
(xi) Animals in shoe box cages should be transferred at least once a week to clean sanitized cages, while the pull-papers under suspended wire-mesh cages should be changed at least once a day and the cages sanitized every other week. Test chemicals that cause diuresis or diarrhoea will necessitate alterations in these schedules.
(xii) Preventative maintenance of ventilation systems and other mechanical equipment, should be scheduled and performed at frequently prescribed intervals. Additionally, the air filters and humidifiers should be changed or monitored at frequent intervals.

Unless it is due to latent viruses, an outbreak of disease among animals within 3 days of their arrival is generally attributable to endemic disease present in the supplier's colony. It is unlikely that with such a short incubation time, the animals were exposed to the disease in transit. However, a disease condition that may not have been clinically apparent in the supplier's colony could be activated by such in-transit stresses as cold or heat.

The initial step in implementing a quarantine programme is the establishment of a precise standard operating procedure to be followed upon receipt of the animals. The technicians executing the programme should have a thorough understanding of all the procedures to be followed, including who is to be contacted for problems beyond the technicians responsibility. A procedure to ensure compliance with the prescribed programme or to ascertain whether any deficiencies exist is also required.

Disease surveillance programme for rats and mice

The disease surveillance programme is one of the major responsibilities of the veterinarians and their staff. Depending upon the nature of the experiment and the quality of a particular supplier's animals, the investigator should order additional animals, approximately 10% in excess of requirements, to be used exclusively for the disease evaluation programme. Obviously if some animals in the shipment appear to be ill upon arrival, they should be removed immediately and prepared for the examination described below. If the animals appear healthy when received, they will be taken to the quarantine room. The animals used for the disease surveillance programme should then be randomly selected from all the animals available and divided into two groups. One half of the animals selected are to be used in connection with the initial disease surveillance programme conducted during the quarantine period, while the remaining half are to be distributed equally among all of the treatment groups for subsequent disease surveillance. Although these animals are housed within the same room and racks as the test animals, they are designated as animals to be used only for purposes of disease surveillance. At preselected intervals (possibly quarterly or semi-annually as appropriate) some proportion of these animals should be killed and examined in the same manner as those killed upon receipt Any or all of the following procedures may be included in the examination of the animals for disease:

(a) *Examination for ectoparasites* (in/on skin or hair)

Organism	*In mouse*	*In rat*
Mites		
Bdellonyssus bacoti	x	x
Myobia musculi	x	
Myocoptes musculinus	x	
Myocoptes romboutsi	x	
Notoedres muris		x
Psorergates simplex	x	
Radfordia affinis	x	
Lice		
Polyplax serrata	x	
Polyplax spinulosa		x

(b) *Examination for endoparasites* [in bladder (B), caecum (CA), colon (C), duodenum (D), ileum (I), jejunum (J), kidney (K), liver (L) and/or ureter(U)]

Organism	*Location*	*In mouse*	*In rat*
Aspiculuris tetraptera	C, CA	x	x
Capillaria hepatica	L	x	x
Cysticercus fasciolaris	L (larval stage)	x	x
Eimeria spp.	Epithelial cells of CA, C, I	x	x
Entamoeba muris	C, CA, D, I, J	x	x
Eperythrozoon coccoides	Blood	x	
Giardia muris	CA, D, J	x	x
Haemobartonella muris	RBC	x	x
Hepatozoon muris	L	x	x
Heterakis spumosa	C, CA	x	x
Hexamita muris	CA, D, I, J	x	x
Hymenolepis diminuta	D, J	x	x
Hymenolepis microstoma	D, L (bile ducts)	x	
Hymenolepis nana	D, I, J	x	x
Klossiella muris	K	x	
Syphacia muris	C, CA	x	x
Syphacia obvelata	C, CA	x	x
Toxoplasma gondii	*	x	x
Trichosomoides crassicauda	B, K, U		x
Trypanosoma duttoni	Blood	x	
Trypanosoma lewis	Blood		x

(c) *Haemogram.* Haemoglobin, haematocrit, RBC, WBC, differential counts, platelet counts and coagulation screening test.

(d) *Biochemistry profile.* Glucose, urea, creatinine, bilirubin (direct, indirect and total), cholesterol, serum glutamic–oxalacetic transaminase (SGOT), serum glutamic–pyruvic transaminase (SGPT), alkaline phosphatase and LDH. LDH may be useful for detecting a latent infection in mice, LDH virus, which will induce an elevation of plasma LDH as well as other enzymes such as isocitric dehydrogenase, malic dehydrogenase, phosphohexose isomerase, GOT and glutathione reductase (Notkins, 1965).

(e) *Pathogenic bacteria profile*

Organism	*Location*	*In mouse*	*In rat*
Bacillus piliformis	Histological demonstration in liver sections	x	x
Bordetella bronchiseptica	Lungs, bronchi, middle ear	x	x
Citrobacter freundii	Colon	x	
Corynebacterium kutscheri	Nasopharynx, lungs, kidney, liver, lymph nodes	x	x
Klebsiella pneumoniae	Lungs, other organs	x	x
Pasteurella multocida	Lungs, conjunctiva, bladder, skin, brain	x	x
P. pneumotropica	Lungs, nasopharynx, conjunctiva, uterus	x	x
Pseudomonas aeroginosa	Majority of tissues, middle ear	x	x
Salmonella (all types, esp. *S. typhimurium* and *enteriditis*)	Lungs, liver, spleen, other organs	x	x
Staphylococcus aureus (coagulase +)	Cornea, lungs, conjunctiva	x	x
Streptococcus Group A Type 50 or Type D	Lungs, liver, digestive tract, cervical lymph nodes	x	x
Streptococcus pneumoniae	Lungs, nasopharynx, middle ear	x	x
Streptobacillus moniliformis	Nasopharynx, middle ear, conjunctiva, articulations	x	x

*Cysts in reticuloendothelial and central nervous systems; trophozoites in epithelial cells and gut.

(f) *Examination for Mycoplasma. Mycoplasma pulmonis* (in lungs, trachea, uterus, middle ear) by culture and fluorescent (FA) antibody techniques, *Mycoplasma arthritidis* by culture and other Mycoplasma spp.

(g) *Pathogenic Fungi Profile.* Microsporum spp. and Trichophyton spp. on the skin and hair of both the mouse and rat.

(h) *Viral Profile.* Serological testing by complement fixation (CF) or the haemaglutination inhibition (HI) technique for the following viral agents:

Organism	*In mouse*	*In rat*
Ectromelia	x	
Encephalomyelitis (GDVII)	x	x
Kilham rat virus (KRV)		x
Lymphocytic choriomeningitis (LCM)	x x	x
Minute virus of mice (MVM)	x	x
Mouse adenovirus	x	
Mouse hepatitis (MHV)	x	
Papova virus (K virus)		
Pneumonia virus of mice (PVM)	x	x
Polyoma	x	
Rat corona virus and sialodacryoadenitis virus		x
Reovirus type 3	x	x
Sendai	x	x
Toolan H-1		x

(i) *Histopathological examination of all major organs.* Skin, eye, brain, trachea, lungs, heart, liver, kidney, spleen, intestine, reproductive tract, skeletal muscle, bone, pancreas, adrenal, urinary bladder, central nervous system and all grossly abnormal areas of dermal, supportive or skeletal tissues (Cotchin & Roe, 1967).

(j) *Special techniques.* Some inapparent infections may be activated by certain types of experimental manipulation such as the use of surgical techniques or immunosuppressive agents.

(i) Pseudomonas infection in mice and rats may be activated by irradiation or immunosuppressive chemicals (Flynn, 1963; Lattuada, 1977; Wensinck, Ven Bekku & Renaud, 1957).

(ii) *Hemobartonella muris* and *Eperythrozoon coccoides* are usually inapparent infections of limited pathogenicity which may be more apparent following splenectomy, concurrent viral infection (e.g. MHV) or daily examination of Giemsa stained blood smears.

(iii) Inapparent murine leukemia, cytomegalovirus, *Pneumocystis carinii* and *Bacillus piliformis* may be activated by irradiation, prolonged administration of corticosteroids and immunosuppressive agents (e.g. azathioprine).

Ideally, the supplier should be able to ensure that his stock is free from such inapparent diseases, but present indications are that such a supplier would be the exception rather than the rule.

(k) *Disease transmission studies.* In some instances a disease process may have advanced to the point where macrophage invasion is so extensive that the organism responsible for the condition cannot be identified histopathologically or by typical clinical laboratory techniques. One such instance followed an infection by Cytomegalovirus. In this case, the agent was identified by inoculating an aliquot of a homogenate of the diseased tissue into a member of the same species that was free of the suspected organism. Subsequently, the inoculated animal was killed and a definitive diagnosis was made. The organ homogenate may be used immediately after it is made or it may be stored at $-80°F$ to await histopathological and clinical laboratory reports, at which time it can be thawed and then used for the inoculation.

Other aspects of disease surveillance

The only animals that might not be examined in such detail are those to be used for an acute study with a short observation period. Ideally, however, it would be preferable to incorporate a disease surveillance programme in all experimental protocols.

Equally important aspects of any disease surveillance/prevention programme include:

(i) Ascertaining that various perishable supplies and reagents have not deteriorated or become contaminated and that they are reordered in a timely and efficient manner, using the correct forms.

(ii) Maintaining a systematic programme for cleaning the physical plant (e.g. animal rooms, receiving areas etc.) and laboratory equipment (e.g. scales, pH meters etc.), as well as sterilizing equipment that is used in more than one animal room.

(iii) Maintaining a daily log of all chemical and clinical laboratory work undertaken, with details of who performed each test along with the results of all tests, including blanks, standards and controls, and concurrently ascertaining that all required work sheets are properly filled out and specimens are properly labelled and stored.

(iv) Undertaking an internal study review programme in which qualified individuals ascertain that all procedures have been performed according to the protocol or acceptable standards and that the results were reliable and properly recorded.

Allocation of animals to treatment groups

Conventional studies

Proper randomization is essential in order to ensure an unbiased allocation of the available experimental animals to the various treatment groups (Cochran, 1974; Kempthorne, 1977). A completely randomized design involves the random assignment of animals to treatments without regard for possible blocking factors such as litter status or body weight. Results from such a study may be assessed using statistical permutation tests, which provide a means of determining whether any observed differences among treatments are larger than would be expected if the experimental variation were due to randomization alone (Gart, Chu & Tarone, 1979; Scheffé, 1959).

Alternatively, a litter-matched design in which pups within litters culled to equal size are randomly allo-

cated among the various treatment groups may be employed as a means of controlling inter-litter variation. Since treatment comparisons are then between littermates, this design may be expected to be more efficient than the completely randomized design in the presence of appreciable litter effects. However, special statistical techniques are required in the analysis of time-to-tumour data if the litter-matched design is to remain advantageous in this case (Mantel, Bohidar & Ciminera, 1977). If litter effects are negligible, the resulting inefficiency of the litter-matched design compared with the completely randomized design may be minimal in a large scale study (Fleiss, 1973; Scheffé, 1959), although information on this is lacking in the case of time-to-tumour data.

Although apparently not used in chronic toxicity studies, another allocation procedure that may be mentioned involves the random assignment of entire litters (culled to equal size) to the various treatment groups. This design may be expected to be less efficient than the completely randomized design since any treatment comparisons will be entirely between animals from different litters (Mantel, 1969).

Since caloric restriction and dietary modification have been shown to alter tumour incidence in both rats (Ross & Bras, 1971 & 1973) and mice (Gilbert, Gillman, Loustalot & Lutz, 1958; Tannenbaum & Silverstone, 1957), differences in body weight may also be taken into account in assigning animals to treatment groups. It has been suggested (Sontag *et al.* 1976) that the available experimental animals be divided into a number of weight classes, within each of which animals are assigned to treatment groups completely at random. As in the case of the litter-matched design, however, this block structure must then be taken into account in the analysis of the results obtained.

In addition to the general approach to be taken in allocating animals to treatments, consideration also needs to be given to the number of animals to be housed in each cage. Since studies have shown that individual housing can lead to increased stress in rodents (Hatch, Wiberg, Zawidzka, Cann, Airth & Grice, 1965; Sigg, Day & Colombo, 1966), it may be felt that single caging might enhance the induction of the particular toxic response under study. Conflicting evidence on this point is available, however, in the case of mammary (Andervont, 1944) and skin tumours (Fare, 1965) in the mouse. While the animals were not handled on a regular basis in any of these studies, our experience indicates that the amount of handling recommended in part III of this series (to be published) will reduce the stress of individual housing significantly.

If multiple housing is used, food and water consumption cannot be controlled or monitored on an individual basis and animals may be lost due to cannibalism or the more rapid spread of communicable disease. In addition, the loss of some animals within a cage may affect the remaining animals in that cage. Thus, some adjustment for possible imbalance within cages may be necessary when assessing the experimental results.

It is also possible that interactions among animals within the same cage or in a common environment could result in various types of caging effects. Gart (1976), for example, found some indication of such effects in the positive control groups for a large skin-painting study in mice, although these effects did not appear to be present in the majority of the treatment groups. Since different effects may be expected with various protocols and experimental environments, the analysis of experimental results when multiple caging is used should, in the absence of firm evidence to the contrary, provide for potential caging effects.

A satisfactory source of random numbers, either computer generated or in tabular form, is essential in the implementation of any experimental design. As an example of how randomization is actually carried out, suppose that 100 animals of the same sex are to be allocated to either a control or test group using a completely randomized design. For simplicity, suppose in addition that individual caging is to be used. After arbitrarily assigning these animals to cages identified by sequence number, fifty distinct random numbers between one and 100 may be selected to designate the test animals. This act of randomization alone will provide a basis for valid comparisons between the control and test groups.

Although this approach will ensure that animals will be randomly assigned to treatments, it does not ensure that these animals have been randomly situated within the experimental environment. The latter concern may be satisfied by randomizing cage positions within the experimental environment, either before or after assignment to treatments has been completed. This will allow any observed variation in response within the experimental environment to be related to spatial differences in factors such as ventilation, lighting, temperature and humidity rather than to differences among the experimental animals themselves. The actual randomization of cage positions in this example is easily accomplished through the selection of a random permutation of the numbers 1–100 (Moses & Oakford, 1963).

Similar randomization schemes may also be used in the case of experimental designs involving several treatment groups, including separate groups of males and females at the same dosage level. Simultaneous randomization of all treatment groups within the experimental environment will provide for comparisons between sexes, while separate randomization schemes with separate racks for housing males and females will result in any sex and rack differences being confounded.

Since adjacent cages may be assigned to different treatments using the randomization schemes described above, alternative approaches may be more appropriate in cases where cross-contamination of the test chemical (Sansone & Fox, 1977; Sansone & Losikoff, 1978; Sansone *et al.* 1977) is of concern. In addition, other randomization schemes for designs involving litter-matching, multiple caging or blocking by body weight may also be worked out, although these will not be considered here.

In contrast to the randomization techniques discussed here, systematic procedures for assigning animals to treatment groups have also been used. In the simple case of a control and single test group, for example, every other cage might be assigned to the test group. Because of the systematic allocation of animals to treatments, statistical analysis of such a

study cannot be based on randomization theory (Cochran, 1974). In addition, systematic positional effects within the experimental environment may bias the comparisons between the control and test groups. Randomization could be used to determine the ordering of the control and test groups within pairs of adjacent cages, although this approach would introduce a blocking factor (position) which would have to be taken into account in the analysis of the results obtained.

Two-generation studies

Since the conventional lifetime feeding study does not take into account the potential risk associated with perinatal exposure, considerable effort has recently been devoted to the development, implementation and interpretation of a two-generation chronic toxicity study. By feeding the test compound to the parent generation, the offspring will be exposed both prenatally during gestation and postnatally during lactation. After weaning, the offspring are subjected to lifetime exposure through the diet exactly as in the single-generation study.

Selection of animals to continue on test in the second generation should be done in such a way as to ensure that within each litter, each animal has an equal chance of selection. One proposed procedure (Grice, 1978) involves culling the litters to a maximum size of eight on day 4 or 5 after birth in order to prevent the natural selection of the more vigorous offspring and balance the burden placed on the dams. At weaning, an equal number of pups of each sex may be selected randomly from each litter to continue on test in the second generation. Although the selection of several pups of each sex per litter will reduce the number of dams required in the parent generation to achieve specified group sizes in the second generation, the selection of one pup of each sex per litter may be expected to ensure maximal statistical sensitivity for the study. When more than one pup is selected, the statistical analysis of the results obtained should provide for potential litter effects (Haseman & Kupper, 1979) due to inter-litter differences in transplacental exposure (Emmerson & Krewski, 1978) and geneology.

Microenvironment

Selection of caging systems

The selection of the appropriate caging system in toxicological studies is critical, since the microenvironment within the cage has a direct influence on the animal's response to the test compound.

Laminar flow caging. Caging systems have become more sophisticated in the last few years. Newer designs include the laminar flow caging systems, the attractive feature of which is the ability to provide a unidirectional filtered air flow. The air is passed through a high-efficiency particulate aerosol (HEPA) filter, to remove potentially pathogenic microorganisms, aerosols and particulate matter, before it circulates to the animal cages. Therefore, the animals are exposed only to minimal amounts of these substances during the experiment. Various filter-rack designs will produce varying degrees of isolation between cages within a rack. Experimental work corroborating these features frequently involves the artificial exposure of the racks to micro-organisms and the subsequent recording of the latter's recovery on agar plates situated throughout the area. Although such microbiological sampling can yield useful information, the most meaningful data come from in-use studies under controlled conditions (McGarrity & Coriell, 1973).

These caging systems reduce the microenvironmental variability and may enable animals to maintain homoeostasis, thereby minimizing variation in physiological parameters and making the animal more resistant to disease processes. There is some evidence to support this contention. The influence of aerosolized particulate matter in the incidence of benzo[*a*]pyrene-induced pulmonary tumours was compared in hamsters housed in a conventional holding room and in similarly treated hamsters housed in laminar flow units (Smith, Rogers & Newberne, 1975). The hamsters housed in laminar flow units developed tumours more slowly than did hamsters in conventional housing. The lower incidence of pneumonia in hamsters housed in laminar flow units may have contributed to this effect. A study with rats indicated that respiratory-tract infections may be co-carcinogenic for pulmonary tissue (Schreiber, Nettesheim, Lijinsky, Richter & Walburg, 1972). Another study with C3H/He mice documented the effect of specially ventilated caging of the authors' design and the reduction of stress in the incidence of viral-induced mammary tumours (Riley, 1975). Various groups of mice, each carrying the Bittner oncogenic virus, were subjected to different degrees of chronic stress. The incidence of mammary tumours after 400 days on test ranged from 92% for mice in conventional housing and under stress to 7% for those housed in the specially ventilated enclosed environment.

These systems do not protect the animal from volatile chemicals within the animal room environment. Furthermore with this type of animal caging, the air movement across the top of the cages may distribute noxious chemicals administered to the animal into the general environment of the room. Laminar air flow systems are also available with a negative air flow that moves across the animal cage and then through the HEPA filter before circulating into the animal room or being exhausted to the outside.

Solid-bottom (shoe-box) caging. This type of caging can be made from several types of material including stainless steel, polycarbonate or polypropylene. Filter-top systems placed on top of these cages decrease the likelihood of exposure to aerosolized microorganisms. However, using filter tops will alter the microclimate within the animal cage, increasing the humidity, temperature and levels of ammonia and carbon dioxide (Serrano, 1971; Simmons, Robie, Jones & Serrano, 1968). The degree of accumulation of gases such as carbon dioxide, ammonia and volatile compounds from wood contact bedding depends upon many factors, such as the number of animals per cage, the cage design, the type of bedding, the amount of urease positive enteric flora and the frequency of changing (Briel, Kruckenberg & Besch, 1972). Broder-

son, Lindsey & Crawford (1976) reported that levels of 25 ppm ammonia or more within the rat cage consistently increased the severity of the rhinitis, otitis media, tracheitis and pneumonia characteristic of murine respiratory mycoplasmosis due to *Mycoplasma pulmonis*.

In consideration of worker safety, the US National Cancer Institute (1976) has recommended the use of filter tops with shoe-box cages for work involving chemical carcinogens.

Wire-bottom caging. The stainless steel wire mesh cage offers the conveniences of durability and individual or group housing while still conserving space and maximizing the visibility of the animal. This type of cage provides only minimal protection to the animal from microbial agents, and maximizes the chances for contamination of the general environment from toxic substances being administered to the animals (Sansone & Fox, 1977). Other disadvantages of this type of caging include the possibility that the wire-mesh floor can cause decubitus ulcers on the plantar surface of rodents' feet after long-term housing, and the animals will occasionally break their mandible while attempting to extract their teeth from the wire grid. Mice housed in this type of caging may require a higher ambient temperature than those housed in solid-bottom caging or than other rodents housed in wire-mesh cages (Murakami & Konoshita, 1978).

Bedding

Bedding for laboratory animals consists of a variety of materials, including different types of wood. Certain types of wood bedding affect the hepatic microsomal enzyme activity of rodents and have the potential to influence the experimental animal. For example, the levels of two hepatic microsomal drug metabolizing enzymes, ethylmorphine *N*-demethylase and aniline hydroxylase, were raised in mice housed on red cedar or white-pine bedding when compared to mice housed on a mixture of hardwoods consisting of beech, birch and maple (Vessel, Lang & White, 1976). Cedrol and cedrene, volatile compounds in the cedarwood bedding, have been shown to be active agents in the induction of hepatic microsomal enzymes (Wade, Holl, Hilliard, Moulton & Greene, 1968). The use of cedar bedding has also been implicated in the high incidence of liver and mammary tumours in C3H-A^{vy} and C3H-A^{vy}fb mice (Sabine, Horton & Wicks, 1973). However, it was suggested that the reduced incidence of these tumours observed when animals were bedded on sawdust was due to the lower weight gain, resulting from ectoparasitic infestation, rather than to food or bedding (Heston, 1975).

It should be noted also that the *p-O*-methyl derivatives of sinapaldehyde and 2,6-dimethoxy-1,4-benzoquinine are carcinogenic for the rat, suggesting that the β-saturated carbonyl compounds in wood shavings are potentially carcinogenic (Schoental, 1973). Additionally, workers exposed to fine particulates of hardwood, or to its volatile products generated during machine processing, develop a high incidence of nasal tumours (Acheson, Cowdell & Hadfield, 1968). Sinapaldehyde (present mainly in angiosperms), whose *p-O*-methyl derivative has been prepared synthetically, has proved carcinogenic to rats, inducing a variety of tumours including nasal squamous carcinomas (Schoental, 1973; Schoental & Gibbard, 1972; Schoental, Hard & Gibbard, 1971). The oxidation product of sinapaldehyde, 2-6-dimethoxy-1,4-benzoquinone, has induced sarcomas at the site of subcutaneous injections in mice and rats (Schoental, 1973). In evaluating the incidence of 'spontaneous' tumours in a given strain of mice or rat housed for long periods on hardwood bedding, the specific type of bedding used may be of importance, particularly when solid-bottom, filter-top cages, in which volatilization products can reach high concentrations, are used.

In addition to the above considerations, contact bedding must have certain characteristics such as moisture absorbance, and must be non-edible and non-odoriferous, possess nest-building qualities, be reasonably dust free, and not contain extraneous contaminants such as pesticides that might alter an animal's response to the test chemical(s). While there are products available that fulfil these criteria, some commercial vendors' lack of a quality control programme can lead to significant batch-to-batch variation.

Macroenvironment

Animals used for long-term studies are housed for the duration of the study in a designated room. It is important to monitor critical environmental factors within the animal room, for when they vary beyond normal tolerances they may produce biochemical, behavioural and physiological effects on the animal. To assess properly the results of an experiment, records of environmental conditions should be carefully maintained (Table 1).

Engineering requirements

All animal rooms must be constructed to provide the proper control that will prevent fluctuations of environmental parameters beyond suggested tolerance (Runkle, 1964). Feed consumption and body weight changes have been observed in rats housed in rooms where inside temperature and humidity levels reflected the outdoor climatic variations; the feeding patterns during the night showed cyclical weather-dependent variations around a 10-day mean (Weihe, 1971). With a decrease in ambient temperature and humidity the rats increased their weight gain because of increased food intake; conversely, with higher temperatures and humidity, the animals consumed less feed, and this resulted in a body weight below their previous mean. Thus the irregular weight gains reflected climatic variations with concurrent change in the energy/caloric requirements of the rat (Hamilton, 1967).

Temperature and humidity

When defining temperature and humidity, the micro(cage)environment and its relationship to the macroenvironment of the animal holding room should be taken into account. Several factors will influence the ambient thermal conditions within the caging environment (cage size, cage construction

Table 1. *Environmental monitoring**

Item to be monitored	Monitoring method†	Frequency of checking	Corrective action
Temperature (space dry-bulb temperature)	7-Day chart recorder	Daily	Verify recorder accuracy and correct thermostat set-point.
Humidity (space relative humidity; wet-bulb temperature; or dew-point temperature. If wet-bulb temperature, check wick daily.)	7-Day chart recorder	Daily	Same as above.
Pressure (differential static pressure; relative to ambient pressure(s))	Incline manometer (differential pressure gauge)	Daily	Verify meter accuracy. Check for blockage of supply and return air grilles. Check for open doors; check air filter.
Air flow	Air-flow meter or pressure gauge	Daily	Same as above.
Illumination (intensity-only lighting system should be on 24-hr timer system)	Light meter	Variable	Check to see if all lamps and/or ballasts are operational. Check timers for correct light-dark cycle. Verify that manual switches do not override timer.
Contamination (particulate or gaseous)	Depends upon contaminant	Depends upon physical properties of contaminant	

*From: Long-term Holding of Laboratory Rodents. A Report of the Committee on Long-term Holding of Laboratory Rodents. Institute of Laboratory Animal Resources, Assembly of Life Sciences, National Research Council (*ILAR News*, 1976).

†Each of these variables should also be monitored through an emergency system.

material, type of bedding, use of filter tops and the animal density, as well as the temperature and humidity within the animal room itself). Another factor will be the actual placement of the animal cages within the room; for example, a rack/cage may be placed in a stagnant zone, thereby deriving very little effect from air movement. Placement of cages, air inlets and exhausts must provide proper temperature, humidity and air-velocity gradients within the room (Nevins, 1971).

The temperature and humidity selection for various laboratory animal species falls within a narrow range, called the thermoneutral zone. This zone is the range of ambient dry bulb temperature readings within which the animal is able to avoid metabolic and insulative adaption. Temperatures and relative humidity within the animal room should be regulated within a specified range, according to the requirement of the particular species of laboratory animals (Table 2).

By astute observation of the animal, one can detect subtle thermoregulation adaptive behaviour, such as increased physical activity, alteration of body position to alter the ratio of surface area to mass, huddling of the animals, increased grooming, and use of bedding to increase or decrease insulation (Bligh & Johnson, 1973). These responses may be more marked in toxicology studies where rodents are often placed singly in cages. It was ascertained that one mouse housed alone in a cage consumed nearly 30% more food than a mouse housed with four others (Prychodko, 1958). It is also important to note that the thermoregulating capacity of the animal may be compromised by ex-

Table 2. *Summary of ranges of temperature and relative humidity (thermoneutral zone)**

Species	Temperature °F	Temperature °C	Relative humidity (%)
Rat	65–73	18–23	45–55
Mouse	68–75	20–24	50–60
Guinea-pig	65–75	18–24	45–55
Rabbit	60–75	16–24	40–45
Hamster	68–75	20–24	40–55
Dog	65–75	18–24	45–55
Cat	70–75	21–24	40–45
Monkey	62–85	17–29	40–75

*Modified from Runkle (1964).

perimental dosing with drugs (Weihe, 1973) or toxic compounds, making the animal more susceptible to the ambient temperature within the animal room. The toxic response of mice and rats to some drugs has been found to be dependent upon cage temperature (Fuhrman & Fuhrman, 1961). When the drug was administered under conditions around the thermoneutral zone there was minimal toxicity, but a greater toxic response was observed whenever the temperature was either raised or lowered. The temperature and humidity can also influence the susceptibility of animals to experimentally induced infectious diseases (Baetjer, 1968), and temperature has also been shown to affect the incidence of spontaneous mammary tumours in mice (Tannenbaum & Silverstone, 1949).

Other environmental variables

Ventilation. If the ventilation system is to be adequate for modern experimental designs, it must provide uniformity of temperature, humidity and rate of air flow, with adequate air changes per hour, and remove excess odours. However, both good husbandry technique and a good ventilation system are required to keep animal odours at the appropriate level. A general, but excellent description of criteria needed for the design of an adequate air distribution and ventilation system is available (*ILAR News*, 1976). Room ventilation recommendations for common rodents are provided in Table 3. Currently recommended are 10–15 complete (100%) fresh air changes per hour (Institute of Laboratory Animal Resources—ILAR, 1974). Recirculation of room air is not advised, unless appropriate filters, scrubbers or incinerators have been added to the ventilation system. Recent advances in high-velocity mass air-displacement systems utilize air that has been recirculated through HEPA filters. The system mixes-in 10% outside air, which, coupled with 120 air changes per hour, provides adequate ventilation (Coriell & McGarrity, 1973).

In conventional one-corridor animal facilities, non-infected animals should be maintained in rooms with a slightly positive static pressure, unless infectious agents or toxic/carcinogenic chemicals are being used. Negative pressure should be maintained in rooms used for quarantine or isolation. However, in toxicological studies, consideration should also be given to having a negative pressure in rooms housing long-term animal studies where the toxic compound is incorporated in the diet (Sansone & Fox, 1977; Sansone *et al.* 1977). In multicorridor systems, air flow should always be directed from the clean access corridor through the animal room to the egress or dirty corridor (National Cancer Institute, 1976).

Table 3. *Room ventilation recommendations**

Rodent	Body weight (g)	Ventilation	
		m^3/hr/animal	ft^3/min/animal
Mouse	21	0·25	0·147
Hamster	118	0·69	0·406
Rat	250	1·38	0·815
Guinea-pig	350	1·97	1·15

*Adapted from Runkle (1964).

Lighting. Lighting in animal facilities can be an important variable in the biologic response of the test animal. All animals manifest a cyclic diurnal and seasonal change in terms of physiological and behavioural functions. Studies revealed that most biological rhythms are free, running without environmental stimuli, and the term circadian rhythm (around a day) evolved. Light plays a key role as an environmental stimulator and synchronizer of these rhythmic functions. A great number of behavioural, physiological and biochemical parameters are known to have this 24-hour periodicity. Light exerts its effect on the animal's biological response via retinal photoreceptors (Wurtman, 1975).

While controlled lighting with proper light-dark cycles (i.e. 12/12–14/10 hr) is an important consideration in animal experiments, there is no definitive information regarding the proper spectra for light to be used during a toxicological study. The current recommendation (ILAR, 1974) concerning light intensity requirements is 50–100 foot-candles at the cage level. Foot-candles are units of illumination (measured in lumens, a unit of brightness per square foot) and do not measure the light emissions in absolute irradiance units, depending largely on the yellow-green emissions of light. However, continuous 24-hour lighting at levels of 50–100 foot-candles can cause retinal photo-receptor cell degeneration in the albino and pigmented rat, becoming more pronounced in animals of increasing age (Noell & Albrecht, 1971; Weihe, 1973). Until more data are available, it would seem prudent to maintain a constant light/dark cycle (i.e. 12/12, 13/11 or 14/10 hr) throughout an experiment, with levels of illumination at the cage level within the 50–100 foot-candle range.

Noise. There is a serious lack of definitive information on the effects of noise upon the auditory capacity of laboratory animals or on whether noise may affect chronic toxicity studies. Though information on the deleterious effects of noise (including frequency, intensity, duration and time pattern of exposure) is being accumulated, the appropriate audibility level for many animals is not known (Fletcher, 1976). It is known that some inbred strains of mice respond to certain noise levels with audiogenic seizures (Fuller & Wimer, 1975). More is known regarding the non-auditory effects (stress effect) of noise. Biochemical changes due to noise are significant and every effort should be made to minimize high noise levels in animal rooms during experimental studies (Friedman, Byers & Brown, 1967). The greatest source of noise, in a well constructed and insulated animal facility, is generated by the animal technician during the feeding and cleaning operation, and by vocalizations of the animals during this period. It is important during the training of animal technicians that the harmful effects of noise on animal experiments be emphasized.

Chemicals in the animal facility

In addition to purposeful dosing of chemicals to animals during a toxicological study, the technical

staff and management must also be cognizant of the animal facility's chemical environment. Many of the chemical substances occurring in our biosphere are potentially hazardous and can enter the animal facility via the air, water supply, animal feed and bedding. In each of the general work areas within the animal facility there is potential for contamination from a variety of chemicals. The identification and safe use of chemicals during the cleaning operation or active rodenticide programme must receive primary consideration in the planning of an animal experiment. However, the unintentional introduction of chemicals into the animal facility is a more difficult problem. Whether contamination is the result of man's activities, such as lead in feeds, spilled insecticides, carcinogenic feeding studies or a natural phenomenon such as mycotoxins in feeds, the end result is the same—outright loss of animals or, more insidiously, a biased interpretation of experimental results (Newberne & Fox, 1978).

Several articles and reviews have been published on the carcinogenic and teratogenic effects of insecticides in animals and their acute toxic manifestations in man (Aldrich & Gooding, 1976; Hamilton & Hardy, 1974). Insecticides commonly used in the animal facility, particularly chlorinated hydrocarbons, are potent inducers of hepatic microsomal enzymes in rodents (Kolmodin, Azarnoff & Sjoqvist, 1969; Poland, Smith, Kuntzman, Jacobson & Conney, 1970). Several reports have demonstrated the deleterious effect of insecticides on the immune system in laboratory rodents (Keast & Coales, 1967; Wassermann, Wassermann, Gershon & Zellermayer, 1969).

The evidence now indicates that the 'innocuous' animal room deodorizers occasionally used to mask animal odours, contain volatile hydrocarbons (Cinti, Lemelin & Christian, 1976) and the disinfecting sprays which contain oils and vinyl chloride, are also capable of inducing or inhibiting the hepatic microsomal mixed-function oxidase systems in the laboratory animal (Jori, Bianchetti & Prestini, 1969; Vesell *et al.* 1976).

Diet

Unwanted variables in the diet (i.e. the presence of chemicals and naturally occurring toxins/contaminants, or variations in the concentrations of essential nutrients) can markedly influence the biological response of animals and thus alter the interpretation of experimental data. Analyses conducted on several standard commercially prepared diets for rats have shown widely variable concentrations not only of essential nutrients but also of biologically active contaminants (Newberne, 1975).

It is advisable for investigators conducting chronic animal studies to monitor pesticides, mycotoxins, trace minerals, nitrosamines and other suspect chemical contaminants in each batch of animal diet in view of the biological effects they may have on the experimental regime (Edwards, Fox, Policastro, Goff, Wolf & Fine, 1979; Fox, Aldrich & Boylen, 1976; Fox & Boylen, 1978; Lillehoj, Fennel and Kwolek, 1976; Newberne, 1975; Yang, Mueller, Grace, Golberg & Coulston, 1976). Many additional toxic or potentially detrimental health factors are known to occur naturally in plants and plant products, and these substances may be incorporated into animal feeds (Boyd & Shapleigh, 1954). These substances include toxic proteins and peptides, compounds involved in flavism (Liener, 1966), vasoactive and psycho-active substances (Hodge, Nye & Emerson, 1964; Udenfriend & Zaltman-Nirenberg, 1963), antivitamins (Somogyi, 1973), enzyme inhibitors (Feeney, Means & Bigler, 1969) and oestrogenic substances (East, 1955).

Also of practical consideration is the routine monitoring of milling dates when animal feed is received from the vendor and again prior to its use, to ensure nutritional integrity. Additionally, each container of feed should be examined, upon receipt, for damage. All faulty containers should be rejected since damaged containers may serve as a vehicle for unwanted pathogens and vermin to enter the facilities. Once feed has been dispensed from its shipping container, the remaining feed should be maintained in a closed container to prevent possible contamination by vermin.

Potentially toxic contaminants also occur in tapwater and should be considered when long-term animal studies that utilize municipal-grade water are performed. Chloroform is commonly found in sources of municipal water, according to EPA reports, as well as known or suspected carcinogens such as carbon tetrachloride, polychlorinated biphenyls, benzene, benzo-[*a*]pyrene, trichloroethylene, bis-(2-chloroethyl) ether, and diphenylhydrazine. This list only partially covers the organic contaminants detected in drinking-water; studies have identified approximately 90% of the volatile organics that are present, but these contaminants represent only 10% of the total organics (National Academy of Sciences, 1977).

Conclusion

The need for a high quality research animal is self evident to most toxicologists. However, few laboratories performing toxicological testing currently conduct in-depth disease surveillance programmes to ascertain the health status of their animals. While the programme outlined above may be extensive for some facilities, implementation of a surveillance programme tailored to specific research needs should help in reducing inter-laboratory variation in test results.

The acquisition of quality research animals does not, by itself, guarantee a successful toxicological study. Evidence of improperly executed studies are as evident today, in the scientific literature and in submissions to regulatory bodies, as they were several years ago (Roe & Tucker, 1973). However, the selection of appropriate methods for allocating test animals among treatment groups, the choice of husbandry techniques and strict disease surveillance protocols that best suit the animal model will help to preclude factors that contribute to a poorly designed and executed toxicological study.

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REVIEWS OF RECENT PUBLICATIONS

IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Vol. 18. Polychlorinated Biphenyls and Polybrominated Biphenyls. International Agency for Research on Cancer, Lyon, 1978. pp. 140. Sw.fr. 20.00 (available in the UK through HMSO).

It is an unfortunate fact that the identification of chemicals posing a hazard to man has often resulted directly from cases of accidental human poisoning. Perhaps with the new chemical registration schemes that are now being adopted by many countries, we may see a reduction in such happenings. Two groups of chemicals that featured in major incidents of accidental exposure were the polychlorinated biphenyls (PCBs) and the polybrominated biphenyls (PBBs), and as a result numerous experimental and human investigations were initiated. The available data on these compounds have now been examined by the International Agency for Research on Cancer as a basis for assessing their carcinogenic risk to man.

The preamble to the document, which accompanies all volumes in the series, provides a useful insight into the IARC's current philosophy in relation to carcinogenicity assessment, and will aid the reader's understanding of the conclusions put forward in the monographs.

PCBs, which have been considered previously in this series (Vol. 7, 1974, p. 261), first gained toxicological fame about 10 years ago following the 'Yusho' incident in Japan, when many individuals were accidentally exposed to rice oil contaminated with the compounds. Nevertheless PCB effects on health in an industrial context had given rise to concern long before that. A toxicological examination of the compounds rapidly followed the Yusho incident, which provided a considerable amount of human data. From the experimental studies and epidemiological data, the IARC has concluded that PCBs should be regarded as if they were carcinogenic to man. However, it is stressed that the role of the polychlorinated dibenzofurans, which contaminate almost all samples of PCB and which were present at unusually high levels in the samples responsible for the Yusho episode, still remains to be clarified. Continued monitoring of the Yusho survivors is recommended to promote a better understanding of the toxicology of the compounds.

PBBs first came into the limelight in 1973 following an incident of poisoning in Michigan dairy cattle fed protein-concentrate into which a commercial hexabromobiphenyl had been accidentally mixed. Human exposure to the compound followed as a result of consumption of PBB-contaminated meat, milk and eggs. Despite numerous experimental and human investigations, the toxicological status of the PBBs remains less clear than that of the PCBs and the IARC was unable to make an evaluation on the basis of the data available.

IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Vol. 19. Some Monomers, Plastics and Synthetic Elastomers, and Acrolein. International Agency for Research on Cancer, Lyon, 1979. pp. 513. Sw.fr. 60.00 (available in the UK through HMSO).

Over the past 30 years, the annual production of monomers and polymers has increased greatly, and yet there has not been a proportionate increase in the toxicological testing of these compounds. We have, however, developed a much better understanding of the potential hazards of plastics, and it can no longer be claimed that polymeric materials are biologically inert. It is recognized that the biological effects of the polymer may include those of the residual monomer, of low-molecular-weight oligomers, of adsorbed and absorbed substances, and of manufacturing contaminants.

According to the general comments within this IARC volume, a polymer may present a cancer risk to animals and man because of a carcinogen present in the material, because of a carcinogen generated by polymer degradation, as a direct result of physical effects or because of other chemicals concentrated in the polymer. For at least one representative of the first group (vinyl chloride), there is unequivocal evidence of carcinogenicity in man; for another (acrylonitrile) there is evidence of probable carcinogenicity and for a third (chloroprene) a suspicion of carcinogenicity in man has been raised. However, for the many remaining chemicals included in this latest IARC volume, there is a paucity or complete absence of human data bearing on carcinogenesis.

This volume is composed of 17 separate monographs covering 40 different monomers and polymers, including acrylics, olefines, diisocyanates, polyurethanes, polyamides and a variety of vinyl compounds and copolymers. Another five draft monographs were considered but not included because of a lack of carcinogenicity data.

IARC monographs are distinguished primarily by their well-organized tabulation of all data relevant to the question of carcinogenicity. Thus, the monograph on vinyl chloride and polymers has separate sections for the monomer and the polymers, comprising lists of "synonyms and trade names", followed by summaries of physical and chemical data, production, use, occurrence and analysis, and biological data. The latter are organized into sections on carcinogenicity studies in animals, other relevant biological data and epidemiological information. In interpreting the results of animal studies, the need for caution in extrapolating to human populations has always been a ruling principle of IARC deliberations. The demonstration of an increased incidence of malignant tumours in multiple species or strains, and/or in multiple experiments and/or to an unusual degree is considered sufficient evidence of carcinogenicity. Ad-

ditional evidence may be provided by data concerning dose-response, mutagenicity or structure.

The cumulative index to the existing 19 volumes in this series now extends over 16 pages, and has become an invaluable reference source for the toxicologist who wishes to examine the facts on available carcinogenicity studies.

Toxic Substances Sourcebook 1: The Professional's Guide to Information Sources, Key Literature, and Laws. Edited by S. S. Ross. Environmental information Center, Inc., New York, 1978. pp. 554 & microfiche appendix. $76.00

This is the first of an annual series, intended to "paint a broad but detailed picture of the use and abuse of toxic substances in the United States and throughout the world". To this end the greater part of the book is devoted to abstracts of more than 2000 articles published in the period 1974–1977 selected from diverse sources ranging from scientific journals to newspaper articles.

Although the title may imply some concern with industrial hazards, the vast majority of abstracts deal with environmental pollutants, somewhat arbitrarily classified into subsections with titles such as "chemical and biological contamination", "air pollution" and "water pollution". A mere two pages are allocated to "food and drugs", although a number of abstracts on contaminants in food appear in other subsections. An index helps the reader to track down papers on specific chemicals, but its value is diminished by a tendency to arrangement by class of compound, so that a paper on 1,1,1-trichloroethane is indexed under "chlorinated hydrocarbons" and another on chlorinated ethylenes appears under "ethylene".

The remainder of the book includes a detailed outline of the Toxic Substances Control Act, and a brief discussion of the value of the Ames test. Useful lists are provided of books, films, NIOSH publications, periodicals, US data bases and 1975–1978 conferences concerned with toxic chemicals. The NIOSH recommendations for occupational health standards are conveniently summarized in tabular form and are followed by the ACGIH list of threshold limit values. A synopsis of major federal environmental bills for 1977 may be of little interest outside the USA, although this is less likely to be true of four major 1977–1978 federal actions (the FDA proposal to ban saccharin, the CPSC ban on TRIS, the OSHA emergency standard for 1,2-dibromo-3-chloropropane and the FDA ban on acrylonitrile bottles), all of which are reprinted in full from the Federal Register with their accompanying preambles. The microfiche appendix includes the full text of major toxic substances laws and regulations now in force.

The book is likely to be of most value to those concerned with the environmental impact of chemicals, for whom it will provide some useful leads into the literature. The details of NIOSH publications should also aid those whose task it is to evaluate hazards in the workplace. As the editor himself acknowledges, it should be considered a beginning rather than an end for most users, and this modest objective may be regarded as having been fulfilled.

Carcinogenesis and Mutagenesis: Predictive Tools in Application to Safety Evaluation/The Mutagenic Properties of Chemicals/Report of the Subcommittee on Inhalation Toxicology. Edited by M. A. Mehlman, M. F. Cranmer and R. E. Shapiro. Pathotox Publishers, Inc., Park Forest South, IL, 1977. pp. x + 388. $18.50.

This book is made up of three separate items, each published previously during 1977 in the Journal of Environmental Pathology and Toxicology. The first two parts—one the proceedings of a conference and the other the report of a DHEW committee—are concerned with tests for carcinogenicity and/or mutagenicity, while the third is another DHEW committee report enumerating the facilities available in the USA for the study of inhalation toxicology, including those currently suitable or adaptable for work with possible carcinogens.

Most toxicological tests are designed to provide information on the biological activity of a particular compound to assist in predictions of its likely effect on man. These effects are usually classified conventionally as toxic, mutagenic, carcinogenic and teratogenic. The number of conventional toxicological tests carried out has increased steadily during the past decade and many new tests have been added to the range, particularly in the realm of short-term tests for mutagenicity and carcinogenicity. At the same time, the influence of other disciplines, particularly statistics, epidemiology and metabolic studies, has increased.

A critical review of the methodology involved in such tests and a retrospective assessment of their predictive value, particularly in the fields of mutagenicity and carcinogenicity, is timely, not only because of the medical importance of these effects but also because of their emotional overtones.

In general, the book's contributors address themselves to the task of evaluating the methodology of the test, but a few attempt to assess the predictive value—and draw some interesting conclusions. The epidemiologists accept the limitations of the current methods of epidemiological investigation and recognize that cancer statistics by themselves cannot predict whether a substance about to be introduced into the environment for the first time is likely to prove carcinogenic. What we are not told is whether epidemiologists consider that positive results from laboratory animals give sufficient lead for an epidemiological study on a substance that has long been in the environment.

Reviews of mutagenicity testing cover numerous tests ranging from those involving bacteria and yeast cells to studies in mammalian cells. The scientific basis for the performance of each test and for recognizing a positive or negative result is given in detail. Some attempt is made to assess the predictive value of each test, and it is refreshing to note that this task is approached with commendable caution. However, no comparison is made between the merits of the different tests, an exercise that could have been of great benefit to the harassed toxicologist. Furthermore, many readers would appreciate some enlightenment about which tests require a minimum of expertise and which demand a high degree of skill and sophisticated

equipment, or some indication of those that can be carried out rapidly and those that involve laborious techniques. Perhaps in some future work, these points may be given more attention.

The sections dealing with metabolism and pharmacokinetics make interesting reading. The authors provide a good insight into the methodology used and support their points with ample experimental data. None really grasp the nettle, however, so that at the end of many erudite chapters the reader is still in doubt as to whether such investigations are of much assistance in the conduct of toxicity studies in the same, let alone in different, species. The participating statisticians go one better, leaving a poor biologist bewildered by a mass of mathematical formulae, on which he is not qualified to comment. It does seem, however, that at the end of these communications there is no indication that all this mathematical treatment can predict with any degree of assurance the effect of small levels of carcinogens in man or even in the species under test.

The most disappointing section is the one dealing with chronic toxicity studies. The contributions are full of pious admonitions about what to do, what to look for and what to avoid in conducting long-term toxicity tests so that guidelines are satisfied, but there is little hint of how useful the results of well-conducted tests in animals may be in predicting the hazard to man. Do we assume that the results can be directly extrapolated from the rat (or mouse) to man? Or are we in danger of losing sight of the objective of these expensive tests?

Suspected Carcinogens. A Sourcebook of the Toxic Effects of Chemical Substances. Edited by E. J. Fairchild. Castle House Publications Ltd., Tunbridge Wells, 1978, pp. xxxi + 253. £20.00.

The zodiacal crab, Cancer, lies resplendent within an orange sphere on the front cover of this book. Perhaps its presence is meant to emphasize the dangerous nature of the chemical substances listed within. However, examination of the listed compounds and of the book's introduction reveals that it may be of doubtful significance.

Although published data suggesting that certain substances have caused carcinogenic or neoplastic effects are listed, the book does not attempt to evaluate the adequacy of these data or examine other studies that have produced contradictory results. It is for this reason that compounds such as sodium chloride appear. Nevertheless the book does serve as a useful introduction to the literature, although it is far from comprehensive. In addition, while not necessarily indicting a substance as a human carcinogen, it gives an indication of materials that may require further research and evaluation.

Unfortunately the book is somewhat out of date. An identical list was published by the US Department of Health, Education, and Welfare in December 1976 as a subfile of the NIOSH Registry of Toxic Effects of Chemical Substances. The suspected carcinogen list has therefore been in circulation for some time, although its renewed publication by a UK publishing house may make it more generally available in this country. It is doubtful, however, whether the rather high cost truly reflects the value of the contents.

BOOKS RECEIVED FOR REVIEW

Environmental Health Criteria 8. Sulfur Oxides and Suspended Particulate Matter. Published under the joint sponsorship of the United Nations Environment Programme and the World Health Organization. WHO, Geneva, 1979. pp. 107. Sw.fr. 10.00 (available in the UK through HMSO).

Pesticide Residues in Food—1977. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues and Environment and the WHO Expert Committee on Pesticide Residues, Geneva 6–15 December 1977. FAO Plant Production and Protection Paper 10 Rev. FAO, Rome, 1978. pp. vii + 81.

Pesticide Residues in Food: 1977 Evaluations. The Monographs—Data and Recommendations of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues and the Environment and the WHO Expert Group on Pesticide Residues, Geneva 6–15 December 1977. FAO Plant Production and Protection Paper 10 Sup. FAO, Rome, 1978. pp. ix + 459.

Pesticide Residues in Food—1978. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues and Environment and the WHO Expert Group on Pesticide Residues, Rome 27 November–December 1977. FAO Plant Production and Protection Paper 15. FAO, Rome, 1979. pp. vii + 42.

Environmental Health Criteria 7, Photochemical Oxidants. Published under the joint sponsorship of the United Nations Environment Programme and the World Health Organization. WHO, Geneva, 1979. pp. 110. Sw.fr. 10.00 (available in the UK through HMSO).

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Information Section

ARTICLES OF GENERAL INTEREST

VINYL CHLORIDE—PART 3: MACROMOLECULAR BINDING

Chloroethylene oxide and chloroacetaldehyde are probably the principal metabolites of vinyl chloride (*Cited in F.C.T.* 1979, **17**, 403). Evidence of the binding of these metabolites to cellular macromolecules, particularly the alkylation of the nucleic acids, may explain the mutagenicity of vinyl chloride (*ibid* 1979, **17**, 542).

In vivo *studies*

The irreversible binding of VC metabolites to nucleic acid and protein has been demonstrated *in vivo* (Bolt *et al.* in IARC Scientific Publn no. 13, IARC, Lyon, 1976, p. 151). In rats exposed to atmospheres containing about 140 ppm [^{14}C]VC, roughly half of the radioactivity found in the liver microsomes immediately after the 5-hr exposure was irreversibly bound to protein, although the major proportion of labelled material in the liver cells was present in the cytosol. Immediately after exposure to 44 ppm [^{14}C]VC, from 10 to 40% of the total activity in the tissues was irreversibly bound, while at 48 hr the proportion of bound activity had increased to 70%, although the amount of irreversibly bound VC metabolites stayed the same. Radioactivity was also found in the liver nucleic acids. The peak incorporation of activity into DNA (0·23% total liver activity) occurred immediately after exposure, whereas the specific labelling of the RNA (0·8% of initial activity of the liver) was at a maximum after 24 hr and decreased more slowly. Bolt *et al.* (*loc. cit.*) calculated that as no more than 1% of the labelled VC would enter the one-carbon pool to be incorporated into protein and nucleic acid, this mechanism could not explain the total high level of binding observed in the study. It was possible that the one-carbon pool could account for the amounts of label found in the nucleic acid of the liver. However, the authors thought this unlikely since the speed by which this activity decreased was faster than normal DNA turnover and the rate of incorporation of label was very different in RNA and DNA derived from the same cellular nucleotide pool.

In further studies at the same institute (Laib & Bolt, *Arch. Tox.* 1978, **39**, 235), liver RNA isolated from rats exposed to VC atmospheres was shown to contain labelled 1,N^6-ethenoadenosine and 3,N^4-ethenocytidine, the alkylation products of adenine and cytosine by chloroethylene oxide and chloroacetaldehyde, as well as the physiological bases. The time-course of dealkylation was found to differ in the two bases. Ninety-two hours after exposure, the level of labelled ethenoadenosine was only one-fifth of its original value; ethenocytidine proved more resistant to repair and the levels of radioactivity associated with this component remained constant over the same period—possibly an indication of the relative importance of cytidine alkylation.

Watanabe *et al.* (*Toxic. appl. Pharmac.* 1978, **44**, 571) exposed Sprague-Dawley rats to 1–5000 ppm ^{14}C-labelled VC for 6 hr. The amount of radioactivity bound to macromolecules in the liver did not increase in proportion to the increase in exposure concentrations. Covalent binding to hepatic macromolecules was related to the amount of VC metabolized. There was no detectable binding of ^{14}C to either DNA or RNA in the liver, in contrast to the results of Bolt *et al.* (*loc. cit.*). The authors thought this difference in results might be explained by the higher specific activity used by Bolt *et al.* They also offered the suggestion that the incorporation observed in the earlier work might have originated from the one-carbon pool, but this would not explain the alkylated bases isolated by Laib & Bolt (*loc. cit.*). Watanabe *et al.* (*loc. cit.*) concluded that covalent binding to hepatic nucleic acids occurred to only a limited degree under their experimental conditions but they pointed out that this did not exclude the possibility of other interactions which might result in the loss of the ability to control replication. Hepatic glutathione content was depressed at exposure concentrations above 100 ppm, and according to these authors reactive metabolites of VC may be detoxified by glutathione, suggesting that the carcinogenicity of VC may be related to a decreased ability to detoxify reactive metabolites.

The *in vivo* binding of VC metabolites to DNA has also been investigated in mice (Osterman-Golkar *et al. Biochem. biophys. Res. Commun.* 1977, **76**, 259). BALB mice took up radioactivity about twice as fast as the CBA and ATL strains. Levels of *S*-(2-hydroxyethyl)cysteine, N^1- and N^3-hydroxyethylhistidine or N^7-hydroxyethylguanine were measured in the hydrolysates of proteins and nucleic acids taken from mice exposed to 98–302 ppm; these compounds were formed from the chemical (sodium borohydride) reduction of 2-oxoethyl groups introduced into the cell macromolecules as a result of the VC treatment. The histidine and guanine derivatives and small amounts of hydroxyethylcysteine were detected in hydrolysates of haemoglobin and liver DNA. The authors calculated that the absolute and relative amounts of alkylated products supported the hypothesis that the main reactive metabolite of VC is chloroethylene oxide. In the BALB mice, the two hydroxyethylhistidines could be detected in small amounts in the protein from the testes.

In vitro *studies*

The binding of the metabolites of VC to cellular macromolecules has also been studied by incubating rat-liver microsomes with NADPH, polyadenosine and [1,2-^{14}C]VC (Laib & Bolt, *Toxicology* 1977, **8**, 185). The radioactivity identified in the enzymic hydrolysates of the polyadenosine was irreversibly attached to 1,N^6-ethenoadenosine. An analogous reaction occurred when polycytidylic acid was incu-

bated with VC and the microsomal system, labelled 3,N^4-ethenocytidine moieties being formed (Laib & Bolt 1978, *loc. cit.*).

The reaction of chloroacetaldehyde with calf-thymus DNA at pH 4·5 gave a modified DNA product (Green & Hathway, *Chemico-Biol. Interactions* 1978, **22**, 211). Enzyme hydrolysis of the DNA produced a mixture of naturally occurring deoxyribonucleosides and chloroacetaldehyde reaction products, predominantly ethenodeoxycytidine and ethenodeoxyadenosine. The same products resulted from hydrolysis of liver DNA prepared from rats that had been exposed to 250 ppm VC in drinking-water for 2 yr.

According to the studies of Kappus *et al.* (*Toxic. appl. Pharmac.* 1976, **37**, 461), the alkylation of protein and uptake of VC by rat-liver microsomes is dependent on concentration, incubation time, enzymatic activity, NADPH and oxygen, and is almost completely blocked by CO. These authors found that only about 1% of the VC taken up by the microsomes became irreversibly bound, a finding that contrasts with the corresponding figure of around 50% observed in the *in vivo* studies of Bolt *et al.* (*loc cit.*). Glutathione added to the microsomal incubation mixture decreased the level of irreversible protein binding. When trichloropropene oxide, an inhibitor of epoxide hydrase, was present, the irreversible protein binding increased two-fold, even though VC uptake by the microsomes was unaffected.

Other *in vitro* studies have produced evidence of chloroethylene oxide's participation in the metabolism of VC. The reaction product of chloroacetaldehyde or chloroethylene oxide with adenosine was tentatively characterized by Barbin *et al.* (*Biochem. biophys. Res. Commun.* 1975, **67**, 596) as 3,β-ribofuranosylimidazo-(2,1-*i*)-purine (1,N^6-ethenoadenosine); a product with the same R_F value and elution characteristics on a Sephadex column was formed when VC was incubated with adenosine in the presence of a microsomal fraction. Barbin *et al.* (*loc. cit.*) passed a mixture of VC and air or oxygen into a medium containing both liver microsomes from a phenobarbitone-treated mouse and an NADPH-generating system. The volatile metabolite trapped by reaction with 4-(4-nitrobenzyl)pyridine (4-NBP) in ethylene glycol had a UV absorption spectrum identical to the product formed from the reaction of chloroethylene oxide (but not 2-chloroacetaldehyde) with 4-NBP.

The evidence from these studies strongly supports the view that chloroethylene oxide is the major reactive VC metabolite. While it is clear that metabolites of VC do bind to cellular macromolecules in rat and mouse liver, the extent of nucleic acid binding has not been clearly resolved.

[J. Hopkins—BIBRA]

BLACK MARKS FOR MBK

The industrial solvent methyl *n*-butyl ketone (MBK) has been implicated in cases of peripheral neuropathy among spray-painters and other workers (*Cited in F.C.T.* 1977, **15**, 159) and has been shown to induce axonal degeneration of the central nervous system and polyneuropathy in experimental animals (*ibid* 1977, **15**, 492).

The metabolic fate and disposition of MBK in the rat has been studied by DiVincenzo *et al.* (*Toxic. appl. Pharmac.* 1977, **41**, 547) in male rats dosed by gavage with [1-^{14}C]MBK at 20 or 200 mg/kg. Absorption was rapid, and activity was eliminated in the breath and urine, mostly within 2 days. Unchanged MBK in the expired air amounted to 6·2% of the dose after 200 mg/kg and to 2·2% after 20 mg/kg. Total activity in the breath represented about 44% of either dose, with unchanged MBK and CO_2 as the only labelled compounds. Excretion of ^{14}C in the urine amounted to 35% after 20 mg/kg and to 40% after 200 mg/kg; faecal excretion of ^{14}C was less than 1·5% of the dose. About 15% of radioactivity remained in the carcass after 48 hr and 8% after 6 days. It was widely distributed throughout the tissues with highest concentrations in the blood and liver. The elimination time for MBK in the serum was about 6 hr, and the serum metabolites were 2-hexanol, 5-hydroxy-2-hexanone and 2,5-hexanedione. These three metabolites were also detected in the urine, together with 2,5-dimethylfuran, norleucine, γ-valerolactone and urea. 2-Hexanol was probably eliminated in the form of both the sulphate ester and the glucuronide.

These findings identify the principal metabolic pathways as reduction of the ketone group or oxidation at the α or ω-1 carbon, followed apparently by decarboxylation of the metabolites with an α-keto acid component, the latter stage being the probable source of most of the respiratory $^{14}CO_2$. The α-keto acid intermediates may also undergo transamination to amino acids. Pretreatment of rats with unlabelled MBK or with phenobarbital did not materially alter the metabolism of [1-^{14}C]MBK, but inhibition of the microsomal mixed-function oxidase system by pretreatment with SKF 525A increased $^{14}CO_2$ excretion from 37·6 to 49·6%, after an initial 4-hr decrease, and decreased urinary activity from 39·9 to 22·5%, indicating the involvement of this microsomal-enzyme system in the ω-1 oxidation of MBK

DiVincenzo *et al.* (*ibid* 1978, **44**, 593) continued their studies with an investigation of the respiratory uptake and percutaneous absorption of MBK in dogs and man. Male beagles exposed to 50 or 100 ppm MBK vapour for 6 hr had average breath concentrations of 16 and 35 ppm MBK, respectively, indicating an absorption rate of 65–68%. On cessation of exposure, the concentration of MBK in the breath fell rapidly and was below the level of detection after 3–5 hr. In male volunteers exposed to 10 or 50 ppm MBK for 7·5 hr or to 100 ppm MBK for 4 hr, mean

concentrations of MBK in the breath were 1·4 and 9·3 ppm, respectively, for the two lower concentrations, with a rapid fall after cessation of exposure, and 22 ppm for the highest exposure. In no instance was MBK detected in the expired air 3 hr after cessation of exposure. Absorption was calculated to be 75–92% of the dose. Only after inhalation of 100 ppm MBK was the ketone detectable in serum. No MBK metabolites were detected in expired air or in urine, but 2,5-hexanedione appeared in the serum after exposure to 50 or 100 ppm MBK, although not during the period of exposure.

Skin absorption was determined in pairs of volunteers exposed to [1-^{14}C]MBK or to a 9:1 (v/v) mixture of methyl ethyl ketone and [1-^{14}C]MBK for 60 min (DiVincenzo *et al. loc. cit.*). Individual rates of absorption were 4·8 and 8·0 μg/min/cm^2 for MBK and 4·2 and 5·6 μg/min/cm^2 for the mixture. After administration of an oral dose of 0·1 mg MBK/kg to two volunteers, respiratory excretion was 49·9 and 29·0% and urinary excretion was 27·6 and 25·0%, giving 8-day recoveries of 77·5 and 54·0%. Although most of the respiratory and urinary excretion occurred in the first 24 and 48 hr, respectively, some radioactivity was present in the expired air over the following 3–5 days and in the urine up to day 8. Thus excretion of MBK in man after percutaneous oral exposure seems to be relatively slow, so that repeated daily exposure to high concentrations of the solvent may result in prolonged exposure to its neurotoxic metabolites.

The effects of MBK on nervous system function and behaviour in monkeys and rats have been reported by Johnson *et al.* (*Am ind. Hyg. Ass. J.* 1977, **38,** 567), who exposed both species to 100 or 1000 ppm MBK for 6 hr daily on 5 days/wk. The animals were given neurological tests on a consistent monthly schedule, in which exposure on at least three consecutive days and then a 16-hr time lapse preceded each test period. After 25 wk, animals exposed to 1000 ppm MBK were removed from further exposure, because neurological and clinical observations indicated hind-limb neuropathy. Those exposed to 100 ppm MBK continued for longer periods, the monkeys for 41 wk and the rats for 29 wk.

After exposure to 1000 ppm MBK, motor conduction velocities in ulnar and sciatic-tibial nerves were reduced in both animal species, and the amplitude of evoked muscle potentials decreased. The latency of specific components of evoked visual potentials was lengthened in monkeys, while operant behavioural performance in a bar-pressing test was impaired in rats. Both species showed a reduction in body weight. After exposure to 100 ppm MBK there were less marked reductions in nerve conduction velocity and, in rats, also in evoked muscle action potential. At this level of exposure, it took 9 months to induce neuropathy in monkeys. Nerve conduction velocity returned to pre-exposure levels 6 months after cessation of exposure to 1000 ppm MBK and 2 months after 100 ppm MBK.

Anger & Lynch (*Envir. Res.* 1977, **14,** 204) tested rats daily (in a 1-hr test period) on a multiple fixed-ration fixed-interval 3-min reinforcement schedule. The rats were given MBK in an oral dose of 68, 135, 270 or 406 mg/kg on one day each week, control saline on three other days, and no treatment at all on the fifth day. Each animal received each concentration on three to six occasions in the course of the 6-month administration period. The performance response rate after MBK was reduced by 20, 30, 40 and 57%, respectively, by these doses, but there were considerable individual differences. At the highest dose, MBK induced sluggishness of movement and unsteadiness in some animals, but no permanent weakness or foot drop was observed throughout the study.

The effects of MBK (at levels of 0·1 and 0·25%) and two of its metabolites, 2-hexanol (0·1 and 0·5%) and 2,5-hexanedione (0·1%) administered to guinea-pigs in drinking-water for 24 wk were examined by Abdel-Rahman *et al.* (*Am. ind. Hyg. Ass. J.* 1978, **39,** 94). A group given 0·5% 2,5-hexanedione all died by wk 8, so this level of exposure was not studied further. All three compounds decreased pupillary response throughout the first 5 wk and greatly impaired the response by wk 24. Body weight increased and locomotor activity decreased. It was demonstrated that 2-hexanol was excreted in guinea-pig urine as MBK, 2-hexanol and 2,5-hexanedione, while the dione was excreted unchanged. The plasma of animals drinking 2-hexanol contained MBK and the dione. Severe polyneuropathy and mortality occurred in the group drinking 0·5% 2,5-hexanedione for a short period. The effect of 2-hexanol on pupillary response may have been due to its conversion to MBK and 2,5-hexanedione, and it is suggested that the observation of pupillary change may serve as an index in monitoring the degree of exposure of industrial workers to MBK.

[P. Cooper—BIBRA]

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TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS

AGRICULTURAL CHEMICALS

Dieldrin and the monkey

Wright, A. S., Donninger, C., Greenland, R. D., Stemmer, K. L. & Zavon, M. R. (1978). The effects of prolonged ingestion of dieldrin on the livers of male rhesus monkeys. *Ecotoxic. envir. Safety* **1**, 477.

Chlorinated hydrocarbon pesticides persist in the environment, enter food chains and are taken up into human and animal tissues. The effect of uptake of dieldrin by monkeys was investigated in the study cited above.

Groups of five male rhesus monkeys were fed technical-grade dieldrin (88·4% pure) for about 6 yr. Initially the dietary levels of dieldrin administered were 0·01, 0·05, 0·1, 0·5, 1 and 5 ppm. However, two of the animals given the highest level died within 6 months of the start of the experiment and, as at least one of these deaths was probably caused by dieldrin intoxication, the dietary content was lowered to 2·5 ppm 4 months after the start of the experiment and to 1·75 ppm 5 months later. Subsequently the dieldrin level for one of the three animals remaining in this group was gradually increased again until, 23 months after the start of the experiment, it was 5 ppm. Therefore, at the end of the experiment only one animal was receiving 5 ppm dieldrin and only two were being given 1·75 ppm. The animals were killed over a 6-month period beginning at month 69.

No effects of dieldrin on the macroscopic appearance, weight or DNA and RNA contents of the livers were detected, but there were dose-dependent increases in the dieldrin concentrations of the livers. In assays of liver enzymes no significant differences were found in the activities or intracellular distributions of glucose-6-phosphatase, succinic dehydrogenase or alkaline phosphatase after dieldrin treatment. However, tests on several substrates demonstrated increases in the activity of enzymes of the microsomal monooxygenase system obtained from the livers of animals dosed with 1·75 or 5 ppm dieldrin. These increases corresponded to increases in the cytochrome *P*-450 content of the microsomal fractions. The dietary-dieldrin threshold for liver microsomal monooxygenase activity was about 1 ppm. This is equivalent to a dietary intake of 25–30 μg/kg body weight/day—many times greater than the exposure level of the general human population—and was reflected in hepatic concentrations of 6–7 ppm dieldrin. In rats and mice dosed with similar dieldrin levels, relatively less dieldrin accumulates in the liver but more microsomal-enzyme induction occurs (Wright *et al. Fd Cosmet. Toxicol.* 1972, **10**, 311).

The authors consider that the results of this study, and of others concerning dieldrin metabolism in the human liver (*Cited in F.C.T.* 1972, **10**, 95), indicate that primate livers are not very sensitive to dieldrin and metabolize this pesticide only slowly. In these respects they differ from the livers of most of the mammals that have been studied.

THE CHEMICAL ENVIRONMENT

How does carbon disulphide impair glucose tolerance?

Kujalová, V., Lukás, E., Šperlingová, I. & Frantík, E. (1979). Glucose tolerance and occupational exposure to carbon disulphide. *Lancet* **I**, 664.

Exposure to carbon disulphide (CS_2) has been linked with a number of toxic effects, including diabetes and polyneuropathy (*Cited in F.C.T.* 1978, **16**, 495). The evidence with regard to diabetes has recently been strengthened by a report of decreased glucose tolerance in 73% of 66 Italian CS_2-exposed workers but in only 17% of the same number of controls (Franco *et al. Lancet* 1978, **II**, 1208). It is now suggested that both the diabetes and the polyneuropathy may share a common biochemical origin.

Of 34 workers exposed to CS_2 for more than 10 yr at concentrations of 20–30 ppm, 59% (after exclusion of two subjects with manifest diabetes) gave abnormal results in triamcinolone oral glucose tolerance tests. The curve of immunoreactive insulin showed a shift to the right with increased peak values, a pattern resembling that seen in diabetes, but lipid metabolism was unaffected. The authors report that similar effects on glucose tolerance can be produced in monkeys and rats, but they do not specify the conditions of CS_2 exposure.

Men and laboratory animals with CS_2-induced polyneuropathy were previously found to excrete abnormally high levels of xanthurenic acid, due to disorders in tryptophan degradation following a change in pyridoxine metabolism (Kujalová *et al. Medna Lav.* 1970, **61**, 227; Lukás, *ibid* 1970, **61**, 302) and a xanthurenic acid–insulin complex has been found in diabetics (Kotake & Murakami, *Am. J. clin. Nutr.* 1971, **24**, 826). This complex has now been isolated from the serum of CS_2-exposed monkeys and workers. As no direct effect of CS_2 on insulin could be demonstrated, it is suggested that CS_2 affects insulin activity indirectly via impaired degradation of tryptophan.

Ethylene oxide and leukaemia

Hogstedt, C., Malmqvist, N. & Wadman, B. (1979). Leukemia in workers exposed to ethylene oxide. *J. Am. med. Ass.* **241**, 1132.

Ethylene oxide (EO) is widely used as an intermediate in the production of ethylene glycol, in the manufacture of surface-active agents, as a fungicidal fumigant and for sterilizing foodstuffs and hospital equipment. Recently, however, concern about the safety of the compound has increased. Following demonstrations of general point (gene) mutations in lower organisms and reports of chromosomal mutations not only in rats but also in humans accidentally exposed to high concentrations of EO (*Federal Register* 1978, **43**, 3800), the compound now qualifies as a suspected carcinogen. Further indication of EO's possible carcinogenicity comes with a report of three cases of leukaemia that occurred between 1972 and 1977 in one male and two female workers in a Swedish factory where 50% EO and 50% methyl formate had been used since 1968 for the sterilization of hospital equipment.

The women, who worked in a hall in which boxes of sterilized equipment were stored, were exposed to EO leaking from the boxes. The third case was the manager of the plant, whose estimated exposure to EO was 3 hr/wk. Seventy individuals had been employed at some time between 1968 and 1977 in the storage hall and another 160 had been employed in the neighbouring rooms or as sterilizing operators. Without considering any latency time or minimum exposure, the expected leukaemia incidence among these workers would have been 0·2, in contrast to the three cases actually observed. Exposure measurements were made in 1977 and the 8-hr time-weighted average concentration in the breathing zone of the women working in the storage hall was calculated as 20 ± 10 ppm.

The male victim had had occasional contact with benzene, but the women had not been exposed to any known leukaemia-inducing agents. While no firm conclusions can be drawn from this limited study, the possible implications are clear and the authors stress the urgent need for epidemiological studies of larger populations exposed to EO. A further difficulty with the current findings is the possibility of some combined effect of EO and methyl formate. Nevertheless, partly on the basis of this report, the Swedish TLV for EO has been lowered to 10 ppm as an 8-hr time-weighted average concentration limit.

[According to statements in the American press (*Chemical Week* 26 July 1978), an industry-wide investigation into the effects of EO has been undertaken at the Carnegie-Mellon Institute of Research, under the co-sponsorship of Union Carbide Corp.]

Renal significance of hexachlorobutadiene

Duprat, P. & Gradiski, D. (1978). Percutaneous toxicity of hexachlorobutadiene. *Acta pharmac. tox.* **43**, 346.

Rats fed hexachlorobutadiene (HCBD) at 20 mg/kg/day for up to 2 yr showed a reduction in weight gain and low erythrocyte counts and developed renal tubular adenocarcinomas (*Cited in F.C.T.* 1979, **17**, 97). Damage to the kidney tubular epithelium has also been reported in rats inhaling HCBD at an atmospheric concentration of 25 or 100 ppm for 6 hr/day for 12 or 15 days (Gage, *Br. J. ind. Med.* 1970, **27**, 1). A study of the percutaneous toxicity of this solvent has now been reported.

Pure HCBD in a dose of 0·25, 0·50, 0·75 or 1·00 ml/kg was applied directly to the skin of female rabbits for 8 hr by means of a glass vial that enabled the residue of the dose to be measured at the end of the application. In this study, however, all the amounts of HCBD applied were completely absorbed. No deaths occurred during the exposure period, but during or 1–2 hr after exposure, there was moderate central nervous system depression, indicated mainly by stupor. A few animals exposed to the two largest doses were subsequently dyspnoeic and cyanosed, and these died within 24 hr from respiratory or cardiac failure. Others died 2–9 days after exposure, having developed weakness and anorexia. None of the animals treated with 0·25 ml HCBD/kg died. The calculated percutaneous LD_{50} was 0·72 ml/kg.

After direct exposure to HCBD the skin was haemorrhagic and necrotic to a degree that increased with time, but the damage was repaired within 2 wk. Animals dying within 24 hr of exposure showed lung congestion and haemorrhage, and congestion in the liver and kidney. On days 3–9 the skin lesions showed eschar formation, the liver showed fatty degeneration, and there was pulpy softening in the renal cortex. By wk 2–5 there was slight fibrosis in the kidney, but no liver abnormality remained. The reversible lesions induced by percutaneous absorption of HCBD were thus a progressive fatty degeneration of the liver and necrotizing nephritis. The renal effects seem to be the most important element, and call not only for the establishment of a low TLV in the environment of those working with HCBD but for the careful observation of precautionary measures to prevent skin contact.

[The ACGIH has identified hexachlorobutadiene as having suspected carcinogenic potential for man, but no TLV was given in the 1978 list; the 1977 data supplement proposed a TLV of 0·02 ppm.]

Hexane in the eye

Raitta, C., Seppäläinen, A.-M. & Huuskonen, M. S. (1978). N-hexane maculopathy in industrial workers. *Albrecht v. Graefes Arch. klin. exp. Ophthal.* **209**, 99.

The neurotoxicity of *n*-hexane has been evident in workers in various industries for some time (*Cited in F.C.T.* 1973, **11**, 157), and polyneuropathy has been identified in shoe and leather workers in Italy (*ibid.* 1979, **17**, 313). There have been few reports of impaired vision in exposed workers, although Yamamura (*Folia psychiat. neurol. jap.* 1969, **23**, 45) noted blurred vision as an early symptom of *n*-hexane polyneuropathy and identified optic atrophy or retrobulbar neuritis in a few of the workers he examined. Now the authors cited above have described the solvent's retinal toxicity, apparently for the first time.

Ophthalmological examinations (including ocular

structure and colour discrimination tests) were carried out on eleven male and four female workers, whose ages ranged from 30 to 65 yr and who had been exposed to *n*-hexane for more than 5 yr (5–21 yr). Eight of the workers were from factories manufacturing adhesive bandages, where the *n*-hexane concentration was generally below 500 ppm, the Finnish TLV. [The US/UK TLV (time-weighted average) is 100 ppm.] The other seven workers were exposed during vegetable-oil extraction to concentrations ranging generally from 10 to 50 ppm, although higher levels (2000–3000 ppm) could be measured during disturbances arising mainly during the stopping and starting of the processes.

In all but two of the workers examined the corrected visual acuity for 5 m and 40 cm was normal. One of these exceptions remains unexplained; the other person had no sight in his right eye, because of posterior uveitis and secondary glaucoma, and his left eye had been amblyopic since childhood. There were no significant findings in relation to visual fields or intraocular pressure, but defective colour discrimination was detected in 12 of the 15 subjects by means of the Farnsworth Panel D-15 test and the Farnsworth 100 HUE test and opthalmoscopy revealed delicate macular changes in 11 of the group. These changes were described as slight pigment dystrophy, yellowish dots and, in some cases, absence of the foveal reflex, and in addition the macula was distinctly demarcated from the surrounding retina and appeared dry, with an orange-like appearance. Fluorescein angiography was used to record the macular pigment changes. No optic atrophy was detected in this study.

The authors felt that a toxic effect acting directly on the cone pigments of the receptors was the explanation for the macular changes; as the receptor pigments are surrounded by lipids and bound to lipid-soluble vitamin-A aldehyde, it seemed possible that maculopathy was a result of damage to receptor lipids. It was also suggested that the very high concentrations of *n*-hexane encountered spasmodically could have been the major cause of this lesion.

More styrene metabolites identified

Seutter-Berlage, F., Delbressine, L. P. C., Smeets, F. L. M. & Ketelaars, H. C. J. (1978). Identification of three sulphur-containing urinary metabolites of styrene in the rat. *Xenobiotica* **8**, 413.

The three principal end-products of styrene metabolism are mandelic, phenylglyoxylic and hippuric acids (*Cited in F.C.T.* 1975, **13**, 155). The first stage, however, is the formation of styrene oxide, which is subsequently hydrolysed to phenylethylene glycol (*ibid* 1971, **9**, 303) or conjugated with glutathione and metabolized to the mercapturic acid *N*-acetyl-*S*-(2-phenyl-2-hydroxyethyl)cysteine (James & White, *Biochem. J.* 1967, **104**, 914). Two other mercapturic acids have now also been identified as urinary metabolites of styrene in the rat.

Adult female rats were given 250 mg styrene/kg in sesame oil by ip injection daily, except at weekends, for 3 wk, and the urine was collected daily and analysed by thin-layer chromatography and by infra-red, mass and nuclear magnetic resonance spectrometry. The methyl esters of *N*-acetyl-*S*-(1-phenyl-2-hydroxyethyl)cysteine, *N*-acetyl-*S*-(2-phenyl-2-hydroxyethyl)-cysteine and *N*-acetyl-*S*-phenacylcysteine were isolated in the molar ratio 65:34:1. Each of the first two substances occurred as two diastereoisomers, as a consequence of the introduction of a new asymmetric carbon atom. After a single dose of styrene, the total excretion of mercapturic acids in the urine amounted to 10·7% of the dose, of which all but 0·26% was eliminated in the first 24 hr. These results indicate that styrene oxide is attacked at both the α- and β-positions.

NATURAL PRODUCTS

Nitrosated spermidine and gastric cancer

Kokatnur, M. G., Murray, M. L. & Correa, P. (1978). Mutagenic properties of nitrosated spermidine. *Proc. Soc. exp. Biol. Med.* **158**, 85.

A high gastric pH is associated with an increased risk of gastric cancer, possibly because it favours the conversion of nitrates to nitrites and hence to nitrosamines (*Cited in F.C.T.* 1978, **16**, 627). However, most nitrosamines formed in this manner require metabolic activation by liver microsomes (Ames *et al. Mutation Res.* 1975, **31**, 347) and animals fed nitrite and amines frequently develop tumours in organs other than the stomach (Lijinsky & Taylor, *Fd Cosmet. Toxicol.* 1977, **15**, 269). A nitrosamine that is a direct-acting mutagen would be more likely to produce stomach cancer, and one possible candidate has now been identified.

Spermidine occurs in the grain-and-pulse diet of populations showing a high rate of gastric cancer in Colombia. High levels of nitrite have been found in the drinking-water of these groups and, in association with a high pH, in the gastric juice of Colombians with atrophic gastritis. Nitrosated spermidine was therefore chosen for study in an Ames test. Initial assays gave positive results only with *Salmonella typhimurium* strains TA1535 and TA100, and subsequent tests were conducted with TA1535. Spermidine alone was not mutagenic and nitrite was only weakly active, but tested together the two compounds showed strong mutagenic activity, which increased with increasing concentration. Thiocyanate catalysed mutagen formation, although the extent of this effect varied from one experiment to another. The formation of mutagenic compounds was pH-dependent, with an optimum at about pH 4. The stimulation of mutagenic activity by S-9 microsomal extract did not

exceed 20% at any concentration used. Ascorbic acid inhibited mutagenesis by 77% on average when added to the reaction mixture before the nitrite and spermidine, and by 67% when added afterwards.

As the optimum pH for mutagen formation in this study was very similar to the gastric pH found in Colombian high-risk subjects and the diet of such subjects is known to be deficient in ascorbic acid, the experimental model satisfied many of the requirements for the hypothesis that nitrosated spermidine may be a cause of gastric cancer in man.

[This work suggests the potential value of a feeding test with spermidine and nitrite, to ascertain whether this combination can in fact cause stomach cancer.]

Spices explored

Seino, Y., Nagao, M., Yahagi, T., Yasuda, T. & Nishimura, S. (1978). Identification of a mutagenic substance in a spice, sumac, as quercetin. *Mutation Res.* **58,** 225.

Abraham, S. K. & Kesavan, P. C. (1978). Evaluation of possible mutagenicity of the condiment clove when administered alone or in combination with caffeine in *Drosophila melanogaster*. *Indian J. exp. Biol.* **16,** 518.

Spices and condiments, whether synthetic or natural, are coming under increasingly searching scrutiny by toxicologists. The first paper cited above describes the methanol extraction of sumac spice (the red seeds of a Rhus species) and the use of column chromatography to isolate a fraction that showed mutagenic activity in a modified Ames test. The specific mutagenic activity of the purified sample with *Salmonella typhimurium* strains TA100 and TA98 was similar to that of quercetin. The ultraviolet absorption spectra of the purified sample and of quercetin and the R_F values of the two compounds on thin-layer chromatographs using three different solvent systems were also similar. Low-resolution mass spectrometry showed, for both compounds, a peak at *m/e* 302, which corresponds with the formula of quercetin, and the fragmentation-analysis patterns for the sample and for quercetin were almost identical and were characteristic of flavonoids. It appears, therefore, that the mutagenic activity of sumac demonstrated in bacterial systems may be attributed to the content of quercetin, which has already been reported to have some mutagenic potential (Bjeldanes & Chang, *Science, N.Y.* 1977 **197,** 577).

The second paper cited describes how an extract of clove (*Eugenia caryophyllata*), made by boiling with water and cooling, was fed to adult males of *Drosophila melanogaster* for 48 hr. Four broods of the fly were then studied. There was no increase in the frequency of sex-linked recessive lethal mutations in pre- or post-meiotic germ cells of males fed 0·0625–0·25% clove extract in sucrose solution or of those fed a combination of 0·125% clove extract and 0·125% caffeine. Previous work had shown that clove extract was clastogenic in the Allium test, in which the combined effect of clove and caffeine was synergistic. The absence of any detectable mutagenicity in Drosophila may be due to the ability of the animal to detoxicate small quantities of the spice, or to the failure of the responsible compound to reach germ cells in damaging concentrations.

COSMETICS, TOILETRIES AND HOUSEHOLD PRODUCTS

PCB residues in the water

Williams, D. T. & Benoit, F. M. (1979). The determination of polychlorinated biphenyls in selected household products. *Bull. env. contam. & Toxicol. (U.S.)* **21,** 179.

In the last few years there has been considerable concern about polychlorinated biphenyl (PCB) residues in the environment (*Cited in F.C.T.* 1975, **13,** 574). The authors cited above were concerned about the organic compounds in drinking-water and took for PCB analysis a selection of household products that are normally disposed of through the sewage system.

Hexane extracts of detergents, soap powders, fabric softeners, household cleaners, disinfectants, soaps (grated) and toilet paper were analysed quantitatively by electron-capture gas chromatography (GC) and by gas chromatography–mass spectrometry (GC–MS). Recovery studies carried out on toilet-paper samples spiked with Aroclor 1248 (used as the PCB standard) at concentrations of 1 and 10 μg/g gave yields above 90%.

The GC peaks for the toilet-paper extracts did not correspond exactly to those for standard Aroclor 1248, but the GC–MS selected ion peak patterns showed a greater similarity, although some differences were discernible. Except for one soap containing about 0·4 μg PCB/g and one soap powder with less than 0·1 μg/g, the cleaning products contained no detectable levels of PCBs, but nine of the eleven toilet-paper samples contained detectable amounts, ranging from traces to 21 μg PCB/g. The authors conclude that these findings identify toilet papers as a potential source of PCB contamination for surface-water systems.

More shadows on flame retardants

Van Duuren, B. L., Loewengart, G., Seidman, I., Smith, A. C. & Melchionne, S. (1978). Mouse skin carcinogenicity tests of the flame retardants tris (2,3-dibromopropyl)phosphate, tetrakis(hydroxymethyl)-phosphonium chloride and polyvinyl bromide. *Cancer Res.* **38,** 3236.

Tests with human DNA have shown that tris-(2,3-dibromopropyl)phosphate (TRIS) may have genetic effects in eukaryotic cells as well as in bacteria (*Cited in F.C.T.* 1979, **17**, 98). Moreover, this flame retardant has been shown to migrate from fabrics during laundering (*ibid* 1976, **14**, 512) and to undergo a variable degree of absorption through the skin (*ibid* 1977, **15**, 257). A close scrutiny of this and similar flame-proofing compounds proposed for use in clothing fabrics is, therefore, an obvious necessity, and a variety of work has already been undertaken.

A recent report presents the results of a skin application study in mice. Three flame-retardants—TRIS, tetrakis(hydroxymethyl)phosphonium chloride (THPC) and polyvinyl bromide (PVB)—were applied three times weekly to the skin of female Swiss mice for 420–496 days. TRIS was applied in doses of 10 or 30 mg in 0·2 ml acetone, THPC in 2-mg doses in 0·2 ml 90% aqueous acetone, and PVB in 0.1-ml doses of a 40% suspension. Another group of Swiss mice was given weekly sc injections of 23 mg PVB in aqueous suspension (injection volume 0·05 ml) for 48 wk, followed by a 60-wk observation period.

THPC and PVB were inactive when applied to the skin, but TRIS induced significant numbers of skin tumours at the site of application and also in other tissues. Some of these tumours, notably several squamous-cell carcinomas of the tongue or the gingival area, are rare in these mice and were probably induced by grooming. TRIS applications also increased significantly the incidence of stomach papillomas. In addition, both of the TRIS doses applied were associated with a high incidence of papillary tumours of the lung, and in the 30 mice given the higher dose there was one tubular adenoma of the kidney. Tumours of the latter type were previously found in a TRIS feeding study, although the incidence then was not significant. PVB injections induced liposarcomas in 19 of 30 mice, but this was considered to be a physical response to the injection of the aqueous suspension.

TOXICOLOGY

Trypan blue teratogenesis

Beck, F., Swidzinska, P. & Gulamhusein, A. (1978). The effect of trypan blue on the development of the ferret and rat. *Teratology* **18**, 187.

Although the potency of trypan blue as a teratogenic agent in rodents has been known for some time (*Cited in F.C.T.* 1968, **6**, 663), the mechanisms involved have remained somewhat obscure. In rats, congenital malformations do not occur if the compound is given on or after gestation day 11·5 (Wilson *et al. Anat. Rec.* 1959, **133**, 115), the time at which a functional chorio-allantoic placenta is established. It is thought that the teratogenic effects of the compound at 8·5 days are due to interference with the histiotrophic function of the inverted yolk-sac endoderm (Williams *et al. Teratology* 1976, **14**, 343). The above-cited study compares the effects of trypan blue in rats with those observed in the ferret, in which species histiotrophic nutrition does not involve an inverted yolk-sac system and is still of great importance at a relatively late stage of embryonic development.

Wistar rats received a dose of 100 mg trypan blue/kg sc at either 8·5 or 11·5 days of gestation. The ferrets received either 25 or 50 mg/kg sc on day 13 or 18, so that both species were treated at comparable stages of embryonic development. As expected, administration to rats on day 8·5 of gestation resulted in high abnormality rates, but there was a negligible teratogenic effect when the compound was given at 11·5 days. In contrast, the ferret showed a high malformation (and resorption) rate following trypan blue administration at both 13 and 18 days of gestation. The types of malformation differed in the two species, with absence or deformity of the tail and malformations of the CNS predominating in the rat, and palatal nonclosure and omphalocoele being the most common malformations in the ferret.

On the basis of these results, four suggestions are put forward to explain the mechanism of teratogenic action in the ferret. Considered by the investigators to be the most convincing is the possibility that the presence of trypan blue in tissue fluid inhibits the phagocytic action of the invading trophoblast of the ferret, as it inhibits pinocytosis by the yolk-sac epithelium of the rat. Alternatively pathological changes produced in the mother may be sufficiently severe to produce embryopathy in the ferret but not in the rat. A third suggestion is that the high incidence of cleft palate in the ferret may be part of a general pattern of delayed growth rather than a special effect in itself. Finally, although the dye cannot be seen in embryonic tissue with the light microscope, it is still possible that small protein-bound quantities are present in sufficient amounts to exert a teratogenic effect.

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MEETING ANNOUNCEMENTS

HEALTH ASPECTS OF SYNTHETIC FIBRE PRODUCTION

The Fifth International Symposium on Occupational Health in the Production of Artificial Fibres will be held in Belgirate (Novara), Italy, on 16–20 September 1980, under the auspices of the Permanent Commission and International Association on Occupational Health. The papers, all of which will be presented in English, will include reports of experimental work on carbon disulphide, hydrogen sulphide, acrylonitrile and other materials used in the production of artificial fibres, epidemiological studies of exposed workers, and toxicological problems relating to fibres other than viscose rayon. Papers may also be presented on other health problems encountered in the production of synthetic fibres.

Scientific information on the conference may be obtained from Prof. A. Cavalleri, c/o Clinica del Lavoro, University of Pavia, Via Severino Boezio, 24, 27100 Pavia, Italy. Organizational enquiries should be addressed to Miss K. White (Secretariat), c/o Fondazione Carlo Erba, Via Cino del Duca, 8, 20122 Milano, Italy.

MEDICINAL CHEMISTRY SYMPOSIUM IN SPAIN

The Sociedad Española de Química Terapéutica has announced that the Seventh International Symposium on Medicinal Chemistry will be held in Madrid on 2–5 September 1980. English will be the official language for the symposium, further details of which will be sent later to those indicating their interest in the meeting. Enquiries should be directed to: VIIth International Symposium on Medicinal Chemistry, Juan de la Cierva 3, Madrid-6, Spain.

POSTGRADUATE SCHOOL ON TOXICITY TESTING

A postgraduate school on aspects of toxicological testing methods is being organized jointly by the Department of Pharmacy, Chelsea College and the Pharmaceutical Society of Great Britain. The school will run from 14 to 18 April 1980. Mornings will be devoted to lectures and discussions covering the main aspects of toxicity testing and in the afternoons there will be further lectures and a supporting programme of demonstrations and seminars. Information on the school may be obtained from Mr. R. E. Marshall, School Secretary, Department of Pharmaceutical Sciences, The Pharmaceutical Society of Great Britain, 1 Lambeth Street, London SE1 7JN (telephone no. 01-735 9141, ext. 287).

THIRD INTERNATIONAL SYMPOSIUM ON MIGRATION

Unilever Research Laboratory in Germany will organize an international symposium on migration in Hamburg on 22–24 October 1980. The programme will cover new results in the field of migration of components from commodities, especially packages, into foods, cosmetics, pharmaceutical preparations and other products. English will be the language of the conference. Requests for information should be addressed to Unilever Forschungsgesellschaft mbH, Behringstr. 154, 2000 Hamburg 50, Federal Republic of Germany.

Fd Cosmet. Toxicol. Vol. 17, p. 693. Pergamon Press Ltd. 1979. 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*:

Synthesis of ^{14}C-labelled FD & C Blue No. 1 (Brilliant Blue FCF) and its intestinal absorption and metabolic fate in rats. By J. P. Brown, A. Dorsky, F. E. Enderlin, R. L. Hale, V. A. Wright and T. M. Parkinson.

The metabolic disposition of ^{14}C-labelled Green S and Brilliant Blue FCF in the rat, mouse and guinea-pig. By J. C. Phillips, D. Mendis, C. T. Eason and S. D. Gangolli.

The effects of butylated hydroxyanisole and butylated hydroxytoluene on renal function in the rat. I. Effects on fluid and electrolyte excretion. By S. M. Ford, J. B. Hook and J. T. Bond.

The effects of butylated hydroxyanisole and butylated hydroxytoluene on renal function in the rat. II. Effects on organic acid and base transport. By S. M. Ford, J. B. Hook and J. T. Bond.

N-Nitrosodimethylamine in beer. By R. A. Scanlan, J. F. Barbour, J. H. Hotchkiss and L. M. Libbey.

N-Nitrosamines—contaminants in blood collection tubes. By L. Lakritz and W. Kimoto.

Influence of caprolactam on rat-liver tyrosine aminotransferase and tryptophan oxygenase. By M. A. Friedman and A. J. Salerno.

The induction of rat hepatic microsomal xenobiotic metabolism by *n*-octadecyl β-(3′,5′-di-*tert*-butyl-4′-hydroxyphenyl)propionate. By B. G. Lake, S. D. Gangolli, K. Schmid, W. Schweizer, W. Stäubli and F. Waechter.

The 'carry over' of aflatoxin M_1 into the milk of cows fed rations containing permitted levels of aflatoxin B_1. By D. S. P. Patterson, E. M. Glancy and B. A. Roberts.

A teratology study of topically applied linear alkylbenzene sulphonate in rats. By I. W. Daly, R. E. Schroeder and J. C. Killeen.

The relationship of insoluble nitrilotriacetate (NTA) in the urine of female rats to the dietary level of NTA. By R. L. Anderson.

Effect of quality and quantity of diet on survival and tumour incidence in outbred Swiss mice. By G. Conybeare.

N-Nitrosodimethylamine in human blood. By L. Lakritz, M. L. Simenhoff, S. R. Dunn and W. Fiddler. (Short Paper).

Lack of carcinogenic effect of nitrosochlordiazepoxide and of nitrosomethylphenidate given orally to mice. By A. Giner-Sorolla, J. Greenbaum, K. Last-Barney, L. M. Anderson and J. M. Budinger. (Short Paper).

Tumours induced in Fischer 344 rats by the feeding of disulfiram together with sodium nitrite. By W. Lijinsky and M. D. Reuber. (Short Paper).

Contents continued]

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Index of Authors

The Index of Authors contains the names of all authors of original papers, review articles and Letters to the Editor. For Reviews of Recent Publications, Book Reviews and Abstracts, only the names of all the authors mentioned in the reference are given. For Articles of General Interest the names of all the authors mentioned in the text are provided, including those constituting the words *et al.*

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Index of Subjects*

*The significance of the superscripts P, M, R and L is given in paragraph 2 of the Index of Authors (p. 3).

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