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CONTENTS

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INFORMATION SECTION

ARTICLES OF GENERAL INTEREST

TOXICOLOGY: ABSTRACTS AND COMMENTS

FORTHCOMING PAPERS

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

Activité enzymatique, au niveau des microsomes hépatiques du rat, après administration d'acides phénoliques. By D. Gaillard, S. Mitjavila et R. Derache.

Bidrin: Perinatal toxicity and effect on the development of brain acetylcholinesterase and choline acetyltransferase in mice. By J. S. Bus and J. E. Gibson.

Metabolism of di-(2-ethylhexyl) phthalate by the contents of the alimentary tract of the rat. By I. R. Rowland.

Effect of prolonged ingestion of polychlorinated biphenyls on the rat. By J. V. Bruckner, K. L. Khanna and H. H. Cornish.

Penicillium viridicatum mycotoxicosis in the rat. IV. Attempts to modify the tissue responses. By M. D. McCracken, W. W. Carlton and J. Tuite.

Liver response tests. IX. Cytopathological changes in the enlarged but histologically normal rat liver. By P. Grasso, M. G. Wright, S. D. Gangolli and R. J. Hendy.

Gastro-intestinal absorption and toxicology: Ingesta-exchange and simulated-meal techniques. By J. C. Pekas.

A simple and rapid method for assaying cytotoxicity. By T. F. M. Ferguson and C. Prottey.

Effects of butylated hydroxytoluene alone or with diethylnitrosamine in mice. By N. K. Clapp, R. L. Tyndall, R. B. Cumming and J. A. Otten. (Short Paper).

The lack of significant absorption of methylcellulose, viscosity 3300 cP, from the gastro-intestinal tract following single and multiple oral doses to the rat. By W. H. Braun, J. C. Ramsey and P. J. Gehring. (Short Paper).

Absorption of pesticidal carbamates from perfused intestinal loops in conscious swine. By J. C. Pekas. (Short Paper).

Carcinogenicity of synthetic aflatoxin M_1 in rats. By G. N. Wogan and S. Paglialunga. (Short Paper).

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

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Research Section

EXCRETION AND METABOLISM OF SACCHARIN IN MAN. II. STUDIES WITH ^{14}C-LABELLED AND UNLABELLED SACCHARIN

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(*Received 31 October 1973*)

Abstract—Each of four men took 500 mg [^{14}C]saccharin (9·8 μCi) and collected excreta for intervals up to 96 hr. More than 98% of the ^{14}C was recovered within 48 hr (92·3% in urine, 5·8% in faeces); only a further 0·3% was excreted in the 48–72-hr interval and none in the 72–96-hr interval. On the basis of extrapolation of the 0–8-hr excretion, about one-half of the saccharin was excreted in 6 hr. In most urine samples, gas–liquid chromatographic (GLC) analysis for saccharin gave results corresponding very closely to those calculated from the ^{14}C counts, but in several there were discrepancies exceeding considerably the error of the GLC method. Very high values in two late samples indicated that one subject had inadvertently ingested extraneous saccharin, while low values (especially in two 0–8-hr samples) suggested that some biotransformation of the administered saccharin might have occurred. However, analysis by high-pressure-liquid and thin-layer chromatography failed to reveal the presence in these samples of any labelled compound other than saccharin. Hydrolysis of the aberrantly low samples with strong alkali and determination of the saccharin as *o*-sulphamoylbenzoate did bring the GLC and ^{14}C values into satisfactory agreement. The faecal ^{14}C consisted of unchanged saccharin.

Subsequently the same four subjects, and two others, took a 500 mg dose of unlabelled saccharin. GLC analysis of urine samples collected up to 72 hr indicated excretion rates similar to those obtained with the labelled compound. The mean 72-hr excretion as saccharin was 90·3 ± 2·1% of the dose, but alkaline hydrolysis brought the recovery up to 94·4 ± 3·1%.

INTRODUCTION

An important aspect of the safety evaluation of a food additive is the detection, identification and toxicological study of its metabolites. In the case of saccharin, recent studies have indicated that this food additive is not metabolized in rats or guinea-pigs (Minegishi, Asahina & Yamaha, 1972), nor in any one of six laboratory species, including the rhesus monkey (Byard & Golberg, 1973). Other reports have indicated that up to 1% of ingested saccharin is excreted in the form of *o*-sulphamoylbenzoic acid (*o*-SAMB) and *o*-sulphobenzoic acid in the urine of rats (Kennedy, Fancher, Calandra & Keller, 1972) and monkeys (Pitkin, Andersen, Reynolds & Filer, 1971).

Although little or no biotransformation of saccharin had been reported in laboratory animals, it was obviously desirable to verify the prediction that there would be little or none in man. The first paper in this series reported the gas–liquid chromatographic (GLC) determination of saccharin and one of its hydrolysis products, *o*-SAMB, in the urine from human subjects given oral doses of 0·5 to 1 g saccharin (McChesney & Golberg, 1973). Within 48 hr, more than 85% of the dose was excreted in the urine as saccharin; significant

levels of *o*-SAMB were not detected in any of the urine specimens examined. Alkaline hydrolysis, which converts saccharin to *o*-SAMB, produced levels of *o*-SAMB which were in some cases greater than those predicted on the basis of the amount of saccharin detected in the same urine. This excess was termed 'combined saccharin', but the nature of the combination was not established. The seeming disparity between the results of this early human study and the findings in six species of laboratory animals given [^{14}C]saccharin prompted us to apply both radioisotope and GLC analysis to excreta from volunteers given [^{14}C]saccharin. In addition, the subjects who had received [^{14}C]saccharin were subsequently given unlabelled saccharin to determine whether the observed rates of excretion were reproducible.

EXPERIMENTAL

Preparation and administration of the dose. Unlabelled saccharin, as the free acid, was purchased from Eastman Kodak Co. (Rochester, New York). GLC analysis (McChesney & Golberg, 1973) and high-pressure liquid chromatography (HPLC) both indicated that the purity of this saccharin exceeded 99%. Reference samples of *o*-SAMB and *o*-sulphobenzoic acid were obtained from Monsanto Co. (St. Louis, Mo.). For HPLC analysis, samples containing saccharin were neutralized with NaOH and injected into a Du Pont Model 830 HPLC with a 0·2 × 100 cm column packed with Permaphase AAX anion-exchange material (Du Pont Instruments Co., Wilmington, Del.). The saccharin was eluted with 10 mM-$NaClO_4$ at 1500 psi and was detected by an ultraviolet photometer. Saccharin, uniformly labelled with ^{14}C in the benzene ring, was obtained from Mallinckrodt Inc. (St. Louis, Mo.), and was purified by both preparative and thin-layer chromatography (Byard & Golberg, 1973) and HPLC. Only the centre of the saccharin peak was collected from the column effluent. To separate the purified [^{14}C]saccharin from $NaClO_4$, the pooled column effluents were acidified with HCl to pH 2·0 and extracted twice with 2 vols chloroform–ethyl acetate (1:1, v/v). The organic solvents were evaporated, and the residue was dissolved in water. The solution was adjusted to pH 7 with NaOH and extracted with an equal volume of chloroform. The resulting aqueous solution contained sodium [^{14}C]saccharin, which was more than 99% radiochemically and chemically pure as determined by HPLC. The ^{14}C compounds separated by HPLC were determined by collecting the column effluent while a peak was being indicated by the UV photometer. The effluent was evaporated to dryness and 1 ml water and 10 ml scintillation fluid (Patterson & Greene, 1965) were added. The ^{14}C was determined in a Packard Tricarb Liquid Scintillation Spectrometer. To complete the preparation of the labelled dose, unlabelled saccharin was added to the purified [^{14}C]saccharin to give a specific activity of 9·8 μCi/500 mg. The pH was adjusted to 7·0 with NaOH and water was removed by evaporation at 65°C. The resulting crystals were dried to constant weight at 65°C and stored at 4°C over anhydrous $CaCl_2$. The dose was prepared by weighing 280 mg of sodium [^{14}C] saccharin (250 mg saccharin) into each of two gelatin capsules.

The subjects were six healthy male volunteers who were between 27 and 60 yr old. After authorization to conduct the experiment had been obtained from the Albany Medical Center Radioisotope and Human Experimentation Committees, the subjects were briefed on the possible hazards involved, on how to collect the excreta, on what food and beverages should not be consumed, and on other pertinent matters. Four of the six subjects each ingested two capsules of [^{14}C]saccharin followed by 150 ml water, about 1·5 hr after a

normal breakfast. Between 3 and 6 wk later, doses of 500 mg unlabelled saccharin were prepared and administered to all six subjects in a similar manner.

Collection of excreta. Urine and faeces were collected for 0–8, 8–24, 24–48, 48–72 and 72–96 hr in polyethylene jars and stored at 4°C. All jars for urine collection contained 20 ml chloroform to prevent bacterial growth. Faeces were not collected from the subjects who received the unlabelled saccharin.

Extraction of excreta. Aliquots of urine were acidified with HCl to pH 1·5 and extracted 3 times with 2 vols chloroform–ethyl acetate (1 : 1, v/v). The extractions removed more than 99% of the ^{14}C from the aqueous phase. The faeces were suspended in methanol, poured into a Soxhlet thimble and extracted for 6–8 hr under reflux with methanol.

Analysis of excreta. The volume of urine for each collection period was recorded, and the sample was filtered through Whatman No. 1 filter paper. Duplicate 1 ml filtered aliquots were added to 10 ml scintillation fluid (Patterson & Greene, 1965) and assayed for ^{14}C as before. The quench correction was determined by adding an internal standard of [^{14}C]toluene or of [^{14}C]saccharin. The unfiltered urine and the chloroform used to preserve the urine were also assayed for ^{14}C; all of the ^{14}C was recovered in the filtered urine. The urine samples collected for 0–8, 8–24 and 24–48 hr were assayed directly by HPLC. GLC analysis for saccharin, 'combined saccharin' and *o*-SAMB was carried out on urine extracts as previously described (McChesney & Golberg, 1973). Methanol extracts of faeces and faecal residues that were too deeply coloured for direct assay of ^{14}C were digested for 30 min with an equal volume of 30% H_2O_2 at 95–100°C and then counted in the usual way. Unbleached faecal extracts were analysed directly by HPLC.

HPLC analysis of the excreta was carried out with a 0·8 × 100 cm preparative column, the conditions for chromatography and elution being the same as those described for the preparation of [^{14}C]saccharin. An equal volume of the fraction injected into the column was assayed for ^{14}C to ensure that all the radioactivity injected into the column had been accounted for.

Thin-layer chromatography (TLC) of the ethyl acetate–chloroform extracts of urine was performed on 0·5 mm-thick Kieselgel F_{254} plates (E. Merck, Darmstadt), using two of the solvent systems developed by Minegishi *et al.* (1972). These systems were: (I) conc. ammonia–methanol–chloroform (11·5:50:100, by vol.) and (II) conc. ammonia–ethyl acetate–acetone (1:1:8, by vol.). In system I, the R_F of saccharin was 0·58 and in system II it was 0·49. Other observed R_F values were as follows: for *o*-SAMB, I = 0·39, II = 0·30; for *p*-SAMB (Aldrich, Milwaukee, Wisconsin), I = 0·20, II = 0·19; for *o*-toluenesulphonamide (Badische Anilin und Sodafabrik, Ludwigshaven, Germany), I = 0·94, II = 0·99. As has been noted by GLC analysis (McChesney & Golberg, 1973), TLC analysis of the sample of *o*-SAMB indicated the presence of 2% saccharin. All ^{14}C, TLC, HPLC and GLC analyses were standardized against the sodium saccharin or sodium [^{14}C]saccharin used to prepare the respective doses.

RESULTS

Recovery of ^{14}C

The results of the two human studies are presented in Tables 1 and 2. As indicated in Table 1, the subjects given [^{14}C]saccharin excreted 98·3 ± 0·9% of the dose in the urine and faeces with 48 hr, with more than 90% in the urine and about 6% in the faeces. One-half of the saccharin was excreted in about 6 hr. Since virtually no ^{14}C was excreted by any subject in either urine or faeces during the 48–72-hr period, it was apparent that no

Table 1. *Excretion of saccharin after ingestion of a dose of 500 mg (labelled) by four subjects*

Subject	Time after ingestion (hr)	Excretion (% of dose)						
		Urinary						
		Saccharin		Combined saccharin	Total saccharin	Faecal		
		GLC*	HPLC†	Difference‡	^{14}C§	^{14}C§	HPLC†	Total§
1	0–8	80·2	79·5	−0·2	80·0	3·2	3·2	94·2
	8–24	11·0	11·3	0	11·0			
	24–48	1·5	2·1	0·1	1·7	1·2	1·3	2·9
	48–72	0·4	—	−0·4	0	<0·1	—	0
	0–72	94·7	92·9	−0·5	92·7	4·4	4·5	97·1
2	0–8	71·6	78·3	7·4	79·0	2·2	2·5	93·3
	8–24	10·6	12·1	1·5	12·1			
	24–48	14·0¶	3·0	−11·7	2·3	2·6	2·8	4·9
	48–72	4·6¶	—	−4·5	0·1	<0·1	—	0·1
	0–72	100·8	93·4	−7·3	93·5	4·8	5·3	98·3
3	0–8	64·6	67·4	5·9	70·5	8·4	8·3	93·8
	8–24	13·0	15·2	1·9	14·9			
	24–48	2·5	2·7	0	2·5	0·3	—	2·8
	48–72	1·1	—	−0·8	0·3	<0·1	—	0·3
	0–72	81·2	85·3	7·0	88·1	8·7		96·9
4	0–8	55·0‖	58·8	2·4	57·6	3·1	3·0	88·3
	8–24	27·8	30·7	−0·2	27·6			
	24–48	10·2	10·8	−0·5	9·7	2·1	2·1	11·8
	48–72	0·4	—	0·2	0·6	—	—	0·6
	0–72	93·4	100·3	1·9	95·5	5·2	5·1	100·7
Mean ± SEM	0–8	67·8 ± 5·3	70·6 ± 4·7	3·9 ± 1·5	71·8 ± 5·2	4·2 ± 1·4	4·3 ± 1·4	92·4 ± 1·4
	8–24	16·0 ± 2·3	17·3 ± 4·5	0·4 ± 0·8	16·4 ± 3·8			
	24–48	7·1 ± 5·2	4·6 ± 2·1	−3·0 ± 2·9	4·1 ± 1·9	1·6 ± 0·5	—	5·6 ± 2·1
	48–72	1·6 ± 1·0	—	−1·4 ± 1·1	0·3 ± 0·1	—	—	0·3 ± 0·2
	0–72	92·5 ± 2·2	92·5 ± 3·1	−0·1 ± 3·0	92·6 ± 1·5	5·8 ± 1·0	—	98·3 ± 0·9

* As determined by gas–liquid chromatography.

†As determined by high-pressure liquid–liquid chromatography.

‡Difference between results obtained by gas–liquid chromatography and radioactive count on the same samples.

§Based on radioactive counts only.

¶It seemed apparent that subject 2 inadvertently ingested unlabelled saccharin during the course of the experiment, since repeated analyses for saccharin and for ^{14}C failed to decrease the discrepancies between results obtained by the two methods in the samples indicated.

‖This subject on his own volition subdivided the 0–8 hr period into two equal periods. The ^{14}C excretion in the two sub-periods was almost equal.

Table 2. *Excretion of saccharin after ingestion of a dose of 500 mg (unlabelled)* by six subjects*

Subject	Time after ingestion (hr)	Excretion (% of dose) as		
		Saccharin	Combined saccharin†	Total saccharin
1	0–8	77·4	−0·8	76·6
	8–24	15·4	4·3	19·7
	24–48	1·3	0·4	1·7
	48–72	0·5	−0·1	0·4
	0–72	94·6	3·8	98·4
2	0–8	71·3	3·1	74·4
	8–24	19·0	1·4	20·4
	24–48	0·3	0·3	0·6
	48–72	0·5	0·9	1·4
	0–72	91·1	5·7	96·8
3	0–8	71·2	0·3	71·5
	8–24	13·8	0	13·8
	24–48	2·8	1·3	4·1
	48–72	0·9	0·5	1·4
	0–72	88·7	2·1	90·8
4	0–8	60·2	−1·1	59·1
	8–24	28·0	−0·1	27·9
	24–48	6·6	1·2	7·8
	48–72	0·5	0·1	0·6
	0–72	95·3	0·1	95·4
5	0–8	63·1	6·5	69·6
	8–24	21·0	1·2	22·2
	24–48	6·2	−0·4	5·8
	48–72	1·0	0	1·0
	0–72	91·3	7·3	98·6
6	0–8	53·6	1·2	54·8
	8–24	19·0	0·7	19·7
	24–48	8·0	2·1	10·1
	48–72	0·6	1·1	1·7
	0–72	81·2	5·1	86·3
Mean ± SEM (6 subjects)	0–8	66·1 ± 4·0	1·5 ± 1·7	67·7 ± 3·6
	8–24	19·3 ± 2·0	1·3 ± 0·7	20·6 ± 1·9
	24–48	4·2 ± 1·3	0·8 ± 0·4	5·0 ± 1·5
	48–72	0·7 ± 0·3	0·4 ± 0·2	1·1 ± 0·2
	0–72	90·3 ± 2·1	4·0 ± 1·1	94·4 ± 3·1
Mean ± SEM (4 subjects; 5 and 6 omitted)	0–8	70·0 ± 3·6	0·4 ± 1·0	70·4 ± 4·0
	8–24	19·0 ± 3·2	1·4 ± 1·1	20·5 ± 2·9
	24–48	2·8 ± 1·4	0·8 ± 0·3	3·5 ± 1·6
	48–72	0·6 ± 0·1	0·3 ± 0·2	0·9 ± 0·3
	0–72	92·4 ± 1·5	2·9 ± 1·2	95·3 ± 1·6

* An amount of the sodium salt (598 mg) equivalent to 500 mg saccharin was given in two gelatin capsules with about 150 ml of water.

† Increase in apparent saccharin content noted on alkaline hydrolysis of the samples, followed by acidification, extraction of the sulphamoylbenzoate, and GLC determination of the latter.

further significant excretion would occur thereafter, but this was confirmed by actual counting of the 72–96-hr urine samples. Some of the ^{14}C remained in the faecal residues after extraction, but the total amount present in the samples from all four subjects would account for only about 1 mg saccharin, or 0·05% of the total dosage given.

Chromatography of ^{14}C in excreta by HPLC

Saccharin, *o*-SAMB and *o*-sulphobenzoic acid were well separated by HPLC (Fig. 1). Saccharin was clearly evident as an ultraviolet-absorbing peak in the 0–8-hr urine samples (Fig. 2). The chromatographic distribution of ^{14}C was determined by radioassay of the column effluent collected for the intervals indicated in Fig. 2. Table 1 includes the results of the ^{14}C analyses of the saccharin peak in urine and faeces. Of the ^{14}C injected into the HPLC, significant amounts of radioactivity were recovered only in the saccharin peak.

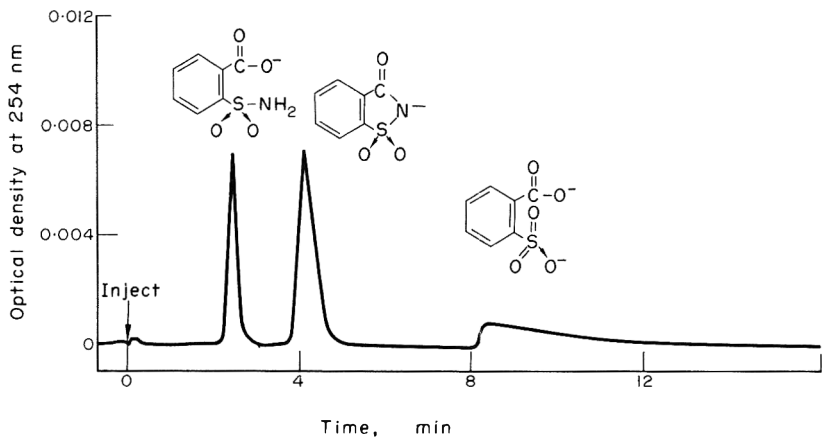

Fig. 1. Chromatography of saccharin, *o*-SAMB and *o*-sulphobenzoic acid by HPLC, involving injection of 10μg of each compound into a 0·2 × 100 cm AAX permaphase anion-exchange column and elution with 10 mM-$NaClO_4$ at 1500 psi.

GLC and TLC analysis of urine from subjects receiving [^{14}C] saccharin

GLC analysis of extracts of the urine specimens containing ^{14}C usually indicated levels of saccharin nearly identical to those found by ^{14}C analysis of the HPLC effluent (Table 1). However, some differences were observed. Subject 2, for example, excreted levels of saccharin in the 24–72-hr collection period which were many times the amount indicated by ^{14}C analysis. This result suggested that he had inadvertently consumed unlabelled saccharin during the course of the experiment. In some samples however, less saccharin was found by GLC than was indicated by either ^{14}C or HPLC analysis. This difference was designated 'combined saccharin', and the implication was that labelled excretory products other than saccharin might be present.

In an attempt to explain these findings, extracts of the 0–8-hr urine samples from the subjects receiving [^{14}C]saccharin were analysed by TLC using the solvent systems of Minegishi *et al.* (1972). With these systems, 99% of the ^{14}C from subjects 2 and 3 (who showed the highest level of 'combined saccharin') chromatographed as saccharin. Most of the remainder did not move from the origin but, since the same was true of chloroform–ethyl acetate extracts of the saccharin preparation administered to the subjects, the fraction at the origin could be disregarded as representing an artefact. Thus, there seemed to

be no tenable explanation for the fact that the 0–8-hr urine samples from subjects two and three invariably gave low results by GLC analysis. This point is considered further in the discussion.

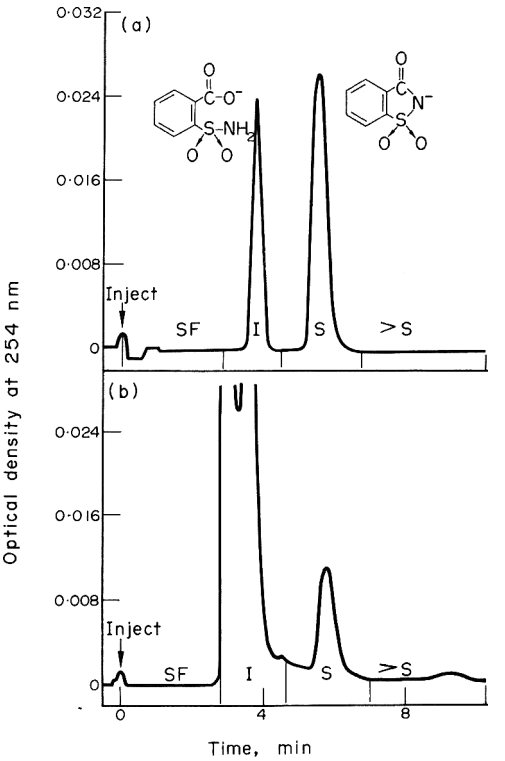

Fig. 2. Chromatography of saccharin, *o*-SAMB and 0–8 hr urine by HPLC: (a) 100 μg [^{14}C]saccharin and *o*-SAMB injected into a 0·8 × 100 cm AAX permaphase anion-exchange column and eluted with 10 mM-$NaClO_4$ at 1500 psi; (b) 50 μl 0–8 hr urine (subject no. 1)—the column effluents were collected and assayed for ^{14}C for the intervals shown: SF, solvent front; I, *o*-sulphamoylbenzoic acid; S, saccharin; >S, after saccharin.

GLC analysis of urine from subjects receiving unlabelled saccharin

GLC analysis of urine specimens from the six subjects given unlabelled saccharin (Table 2) confirmed the results obtained from the four who had received [^{14}C]saccharin, as well as from previously-reported studies in man (McChesney & Golberg, 1973). The data are summarized at the foot of Table 2, both for the four subjects who participated in the radioactive experiment and for all six subjects. It is evident that the composite results are much the same, whether they are based on the first four subjects or on all six. Also, the four subjects excreted 95·3 $\pm$ 1·6% of the dose in 72 hr in this case, as compared to 92·6 $\pm$ 1·5% when they ingested the radioactive saccharin. This is not a significant difference, nor should it be. In this instance, at least 97% of the urinary product could be definitely identified as saccharin, as compared to 96% in the radioactive experiment, a difference which is reflected in the smaller 'combined saccharin' item in Table 2. The four subjects who participated in both studies duplicated their individual excretory patterns to a

satisfactory degree. The duplication was particularly impressive in the following cases: subject 2, 0–8 hr; subject 1, 0–8 and 24–48 hr; subjects 3 and 4, all intervals.

DISCUSSION

The results of these studies are in accord with earlier observations in laboratory animals (Byard & Golberg, 1973; Kennedy *et al.* 1972; Matthews, Fields & Fishbein, 1973; Minegishi *et al.* 1972) and in man (McChesney & Golberg, 1973) in showing that saccharin is very rapidly excreted in the urine, and almost entirely, if not entirely, in unchanged form. Faecal excretion, averaging nearly 6% of the dose, was considerably greater than was anticipated from the value of approximately 1% reported for that route in the monkey by Pitkin *et al.* (1971), but our findings could have been due to the presence of food in the stomach, an effect observed by Matthews *et al.* (1973).

The question whether any degradation product of saccharin was present in the urine revolves precisely about the significance, if any, of the so-called 'combined saccharin' fraction observed by McChesney & Golberg (1973) and seen to some extent in the present studies. Numerically, this fraction reflects the increase in recovery obtained on alkaline hydrolysis. Such a fraction was not found in every urine sample collected following the administration of saccharin (in most cases the 24–48- and 48–72-hr samples contained too little saccharin to make this type of differential analysis meaningful), but it was observed too frequently to dismiss it, without further systematic study, as representing some sort of analytical artefact. McChesney & Golberg (1973) suggested several possible explanations for their observance of this fraction. All these possibilities have since been eliminated as follows:

(1) Since the method of extraction used removed all ^{14}C from the urine of the subjects who ingested the labelled compound, 'combined saccharin' could not simply represent saccharin or related products which failed to extract from the urine in the expected manner.

(2) The 'combined saccharin' could not represent extracted saccharin which failed to methylate completely, since in the case of an especially crucial sample (subject 2, 0–8 hr, Table 1, a sample which repeatedly gave a value well below that calculated from its ^{14}C content), three successive methylation steps, with complete evaporation of the solvents between steps, failed to increase the amount of saccharin found by GLC. Also, saccharin added to this urine sample just prior to the methylation step, or prior to the extraction step, was recovered quantitatively.

(3) The 'combined saccharin' fraction could not represent saccharin which had been hydrolysed to *o*-SAMB and had been conjugated with glycine or glucuronic acid, since TLC and HPLC analysis of the whole urine specimens or of extracts from them failed to reveal the presence of any such fraction. The remote possibility that such a fraction existed and failed to separate from saccharin in any analysis other than GLC may be ruled out, since all radioactivity subjected to TLC or HPLC analysis could be recovered as saccharin, except for the very minor amounts which were present as impurities in the [^{14}C]saccharin administered. The latter point is illustrated in Fig. 2 (lower tracing): following chromatography of an aliquot of the whole urine sample from subject 2, the effluent fractions were analysed separately for ^{14}C and only the fraction labelled 'S' proved to be radioactive. When this fraction was concentrated, acidified, extracted,

methylated and subjected to GLC analysis, the amount of saccharin found was equivalent to the ^{14}C which was put on the column. Therefore, saccharin *per se* was the only labelled compound present in fraction 'S'.

(4) The low values obtained in the several samples mentioned could not be attributed to an artefactually increased internal standard response, since extracts prepared (and methylated) in the absence of the standard showed no urinary constituent coming off the column at precisely the same time as the internal standard.

It is of interest to note that somewhat similar analytical artefacts were observed for saccharin by Matthews *et al.* (1973) and by Miller, Crawford, Sonders & Cardinal (1966) for cyclamate. However, the artefacts observed in the present studies were not of precisely the same type as those reported by Matthews *et al.* since substitution of the solvent system chloroform–methanol for chloroform–methanol–ammonia made no difference in the TLC behaviour of the urine extracts: only saccharin *per se* was detected with either system. Therefore, as matters now stand, no direct evidence has been found for the presence of any chemically-bound form of saccharin in the 0–8-hr urine samples from either subject 2 or 3, and the increase in yield consistently observed on alkaline hydrolysis of these samples has not been explained.

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Excrétion et métabolisme de la saccharine chez l'homme. II. Expériences avec de la saccharine marquée au ^{14}C et de la saccharine non marquée

Résumé—Quatre hommes ont pris chacun 500 mg de saccharine marquée au ^{14}C (9,8 μCi) et recueilli leurs excrétions à différents moments, jusqu'à 96 h après l'ingestion. Plus de 98% du ^{14}C ont été récupérés dans les 48 h (92,3% dans l'urine, 5,8% dans les fèces). Seulement 0,3% ont encore été excrétés dans l'intervalle de 48 à 72 h et rien n'a plus été excrété dans l'intervalle de 72 à 96 h. D'après l'extrapolation de l'excrétion de 0 à 8 h, à peu près la moitié de la saccharine a été excrétée en 6 h. La détermination de la saccharine par analyse chromatographique en phases

liquide et gazeuse (GLC) des échantillons d'urine a donné pour la plupart de ceux-ci des résultats très proches de ceux calculés d'après les comptages du ^{14}C, mais pour plusieurs échantillons les discordances dépassaient la marge d'erreur de la méthode GLC. Les valeurs très élevées de deux échantillons tardifs indiquaient qu'un des sujets avait consommé par inadvertance de la saccharine d'une autre provenance, tandis que de faibles valeurs (surtout dans deux échantillons de 0–8 h) suggéraient que la saccharine administrée pouvait avoir subi une certaine transformation biologique. L'analyse par chromatographie en phase liquide et sous haute pression ainsi qu'en couche mince n'a cependant révélé dans ces échantillons la présence d'aucun composé marqué autre que la saccharine. L'hydrolyse avec une base forte des échantillons à teneur anormalement faible et la détermination de la saccharine comme *o*-sulfamoylbenzoate ont mis en concordance satisfaisante les valeurs GLC et ^{14}C. Le ^{14}C fécal consistait en saccharine inchangée.

Les quatre mêmes sujets et deux autres ont pris par après une dose de 500 mg de saccharine non marquée. L'analyse GLC des échantillons d'urine recueillis jusqu'à 72 h a révélé des taux d'excrétion similaires à ceux obtenus avec le produit marqué. L'excrétion moyenne à 72 h sous forme de saccharine représentait 90,3 ± 2,1% de la dose, mais l'hydrolyse alcaline a porté le taux de récupération à 94,4 ± 3,1%.

Ausscheidung und Stoffwechsel von Saccharin beim Menschen. II. Untersuchungen mit ^{14}C-markiertem und unmarkiertem Saccharin

Zusammenfassung—An vier Versuchspersonen wurden je 500 mg [^{14}C]Saccharin (9,8 μCi) verabreicht und die Ausscheidungen bis 96 Stunden lang gesammelt. Über 98% des ^{14}C wurden innerhalb von 48 Stunden wiedergewonnen (92,3% im Urin, 5,8% in den Faeces); nur weitere 0,3% wurden in dem Zeitraum der 48.–72. Stunde ausgeschieden und nichts in dem Zeitraum 72.–96. Stunde. Auf Grund der Extrapolation der Ausscheidung im Zeitraum 0.–8. Stunde lässt sich sagen, dass etwa die Hälfte des Saccharins in 6 Stunden ausgeschieden wurde. Bei den meisten Urinproben ergab die gasflüssigkeitschromatographische Analyse auf Saccharin Ergebnisse, die recht genau denen der ^{14}C-Messungen entsprachen, aber in verschiedenen Fällen gab es Unterschiede, die beträchtlich die Fehlergrenze der chromatographischen Methode überschritten. Sehr hohe Werte bei zwei späten Proben liessen darauf schliessen, dass eine Person unabsichtlich weiteres Saccharin zu sich genommen hatte, während niedrige Werte (besonders bei zwei Proben aus der Periode 0.–8. Stunde) die Vermutung nahelegten, dass eine biochemische Umwandlung des verabreichten Saccharins eingetreten sein könnte. Jedoch ergaben Analysen durch Hochdruckflüssigkeits- und Dünnschichtchromatographie keine Anzeichen für die Anwesenheit von anderen markierten Verbindungen ausser dem Saccharin. Die Hydrolyse der abweichend niedrigen Proben mit starkem Alkali und die Bestimmung des Saccharins als *o*-Sulfamoylbenzoat brachte die gasflüssigkeitschromatographischen und die ^{14}C-Werte in zufriedenstellende Übereinstimmung. Das faecale ^{14}C war unverändertes Saccharin.

Anschliessend erhielten die gleichen vier und zwei weitere Personen je eine 500-mg-Dosis unmarkiertes Saccharin. Die gasflüssigkeitschromatographische Analyse von bis zu 72 Stunden lang gesammelten Urinproben liess auf Ausscheidungsgeschwindigkeiten ähnlich denen schliessen, die mit der markierten Verbindung erhalten wurden. Die mittlere 72-Stunden-Ausscheidung als Saccharin betrug 90,3 ± 2,1% der Dosis, jedoch brachte die Alkalihydrolyse die Wiedergewinnung auf 94,4 ± 3,1%.

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SHORT-TERM TOXICITY OF METHYLPHENYLCARBINYL ACETATE IN RATS

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Abstract—Groups of rats were given daily oral doses of 0 (control), 15, 50 or 150 mg methylphenylcarbinyl acetate/kg body weight for 13 wk. There were no effects on the rate of body-weight gain, although the food and water intakes were increased in the male rats given 150 mg/kg. The relative liver and kidney weights were increased in male rats given 50 or 150 mg/kg/day. There was an increased excretion of cells in the urine of male rats given 150 mg/kg for 6 wk. No histopathological changes were seen that could be related to treatment with methylphenylcarbinyl acetate. It was concluded that the no-untoward-effect level for methylphenylcarbinyl acetate when given to rats for 13 wk was 15 mg/kg.

INTRODUCTION

Methylphenylcarbinyl acetate (phenylmethylcarbinyl acetate; α-methylbenzyl ethanoate; 1-phenylethyl ethanoate; methylbenzyl acetate; $CH_3-CO_2-CH(CH_3)-C_6H_5$; MPCA) is used as a constituent of flavourings in a variety of foods. There are no specific regulations governing the use of flavourings in the U.K. However, MPCA was not one of the 17 materials that the Food Standards Committee (1965) suggested should be prohibited for use in food. There seemed to be some confusion in the Council of Europe (1970) partial agreement, as the flavouring was listed as temporarily admissible and requiring further studies and also, under methylbenzyl acetate, as admissible with an acceptable daily intake of 5 mg/kg. In the Council of Europe (1972) partial agreement this confusion is resolved and only the listing as admissible remains.

Although no published data exist on the metabolism of MPCA, it is likely that hydrolysis to the carbinol and acetic acid is the initial step. Williams (1959) reports studies in which 50% of a dose of (±)-methylphenylcarbinol in rabbits was excreted as the glucuronide in the urine within 24 hr. There was some evidence of oxidation and demethylation, as mandelic and hippuric acids were found in the urine. These results have been confirmed by El Masry, Smith & Williams (1956), who found that after the oral administration of methylphenylcarbinol to rabbits, 28% of the dose was oxidized to benzoic acid and excreted as hippuric acid, while 50% was recovered as a glucuronic acid conjugate. The oral LD_{50} of the carbinol in rats has been estimated as 0·4 g/kg (Williams, 1959).

In view of the lack of toxicological information on MPCA, a short-term feeding study in rats was undertaken as part of the BIBRA safety evaluation programme and the results are given in this paper.

EXPERIMENTAL

Materials. MPCA was supplied by Givaudan & Co. Ltd., Whyteleafe, Surrey, and complied with the following specification: colourless liquid with a 'green leaf' odour; purity, min. 98%; specific gravity (25/25°C), 1·023–1·026; refractive index (20°C), 1·494–1·496; acid no., max 1·0.

Animals and diet. Rats of the CFE strain obtained from an SPF breeding colony were fed *ad lib.* on ground Spillers' Laboratory Small Animal Diet and allowed unlimited access to water. They were housed in a room maintained at 21 ± 1°C with a relative humidity of 50–60%.

Loss of flavouring from diet. A diet was prepared containing 2·5% MPCA. A sample of this was immediately placed in a sealed container and other samples were exposed to the air in an animal room for 2 or 4 days. The concentration of MPCA was measured in methanol extracts of these samples using a Pye 104 dual-flame gas chromatograph fitted with a 5 ft glass column packed with 10% polyethylene glycol adipate on 100/120 mesh Celite. The column temperature was 145°C. It was found that 15 and 40% of the MPCA was lost from the diet in 2 and 4 days respectively. Due to this loss of flavouring from animal diets, it was decided to give the MPCA by daily oral intubation.

Experimental design and conduct

Four groups of 15 male rats (body weight 82–115 g) and 15 females (body weight 68–104 g), randomized from a single population, were housed five in a cage and given daily oral doses (7 days/wk) of 0 (control), 15, 50 or 150 mg MPCA/kg body weight for 13 wk. Additional groups of five rats of each sex, randomized from the same population, were given 0 (control), 50 or 150 mg MPCA/kg/day for 2 or 6 wk. The flavouring was administered in solution in corn oil with the concentrations adjusted so that each rat was given a daily volume of 5 ml/kg.

Weekly records were kept of body weight. Food and water consumptions were recorded over the 24-hr periods prior to weighing. At the end of the appropriate experimental period the animals were killed by exsanguination under barbiturate anaesthesia, following a 24-hr period without food. Samples of blood were taken from the aorta for haematological examination and serum analyses. An autopsy was conducted during which any macroscopic abnormalities were noted, and the brain, pituitary, thyroid, heart, liver, stomach, small intestine, caecum, spleen, kidneys, adrenal glands and gonads were weighed. Samples of these organs and of salivary gland, trachea, aorta, lung, lymph nodes, urinary bladder, colon, rectum, pancreas, uterus and skeletal muscle were preserved in 10% buffered formalin. Paraffin-wax sections of the tissues from 50% of the control aminals and from all the animals given 150 mg MPCA/kg/day were stained with haematoxylin and eosin for microscopic examination.

Blood taken at autopsy was examined for haemoglobin concentration, packed cell volume and counts of erythrocytes, reticulocytes, total leucocytes and the individual types of leucocyte. Serum was analysed for urea, glucose and activities of glutamic–oxalacetic transaminase, glutamic–pyruvic transaminase and lactic dehydrogenase.

Urine was collected during the final week of treatment and examined for appearance, microscopic constituents and content of glucose, ketones, bile salts and blood. A renal concentration and dilution test was carried out at the same time. At wk 6 and 13 this consisted of measurements of the specific gravity and volume of the urine produced during a 6-hr period of water deprivation, during a 2-hr period following a water load of 25 ml/kg and

from 16 to 20 hr after the water load. At wk 2 these measurements were made only during the 6-hr period of water deprivation. A count of the number of cells in the urine was carried out on the 6-hr urine sample.

RESULTS

There were no obvious abnormalities in the appearance or behaviour of the rats in any of the treated groups. Two rats, one female given 50 mg/kg/day and one male given 15 mg/kg/day died immediately following dosing on days 39 and 46, respectively. Both exhibited a brief period of laboured breathing prior to death and autopsy revealed the presence of oil in the trachea and lungs.

There were no statistically significant differences in the rate of body-weight gain by the treated animals compared with that in the controls (Table 1). Throughout the study the food and water consumption (Table 1) of the male rats given 150 mg/kg/day was slightly increased so that the mean intakes over the 90-day period were significantly greater than the control values. No significant differences in food and water consumption were seen in the females given 150 mg/kg/day or in either sex at the lower dose levels.

There were no consistent or dose-related effects in the results of the haematological examinations (Table 2) and no significant differences between treated and control groups in the results of the serum analyses.

The stomach weights of female rats given 50 or 150 mg MPCA/kg for 2 wk were significantly greater than those of the controls. At wk 6 the testis weight of both treated groups was significantly lower than the control value. In both cases the differences amounted to only 10–12% of the control weight and were not significant when the organ weight was expressed relative to body weight (Table 3). Although there were no statistically significant differences in the organ weights at wk 13, the mean relative liver and kidney weights in males receiving 150 mg/kg/day were higher than those of the controls.

No abnormal constituents were found in the urine of any of the rats and there were no statistically significant differences between treatments in the results of the renal concentration and dilution tests (Table 4). After treatment for 6 wk, the number of cells excreted in the urine of the male rats given 150 mg/kg/day was approximately 70% greater than that of the controls. However, no similar changes were seen in the female group or in either sex at wk 13.

At autopsy, thickening of the pericardium and an adhesion between the heart and pericardium were seen in a female given 50 mg MPCA/kg/day for 13 wk. Histopathological examination revealed evidence of oil droplets in the thoracic cavity, pericarditis and a granulomatous reaction of the pericardium containing oil droplets. Similar histopathological changes without the granulomatous reaction were seen in a male given 15 mg MPCA/kg/day for 13 wk. In addition, one female control rat killed after 6 wk on study had multiple small nodules in the lung, which proved to be a reticulum-cell neoplasm, of type A as defined by Dunn (1954).

The histopathological examination also revealed evidence of a mild pulmonary infection, early changes characteristic of glomerulonephrosis and splenic haemosiderin deposition. However, the incidence and severity of these changes were similar in treated and control animals. In addition, hydrometra was seen in the uteri of two rats given 150 mg MPCA/kg/day for 13 wk.

Table 1. *Mean values for body weight and food and water consumption of rats given daily oral doses of 0–150 mg MPCA/kg for 13 wk*

Dose level (mg/kg)	Body weight (g) at day				Body-weight gain (g) at day 95	Food consumption (g/rat/day) on day				Mean food consumption (g/rat/day)	Water consumption (ml/rat/day) on day				Mean water consumption (ml/rat/day)
	0†	32	60	95		0†	32	60	95		0†	32	60	95	
Males															
0	101	287	376	432	331	11·9	15·3	16·7	14·3	16·1	18·1	21·0	26·3	21·1	22·3
15	101	288	368	429	328	12·3	15·7	15·9	15·6	15·9	18·9	22·2	23·7	27·8	22·9
50	104	302	383	446	342	13·3	14·5	16·9	16·2	16·4	19·2	21·5	29·4	21·9	23·4
150	103	302	381	447	344	12·8	17·5	17·9	17·1	17·5**	19·4	24·3	29·1	22·7	24·5*
Females															
0	88	194	231	258	170	11·3	12·5	14·4	13·7	13·4	18·1	17·9	22·1	19·1	19·4
15	89	199	241	260	171	11·3	11·7	13·5	13·7	13·4	18·6	16·3	22·5	20·2	19·8
50	91	203	238	265	174	11·7	10·9	12·9	12·6	12·5	18·9	16·0	19·2	23·9	18·0
150	92	201	233	265	173	11·8	12·0	14·3	13·8	13·7	18·5	19·1	23·2	20·0	20·2

† First day of treatment.

Values of body weights are the means for 15 animals and those of food and water consumption are the means for three cages of five animals. Values marked with asterisks differ significantly (ranking method of White. 1952) from those of controls: $*P<0.05$; $**P<0.01$.

Table 2. *Results of haematological examinations in rats given daily oral doses of 0–150 mg/kg MPCA for 2, 6 or 13 wk*

Sex and dose level (mg/kg)	No. of rats examined	Hb (g/100 ml)	PCV (%)	RBC (10^6/mm^3)	Retics (% of RBC)	Leucocytes: Total (10^3/mm^3)	Leucocytes: Differential (%) N	E	L	M
Wk 2										
Male										
0	5	11·7	41	4·79	2·4	5·0	10	0	89	1
50	5	11·4	41	4·47	3·0	4·6	6	0	92	2
150	5	11·9	40	4·66	2·8	5·5	8	1	89	2
Female										
0	5	12·1	39	5·04	2·3	7·0	8	1	89	2
50	5	11·6	40	4·54*	1·8	5·6	10	1	88	1
150	5	12·5	41	5·11	1·8	3·5**	11	1	87	1
Wk 6										
Male										
0	5	14·7	45	6·67	1·1	8·3	11	1	87	1
50	5	14·6	45	6·82	1·0	7·4	18	1	80	1
150	5	14·5	44	6·39	1·1	7·6	13	0	86	1
Female										
0	5	13·6	42	6·17	0·9	6·9	16	1	82	1
50	5	14·9*	44	6·21	1·0	5·5	13	1	85	1
150	5	14·4	43	6·12	1·0	4·7	12	2	85	1
Wk 13										
Male										
0	14	14·3	45	7·18	1·0	6·1	15	1	82	2
15	14	14·4	44	7·08	1·1	5·5	15	1	83	1
50	15	14·2	45	7·32	1·0	6·3	18	1	80	1
150	15	14·3	45	7·25	1·0	7·4	13	1	84	2
Female										
0	15	14·0	43	6·13	1·1	3·6	13	2	84	1
15	15	13·8	42	5·98	1·1	4·2	13	1	85	1
50	14	13·9	40	6·17	1·3	4·3	10	1	88	1
150	15	14·0	43	6·12	1·1	4·2	14	1	84	1

Hb = Haemoglobin PCV = Packed cell volume RBC = Red blood cells
Rectics = Reticulocytes N = Neutrophils E = Eosinophils
L = Lymphocytes M = Monocytes

Values are means for the numbers of rats shown and those marked with asterisks differ significantly (Student's *t* test) from those of controls: $*P<0.05$, $**P<0.01$.

DISCUSSION

No effects were seen in rats given daily doses of 15 mg MPCA/kg/day for 13 wk. It is unlikely that the two deaths during the study were due to treatment. In both cases death occurred within minutes of dosing and respiratory distress was seen immediately after intubation. These observations, combined with the presence of oil in the respiratory tract, suggest that death was due to inadvertent intratracheal dosing. Similarly the finding of oil droplets in the pleura and pericardium with an associated pericarditis was probably

Table 3. *Mean relative organ weights of rats given daily oral doses of 0–150 mg MPCA/kg for 2, 6 or 13 wk*

Sex and dose level (mg/kg)	No. of rats examined	Relative organ weights (g/100 g body weight)												Terminal body weight (g)
		Brain	Heart	Liver	Spleen	Kidneys	Stomach	Small intestine	Caecum	Adrenals†	Gonads‡	Pituitary†	Thyroid†	
Wk 2														
Male														
0	5	0·94	0·47	3·90	0·43	0·93	0·68	4·13	0·46	26	1·11	5·0	—	177
50	5	0·91	0·44	3·83	0·41	0·88	0·69	4·23	0·45	29	1·20	4·9	—	181
150	5	0·96	0·44	3·87	0·43	0·92	0·67	4·35	0·44	27	1·19	5·1	—	176
Female														
0	5	1·14	0·53	3·88	0·46	0·94	0·78	4·74	0·56	35	98	7·0	—	143
50	5	1·08	0·44	3·92	0·42	0·90	0·85	4·47	0·49	38	106	7·0	—	153
150	5	1·13	0·64	4·11	0·46	0·94	0·85	4·52	0·57	35	108	6·1	—	147
Wk 6														
Male														
0	5	0·65	0·38	3·01	0·30	0·78	0·54	2·69	0·33	18	1·19	3·1	—	317
50	5	0·66	0·39	2·96	0·33	0·79	0·56	2·94	0·34	20	1·17	3·2	—	291*
150	5	0·69	0·38	3·06	0·30	0·82	0·59	2·81	0·35	19	1·20	3·1	—	278*
Female														
0	5	0·92	0·43	2·92	0·33	0·78	0·69	2·63	0·38	35	135	6·2	—	190
50	5	0·98	0·44	3·09	0·35	0·81	0·70	3·05	0·40	39	141	6·4	—	187
150	5	0·97	0·45	3·18	0·37	0·80	0·66	3·07	0·40	36	158	5·6	—	187
Wk 13														
Male														
0	15	0·47	0·29	2·58	0·19	0·58	0·46	2·32	0·26	15	0·85	2·5	4·0	411
15	14	0·45	0·28	2·53	0·18	0·58	0·46	2·25	0·24	15	0·88	2·3	4·6	405
50	15	0·45	0·30	2·73	0·19	0·62	0·46	2·24	0·25	16	0·87	2·4	4·6	422
150	15	0·45	0·30	2·78*	0·19	0·63**	0·47	2·31	0·25	15	0·85	2·4	4·4	418
Female														
0	15	0·71	0·34	2·48	0·25	0·62	0·56	2·84	0·37	27	56	4·6	6·4	252
15	15	0·71	0·35	2·49	0·26	0·62	0·55	2·81	0·30	27	55	4·1	7·3	255
50	14	0·66	0·34	2·50	0·25	0·62	0·55	2·71	0·27	27	61	4·7	6·2	260
150	15	0·69	0·35	2·51	0·25	0·61	0·54	2·66	0·31	27	59	4·7	6·8	258

†Values expressed in mg/100 g body weight.
‡Values for ovaries expressed in mg/100 g body weight.
Values are means for the numbers of rats shown and those marked with asterisks differ significantly (Student's t test) from those of controls: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$.

Table 4. *Mean values of renal concentration/dilution tests and urinary cell excretion in rats given daily oral doses of 0–150 mg MPCA/kg for 13 wk*

Sex and dose level (mg/kg/day)	Cells (10^3/hr)	Concentration test				Dilution test (2 hr)	
		Specific gravity		Volume (ml)			
		0–6 hr	16–20 hr	0–6 hr	16–20 hr	Specific gravity	Volume (ml)
Male							
0	2·7	1·059	1·061	1·8	0·6	1·006	7·5
15	3·6	1·056	1·071	1·5	0·9	1·006	6·5
50	2·7	1·060	1·065	1·9	1·0	1·006	6·8
150	3·4	1·053	1·070	1·5	0·8	1·008	7·8
Female							
0	2·0	1·061	1·067	0·7	0·5	1·007	5·5
15	2·8	1·049	1·066	0·8	0·5	1·006	5·9
50	2·3	1·055	1·064	0·9	0·7	1·007	5·6
150	1·7	1·054	1·066	0·6	0·4	1·008	5·3

Results are means for groups of 12 rats. No test values differed significantly (ranking method of White, 1952) from control values at wk 13. Renal concentration and dilution tests at wk 2 and 6 and at wk 6, respectively, similarly showed no differences between treated and control rats. Cell excretion was $7{\cdot}3.10^3$/hr at wk 6 in males given 150 mg/kg/day, compared with $4{\cdot}3.10^3$/hr in the control group.

caused by the introduction of the oily dose into the pleural cavity, possibly through small ruptures in the oesophagus.

The increased food and water intake in the males given the highest dose of MPCA (150 mg/kg) was accompanied by a slight but not statistically significant increase in the rate of body-weight gain. Similar slight increases in food and water consumption in rats given oral doses of high concentrations of flavouring agents have been encountered in other studies in these laboratories (Brantom, Gaunt, Grasso, Lansdown & Gangolli, 1972; Gaunt, Agrelo, Colley, Lansdown & Grasso, 1971). The reason for this effect is not known, but it is possible that dosing with highly flavoured materials during the day, when rats do not normally eat, may stimulate them to consume small quantities of food.

The isolated reticulum-cell tumour found in the lungs of a control female has no significance in the assessment of the toxicity of MPCA, but is of interest as it occurred in a very young (approximately 11-wk-old) rat.

The higher stomach weight in female rats given 50 or 150 mg/kg/day for 2 wk represented an increase of only approximately 10% of the control value and was not accompanied by any similar change in the males at the same time or in either sex at the later examinations. In addition, the differences were not statistically significant when expressed relative to body weight. These observations, together with the fact that there were no histopathological changes in the stomachs of the rats treated for 13 wk, suggest that the lower stomach weights found after 2 wk treatment were fortuitous. They were probably due to the small number of rats examined and were of no toxicological significance.

The reduced testis weights found at wk 6 occurred in animals with a body weight lower than that of the appropriate controls. The body weight of the five control animals was comparable with those of the remaining males in the study at the same time. Thus it would appear that the treated groups, randomly selected for these interim studies, consisted of rats that gained slightly less weight. This suggestion is supported by the fact that the relative testis weights were not significantly different from those of the controls. It is considered that the reduced testis weight was not related to treatment with MPCA, a fact supported by the lack of any similar effect at wk 2 or 13 and the absence of any histopathological change in the testis.

After treatment for 13 wk, the absolute and relative kidney weights of the male rats given 150 mg MPCA/kg/day were slightly increased. This was not associated with any histopathological change in the kidney nor was any functional defect detected. However, with a shorter period of treatment (6 wk), there was a slight increase in the number of cells excreted in the urine, an effect which has been related to renal tubular damage (Davies & Kennedy, 1967) and has been found to occur in animals without demonstrable histopathological damage in the kidney (Brown & Hardy, 1968; Gaunt, Colley, Grasso, Lansdown & Gangolli, 1968). Therefore it is possible that MPCA has a slightly toxic effect on the kidney at a level of 150 mg/kg/day. It was noticed that at the 50 mg/kg dose there was an increase in relative kidney weight, although the difference from controls was not statistically significant. In view of the possibility of renal toxicity at the higher level it would seem that there may be some marginal effect at this lower dose.

Increased absolute and relative liver weights were also seen in the males given 150 mg/kg/day for 13 wk. Again no abnormalities were found in histopathology or in the levels of serum enzymes. Elevations of liver weight have been attributed to increased activity of microsomal drug-metabolizing enzymes (Golberg, 1967) and the results of metabolic studies with methylphenylcarbinol (El Masry *et al.* 1956) have suggested that oxidation

and demethylation occur. As these processes would involve microsomal enzymes, it is possible that the slightly elevated liver weights seen in the present study may have been due to an increased metabolic load on the liver. However, in the absence of any confirmatory evidence, this increase in organ weight must, at present, be regarded as a toxic effect of MPCA. As with the kidney weights, the weight of the liver was increased in rats given 50 mg/kg, suggesting a marginal effect at this dose level.

From the results of this study the no-untoward-effect level for MPCA is 15 mg/kg/day. However, the changes seen at 50 mg/kg were not statistically significant and even at 150 mg/kg/day were not marked. This suggests that the true no-untoward-effect level lies somewhere between 15 and 50 mg/kg/day. From data supplied by seven of the leading flavouring manufacturers, it is calculated that the maximum likely intake of MPCA by man is 4·6–8·7 mg/day (0·06–0·15 mg/kg for a 60-kg adult). Thus, even at 15 mg/kg/day the no-untoward-effect level in this study is 100–200 times greater than the calculated maximum intake in man.

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Toxicité à court terme de l'acétate de méthylphénylcarbinyle chez le rat

Résumé—Pendant 13 semaines on a administré à des groupes de rats des doses orales quotidiennes de 0 (groupe témoin), 15, 50 ou 150 mg d'acétate de méthylphénylcarbinyle par kg de poids vif. Le gain de poids n'en a pas été influencé, malgré une plus forte consommation de nourriture et d'eau chez les rats mâles qui recevaient 150 mg/kg. Le poids relatif du foie et des reins a augmenté chez les rats mâles qui recevaient 50 ou 150 mg/kg/jour. Les rats mâles qui avaient reçu 150 mg/kg pendant 6 semaines ont excrété plus de cellules dans leur urine. On n'a observé aucune modification histopathologique qu'on puisse mettre en corrélation avec le traitement à l'acétate de méthylphénylcarbinyle. On conclut que le seuil d'indifférence de l'acétate de méthylphénylcarbinyle administré pendant 13 semaines à des rats se situe à 15 mg/kg.

Kurzzeittoxizität von Methylphenylcarbinylacetat in Ratten

Zusammenfassung—Gruppen von Ratten erhielten tägliche orale Dosen von O (Kontrolle), 15, 50 oder 150 mg Methylphenylcarbinylacetat/kg Körpergewicht auf die Dauer von 13 Wochen. Es war kein Einfluss auf die Geschwindigkeit der Körpergewichtszunahme festzustellen, obwohl der Futter- und Wasserverbrauch sich bei den männlichen Ratten, die 150 mg/kg erhielten, erhöhte. Das relative Leber- und Nierengewicht erhöhte sich bei männlichen Ratten, die 50 oder 150 mg/kg/Tag erhielten. Eine vermehrte Ausscheidung von Zellen war in dem Urin männlicher Ratten zu beobachten, die 6 Wochen lang 150 mg/kg erhalten hatten. Es waren keine histopathologischen Änderungen zu beobachten, die mit der Verabreichung von Methylphenylcarbinylacetat in Verbindung gebracht werden konnten. Daraus wurde der Schluss gezogen, dass die von nachteiligen Wirkungen freie Konzentration von Methylphenylcarbinylacetat bei Verabreichung an Ratten auf die Dauer von 13 Wochen 15 mg/kg betrug.

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INHIBITION OF MOUSE-LIVER MICROSOMAL ENZYME FUNCTION AFTER ORAL ADMINISTRATION OF SODIUM NITRITE

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Abstract—The effect of a single oral dose of sodium nitrite on mouse-liver microsomal-enzyme activity was studied. Sodium nitrite (100 mg/kg) produced a rapid and marked inhibition of both aminopyrine-demethylase and aniline-hydroxylase activity. Inhibitory effects of approximately 50% were observed as early as 15 min after treatment and were still persisting at 90 min. The responses at 45 min after treatment were dose-dependent, no response being observed at 25 mg/kg. Daily oral administration of 25 or 50 mg sodium nitrite/kg for 8 days produced no subacute inhibition of microsomal-enzyme activity. The mechanism of this response involves non-competitive inhibition of enzyme activity. While 300 mg sodium nitrite/kg had little effect on the K_m of aminopyrine demethylase, V was decreased from 2·35 to 1·54 μmols formaldehyde/g liver/hr.

INTRODUCTION

The potential human hazard of dietary exposure to nitrate and nitrite salts has become a matter of recent concern (Shuval & Gruener, 1972). The biological activity of sodium nitrite generally results from reaction between nitrite and iron in haem groups. For example, the acute toxicity of sodium nitrite is attributable to the oxidation of haemoglobin to methaemoglobin (Gruener & Shuval, 1969). Anaemic hypoxia and asphyxia follow excessive methaemoglobinaemia. Similarly, nitrite is used as a colour additive in meat preparations such as frankfurters (Wolff & Wasserman, 1972). Nitrite reacts with myoglobin to form nitrosylmyoglobin, which upon cooking retains its appealing red-pink colour. However, the reactivity of sodium nitrite with haem groups has raised the possibility that other relevant haem-containing enzyme systems may be sensitive to nitrite intoxication.

Liver-microsomal drug-metabolizing enzymes consist of a series of haem-containing proteins (Omura, Sato, Cooper, Rosenthal & Estabrook, 1965). Agents such as carbon monoxide, which interfere with haem groups on these proteins, consequently inhibit microsomal enzyme activity (Kuntzman, Levin, Jacobson & Conney, 1968).

It is the purpose of this communication to report the inhibitory effects of sodium nitrite on the mixed-function oxidase of liver microsomes.

EXPERIMENTAL

Animals. Male Swiss mice of the DUB (ICR) strain (body weight 20–30 g) were maintained on water and Purina chow and housed five in a shoe-box cage prior to sacrifice.

Enzyme assays. The effects of sodium nitrite on mouse-liver microsomal-enzyme activity were determined by assaying hepatic aminopyrine-demethylase and aniline-hydroxylase activities. Livers, from two mice (except for the kinetic study) were pooled and homogenized in 7 vols cold sucrose-phosphate buffer (Friedman, Arnold, Bishop & Epstein,

1971). Crude microsomes were prepared by centrifugation for 15 min at 15,000 **g**. Microsomal aniline-hydroxylase activity was quantitated by measuring *p*-aminophenol production (Kato & Gillette, 1965). Microsomal aminopyrine-demethylase activity was assayed by determining formaldehyde production (Friedman, Greene, Csillag & Epstein, 1972a).

Dose–response study. The dose–response characteristics were measured 45 min after sodium nitrite administration. Aqueous nitrite solutions were prepared in such concentrations that the desired dose would be delivered in a solvent volume of 5 ml/kg. Sodium nitrite was administered by stomach tube in doses of 200, 150, 100, 75, 50 and 25 mg/kg, while negative controls received water. Groups of ten mice were killed by cervical dislocation and the livers were assayed for microsomal-enzyme activity.

Time-dependence study. The time-dependence of the response of liver microsomal-enzyme activity was determined after administration of 100 mg sodium nitrite/kg by stomach tube. Groups of ten mice were killed 15, 30, 60 and 90 min after treatment. Livers were removed and assayed for aminopyrine-demethylase and aniline-hydroxylase activity.

Subacute study. The effect of multiple treatment with low levels of sodium nitrite was determined following daily oral administration of 50 or 25 mg sodium nitrite/kg. Control mice received water. Animals were treated for 8 days and were killed 45 min after the last dose. Livers were then taken for aminopyrine-demethylase and aniline-hydroxylase assays.

Enzyme kinetics. The effect of sodium nitrite on the kinetic constants of aminopyrine demethylase was determined 15 min after treatment with 300 mg sodium nitrite/kg. Livers from five mice were pooled and kinetic constants were determined on the pooled samples (Friedman *et al.* 1972a). These constants were determined, as described previously (Friedman *et al.* 1972a), from plotting v against $v/[S]$.

RESULTS

Dose response to sodium nitrite

The dose–response characteristics of mouse-liver microsomal enzymes are shown in Table 1. Dose–response characteristics measured 45 min after administration of sodium nitrite in a dose of 200, 150 or 100 mg/kg were tested in experiment no. 1. These doses

Table 1. *Dose-response of microsomal-enzyme activity to sodium nitrite given to mice in a single oral dose*

Experiment no.	Dose of $NaNO_2$ (mg/kg)	Enzyme activity (nmols product/g liver/min) Aminopyrine demethylase	Aniline hydroxylase†
1	0	1·90 ± 0·21	1·05 ± 0·14
	200	0·95 ± 0·07*	0·76 ± 0·10
	150	0·80 ± 0·10*	0·50 ± 0·09*
	100	0·90 ± 0·05*	0·66 ± 0·03*
2	0	2·09 ± 0·18	2·29 ± 0·19
	100	1·31 ± 0·30*	1·76 ± 0·09*
	75	1·67 ± 0·06*	1·94 ± 0·11
	50	1·65 ± 0·26	1·83 ± 0·07*
	25	1·95 ± 0·02	2·13 ± 0·09

†Mean activity × 10.

Values are means ± SEM for livers pooled in pairs from groups of ten mice killed 45 min after treatment and those marked with an asterisk differ significantly from the corresponding control value: $*P < 0·05$.

inhibited aminopyrine-demethylase activity to 50, 42 and 47% of the control value, respectively. Similarly, these doses inhibited aniline hydroxylase to 72. 48 and 63% of the control, respectively. All responses, with the exception of that of aniline hydroxylase with the 200 mg/kg dose, were statistically different from the control. Lower doses were tested in experiment no. 2. In the case of aminopyrine demethylase, inhibition to 63, 80, 79 and 93% of the control was observed with doses of 100, 75, 50 and 25 mg/kg, respectively. Of these, only the first two were statistically different from the control. Similarly, these doses produced inhibition of aniline hydroxylase to 77, 85, 80 and 93% of the control, respectively. Only dose levels of 100 and 50 mg/kg were statistically different from the control.

Time dependence of nitrite inhibition

The time course of the inhibition of microsomal-enzyme activity is shown in Table 2. Enzyme activity was measured 15. 30. 60 and 90 min after administration of 100 mg

Table 2. *Time course of effect of a single oral dose of 100 mg sodium nitrite/kg on microsomal-enzyme activity in the mouse*

Time after treatment (min)	Enzyme activity (nmols product/g liver/min)	
	Aminopyrine demethylase	Aniline hydroxylase†
—‡	2·52 ± 0·06	1·43 ± 0·20
15	1·58 ± 0·06*	0·72 ± 0·04*
30	1·34 ± 0·08*	0·64 ± 0·04*
60	1·62 ± 0·10*	0·66 ± 0·07*
90	1·40 ± 0·10*	0·57 ± 0·12*

†Mean activity × 10.
‡Untreated control group.
Values are means ± SEM for livers pooled in pairs from groups of ten mice and those marked with an asterisk differ significantly from the corresponding control value: *$P < 0·01$.

sodium nitrite/kg. In the case of aminopyrine-demethylase activity, the inhibition was maximal 30 min after treatment, when enzyme activity was 53% of the control value. However, inhibition at other times was marked and similar to that at 30 min. Inhibition of aniline-hydroxylase activity paralleled that of aminopyrine demethylase at each of the four times. when enzyme activities were 50. 45. 46 and 40% of the control. respectively.

Subacute effects

The subacute effect of sodium nitrite on microsomal-enzyme activity was determined by daily administration of low-effect levels of nitrite for 8 days prior to killing. This experiment was repeated three times with the results shown in Table 3. Although in experiment no. 1 there was a statistically significant inhibition of amincpyrine demethylase, this was not observed in experiments 2 and 3 and was not seen in the case of aniline hydroxylase.

Enzyme kinetics

In order to determine the mechanism of enzyme inhibition by sodium nitrite, Michaelis–Menton kinetics were determined 15 min after administration of 300 mg nitrite/kg. As can be seen in Table 4, sodium nitrite lowered the V of aminopyrine demethylase by 35%, while the K_m was not statistically different from the control.

Table 3. *Effect of multiple treatment with eight daily oral doses of sodium nitrite on microsomal-enzyme activity in the mouse*

Experiment no.	Dose level of $NaNO_2$ (mg/kg/day)	No. of mice/group	Enzyme activity (nmols product/g liver/min)	
			Aminopyrine demethylase	Aniline hydroxylase†
1	0	10	2·10 ± 0·08	—
	50	10	1·41 ± 0·06*	—
	25	10	1·61 ± 0·11*	—
2	0	12	1·28 ± 0·07	0·79 ± 0·06
	50	12	1·40 ± 0·12	0·70 ± 0·04
	50	12	1·41 ± 0·06	0·85 ± 0·06
3	0	12	3·57 ± 0·27	0·52 ± 0·03
	50	12	3·59 ± 0·04	0·56 ± 0·05
	25	12	3·55 ± 0·02	0·49 ± 0·04

†Mean activity × 10.

Values are means ± SEM for livers pooled in pairs from groups of mice killed 45 min after the eighth dose and those marked with an asterisk differ significantly from the corresponding control value: $*P < 0·01$.

Table 4. *Effect of a single oral dose of 300 mg sodium nitrite/kg on the kinetics of mouse-liver microsomal aminopyrine demethylase*

Treatment	No. of determinations	K_m (aminopyrine concn, mM)	V (μmols HCHO/g liver/hr)
Water	8	1·64 ± 0·19	2·35 ± 0·16
$NaNO_2$	5	1·93 ± 0·68	1·54 ± 0·18*

Values are means ± SEM for the stated number of determinations on crude microsomes isolated from the pooled livers from groups of five mice killed 15 min after oral treatment with water (control group) or 300 mg sodium nitrite/kg. The value marked with an asterisk differs significantly from the control figure: $*P < 0·01$.

DISCUSSION

Oral administration of sodium nitrite in a single dose as low as 50 mg/kg inhibited mixed-function oxidase of mouse-liver microsomes. Inhibition of the activity of both aminopyrine demethylase, a Type I enzyme, and aniline hydroxylase, a Type II enzyme, was observed. Generally, the magnitude of the response of each enzyme was similar, suggesting that nitrite showed little preference between them. Onset of the inhibition was both rapid and marked: within 15 min the inhibitory effects were nearly maximal. This rapid action might have been anticipated since nitrite is rapidly absorbed from the mouse stomach (Friedman, Greene & Epstein, 1972b). Approximately 84% of administered sodium nitrite is absorbed within the first 10 min, and of that 38% is absorbed within 1 min. As in the dose–response experiments, both enzymes responded in a similar fashion to sodium nitrite in the time-dependence study. The suppression of mixed-function oxidase of mouse-liver microsomes does not appear to be cumulative in nature. In two out of three experiments, no inhibition was observed after eight daily oral doses of sodium nitrite. The inhibition

in the third experiment may have been the result of a more sensitive animal population or it may perhaps have represented a variation in the response.

The suppression of aminopyrine-demethylase activity is non-competitive, involving a suppression of V. This non-competitive suppression may result from a direct interaction between sodium nitrite and microsomal haem groups of microsomal protein. This hypothesis is further supported by the observation that the extent of inhibition of aniline hydroxylase and aminopyrine demethylase are quantitatively similar. There appears to be little selectivity among the enzymes. Moreover, the rapidity of the response is in accord with a direct action of sodium nitrite on the microsomal-enzyme system, rather than a secondary action in response to nitrite intoxication. The possibility that nitrite alters the availability of necessary microsomal cofactors and that this may be a potential cause for the inhibition may be eliminated, since all necessary precursors and cofactors were supplied *in vitro*.

An alternative possibility to explain these data is that microsomal-enzyme inhibition is mediated by nitrosamine production. Nitrosamines inhibit microsomal-enzyme activity in a fashion similar to that of nitrite (Couch, McClanahan & Friedman, 1973). Sodium nitrite reacts with amines in the mouse stomach to produce nitrosamines (Friedman, Millar & Epstein, 1973). These nitrosamines may then inhibit microsomal drug-metabolizing enzyme systems. This explanation is unlikely because nitrite inhibits dimethylnitrosamine-induced mutagenicity (Couch *et al.* 1973). If the effects of nitrite were nitrosamine-mediated, a stimulation in dimethylnitrosamine mutagenicity would be anticipated.

The significance of these findings merits further consideration. Young children are exposed to very high nitrite levels as a result of low methaemoglobin-reductase activity and elevated gastric pH, which allows gastric conversion of dietary nitrate to nitrite (Shuval & Gruener, 1972). This exposure may result in impaired drug detoxification and altered steroid metabolism.

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Inhibition de l'activité des enzymes microsomiques du foie chez la souris après administration orale de nitrite de sodium

Résumé—On a étudié l'effet d'une dose orale unique de nitrite de sodium sur l'activité des enzymes microsomiques du foie chez la souris. Le nitrite de sodium (100 mg/kg) a provoqué rapidement une inhibition marquée de l'activité de l'aminopyrine-déméthylase et de celle de l'aniline-hydroxylase. Déjà 15 min après l'administration on a observé un effet d'inhibition d'environ 50%, qui persistait encore à 90 min. Les réactions à 45 min étaient en corrélation avec le dosage, aucune réaction n'étant observée avec 25 mg/kg. L'administration orale de 25 ou 50 mg de nitrite de sodium par kg de poids vif et par jour poursuivie pendant 8 jours n'a pas provoqué d'inhibition subaiguë de l'activité des enzymes microsomiques. Le mécanisme de cette réaction implique une inhibition non compétitive de l'activité enzymatique. Tandis que 300 mg de nitrite de sodium/kg n'ont eu que peu d'effet sur le K_m de l'aminopyrine-déméthylase, V diminuait de 2.35 à 1.54 μmols de formaldéhyde par g de foie et par h.

Hemmung der Mikrosomenenzymfunktion der Mäuseleber nach oraler Verabreichung von Natriumnitrit

Zusammenfassung—Die Wirkung einer oralen Einzeldosis von Natriumnitrit auf die Mäuseleber-Mikrosomenenzymaktivität wurde untersucht. Natriumnitrit (100 mg/kg) verursachte eine schnelle und markante Hemmung der Aminopyrindemethylase- und Anilinhydroxylaseaktivität. Es wurden inhibitorische Effekte von etwa 50% schon 15 min nach der Verabreichung beobachtet, und sie hielten noch 90 min nach der Verabreichung an. Die Reaktionen 45 min nach der Verabreichung waren dosisabhängig, und bei 25 mg/kg wurde keine Reaktion beobachtet. Die 8 Tage lang erfolgende tägliche orale Verabreichung von 25 oder 50 mg Natriumnitrit/kg verursachte keine subakute Hemmung der Mikrosomenenzymaktivität. Der Mechanismus dieser Reaktion involviert eine nichtkompetitive Hemmung der Enzymaktivität. Während 300 mg Natriumnitrit/kg eine geringe Auswirkung auf den K_m-Wert der Aminopyrindemethylase hatten, wurde V von 2,35 auf 1,54 μmol Formaldehyd/g Leber/hr vermindert.

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DIGESTIBILITY OF ACETYLATED DISTARCH GLYCEROL—EFFECT ON GROWTH, SERUM BIOCHEMICAL VALUES AND BODY COMPOSITION OF PITMAN–MOORE MINIATURE PIGS*

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Abstract—Two groups each of eight Pitman–Moore miniature pigs were weaned at 3 days of age and then, for 25 days, were allowed unrestricted access to formula diets identical except for the type of carbohydrate. The diets contained 6% of thin-boiling waxy corn starch or acetylated distarch glycerol. At 14 and 21 days of age, the body weight of pigs fed the control starch diet was significantly higher, while at 28 days, the weight of the empty caecum and the water content of the wet carcass and fat-free wet liver were significantly lower and the carcass-fat and liver-protein contents were significantly greater than the corresponding values for pigs fed the diet containing acetylated distarch glycerol as the sole carbohydrate.

INTRODUCTION

In a recently reported feeding study in miniature pigs, a thin-boiling waxy corn starch or one of three chemical modifications of this starch provided the sole source of dietary carbohydrate (Anderson, Filer, Fomon, Andersen, Jensen & Rogers, 1973). No statistically significant effect related to treatment was observed in respect of growth, biochemical values of serum, or carcass or liver composition. This report is concerned with an evaluation of the digestibility of another chemical modification of the control starch used in our previous study. A similar protocol was used in both studies.

EXPERIMENTAL

Starches and diets

Sixteen 3-day-old Pitman–Moore miniature pigs were randomly assigned to one of two diets (Table 1), provided through the co-operation of Dr. G. A. Purvis, Gerber Products Company, Fremont, Mich. These diets consisted of water, co-precipitated milk protein (Crest-O-Lac, Type CaS8K, Crest Foods Company, Inc., Ashton, Ill.), corn oil, starch, vitamins and minerals. Proximate analyses confirmed that the diets were nearly identical to one another in the distribution of calories from protein, fat and carbohydrate. The content of vitamins and minerals was that recommended by Schneider & Sarett (1966).

The thin-boiling waxy corn starch and the cross-linked modification of the thin-boiling control starch were supplied by Mr. O. B. Wurzburg, National Starch and Chemical Corp.,

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Table 1. *Proximate analysis of diets containing unmodified or chemically modified thin-boiling waxy corn starch*

Dietary component	Content (% w/w)* in diet no.	
	1	2
Water	82·0	81·7
Protein	6·1 (26)	6·4 (27)
Fat	5·0 (48)	5·1 (48)
Carbohydrate†	6·0 (26)	6·0 (25)
Ash	0·9	0·8
Calcium	0·170	0·158
Phosphorus	0·129	0·111
Caloric value (kcal/100 g)	93	96

*Figures in parentheses denote the percentage of total kcal.
†Source of dietary carbohydrate was acid thin-boiling waxy corn starch: unmodified (diet 1); modified with epichlorohydrin cross-links and stabilized by acetylation with acetic anhydride (diet 2).

Plainfield, N.J. The thin-boiling waxy corn starch was made by treating waxy corn starch with hydrochloric acid in accordance with the Code of Federal Regulations (21 CFR 121.1031(a), revised 1 January 1972). The resulting starch had a water fluidity of 80. The acetylated distarch glycerol was prepared in compliance with 21 CFR 121.1031(f) by treatment with 0·05% epichlorohydrin and acetic anhydride to an acetyl content of 1·1%. The resulting cross-linked starch had a degree of substitution of approximately 0·04.

Experimental procedure

Each diet was fed *ad lib.* for 25 days to a group of eight pigs. Because the formula diet containing modified starch was somewhat more viscous than that containing the control starch, all pigs were allowed unrestricted access to water. At 28 days of age, each pig was weighed and bled from the anterior vena cava. All animals were killed by intracardiac injection of sodium pentobarbitone and immediately eviscerated. Methods used for the biochemical analyses of serum and the procedures for preparing the carcass for chemical analyses have been described in detail elsewhere (Filer, Fomon, Anderson, Andersen, Rogers & Jensen, 1973).

Data were analysed by one-way analysis of variance as described by Snedecor (1956).

RESULTS AND DISCUSSION

Performance

As may be seen from Table 2, the body weight of pigs fed the control starch was significantly greater at 14 and 21 days of age than that of pigs fed the diet containing acetylated distarch glycerol. Gain in body weight between 7 and 28 days of age was approximately 15% less in pigs fed the modified starch than in pigs fed the control starch, but this difference was not statistically significant. The slight weight loss observed between 3 and 7 days of age was probably due to the stress of weaning (adapting to a new diet, isolation). As mentioned previously, the viscosity of the formula diet containing acetylated distarch glycerol was greater than that of the control starch diet and may have influenced the acceptability of the diet during the early phases of the study. The greater weight loss between 3 and 7 days in pigs fed the modified starch-formula diet was thought to be due to the rela-

Table 2. *Effect of carbohydrate source on body weights of miniature pigs*

Age (days)	Mean body weights (g) of pigs fed diet no. 1	2	Standard error of the mean
3	1303	1252	65·6
7	1211	1101	43·3
14	1841*	1467	111·2
21	2860*	2318	178·2
28	4099	3530	246·3
Gain (days 7–28)....	2889	2429	228·2

Values are means for groups of eight pigs and those marked with an asterisk are significantly higher than the corresponding value for the other group: $*P < 0.05$.

tive unpalatability of the diet and may have accounted, in part, for the significantly lower body weights.

The gain in body weight between 7 and 28 days of age in both groups of pigs in the present study was greater than the average gain (1900–2000 g) over the same period noted in the previous study (Anderson *et al.* 1973). The depressed weight gain of pigs in the first study was thought to be due to a less than optimum level of dietary calcium.

As mentioned in the previous report, no attempt was made to measure feed efficiency because of the viscous nature of the starch-formula diets and the problems encountered in measuring wasted feed. However, estimates of food intake during the 25-day feeding period suggested that pigs fed the diet containing acetylated distarch glycerol consumed approximately 20% less food than pigs fed the control-starch diet. The relative unpalatability of the more viscous diet apparently influenced growth during the early phases of the study, but later in the feeding period (days 21–28; Table 2) pigs fed diet 2 appeared to have adapted to the diet and grew at a rate comparable with that of pigs fed diet 1.

Serum chemical values

With the exception of a higher serum concentration of inorganic phosphorus in pigs fed diet 1, no differences due to treatment were observed in any of the serum chemical values

Table 3. *Effect of carbohydrate source on serum chemical values of 28-day-old miniature pigs*

Parameter	Mean values (per 100 ml serum) for pigs on diet no. 1	2	Standard error of the mean
Cholesterol (mg)	82	70	5·6
Triglyceride (mg)	93	82	4·7
Calcium (mg)	11·2†	11·2	0·11
Phosphorus (mg)	10·3*	8·9	0·36
Alkaline phosphatase (auto analyser units)	84·2	73·0	9·44
Urea nitrogen (mg)	28·7	28·6	1·43
Total protein (g)	5·2	5·2	0·11
Albumin (g)	3·3	3·2	0·05
Globulin (g)	2·0	1·9	0·09

†Mean value for seven pigs; all others are means for eight animals.

The value marked with an asterisk is significantly higher than that for the other group: $*P < 0.05$.

(Table 3). Although the concentration of phosphorus was approximately 10% higher in diet 1 than in diet 2, it seems unlikely that such a minor difference in diet would have been responsible for a statistically significant difference in serum concentrations. Concentrations of cholesterol and triglyceride in the sera of the starch-fed pigs were lower than in our previously studied sow-reared pigs (Filer *et al.* 1973). In contrast to sows' milk, the starch diets were cholesterol-free and contained highly unsaturated fat. The relationship between dietary cholesterol and serum cholesterol in the young animal has previously been discussed (Anderson *et al.* 1973).

The concentration of calcium in the serum was somewhat higher than that observed in our previous study (Anderson *et al.* 1973), possibly reflecting the higher concentrations of calcium in the diets used in the later study. Concentrations of urea nitrogen in the serum were similar to those reported in the previous study of starch-containing diets (Anderson *et al.* 1973) and were again greater than those anticipated on the basis of our previous study of sow-reared pigs (Filer *et al.* 1973).

Organ weights

Statistically significant treatment-related effects on organ weights, expressed as a percentage of body weight, were demonstrated only for the empty caecum, which was significantly heavier in pigs fed the diet containing acetylated distarch glycerol (Table 4). All organs appeared to be grossly normal when inspected at autopsy. At 28 days of age, liver weight accounted for a slightly smaller percentage of body weight than had been the case in our previous studies (Filer, Fomon, Anderson, Nixt & Andersen, 1974). The weights of other organs expressed as a percentage of body weight were similar in this and the earlier studies.

Table 4. *Effect of carbohydrate source on organ weights of miniature pigs*

Organ	Mean organ weights (% of body weight) of pigs fed diet no.		Standard error of the mean
	1	2	
Thyroid	0·015	0·018	0·0012
Adrenals	0·012	0·014	0·0007
Spleen	0·14	0·16	0·008
Kidneys	0·78	0·80	0·038
Heart	0·51	0·57	0·027
Liver	4·10	4·19	0·177
Caecum			
With contents	0·63	0·82	0·080
Without contents	0·15	0·22**	0·015

Values are means for groups of eight pigs and that marked with asterisks is significantly higher than those of other groups: $^{**}P < 0{\cdot}01$.

Carcass composition

The percentage of water was significantly greater and that of protein significantly less in the carcasses of pigs fed diet 2 than in those of pigs fed diet 1 (Table 5). In our previous study, the concentration of water was significantly higher in the carcasses of pigs fed a diet containing starch modified with sodium trimetaphosphate cross-links and stabilized with sodium tripolyphosphate than in those of pigs fed the unmodified control starch. Although the present diets contained more calcium than those fed in our previous study, carcass cal-

Table 5. *Effect of carbohydrate source on carcass composition of miniature pigs*

Component		Carcass concn in pigs fed diet no. 1	Carcass concn in pigs fed diet no. 2	Standard error of the mean
	Carcass weight			
	Wet (g)	3122	2599	184·2
	Fat-free wet (g)	2613	2257	145·4
	Wet carcass			
Water (%)		67·0	69·9*	0·75
Fat (%)		16·0*	13·0	0·72
Protein (%)		14·2	14·3	0·36
Ash (%)		2·8	2·7	0·11
Minerals (mg/100 g)				
Calcium		726	670	55·3
Phosphorus		480	460	19·2
Potassium		197	211**	3·0
Sodium		125	130	2·8
Magnesium		27	26	1·1
	Fat-free wet carcass			
Water (%)		79·8	80·4	0·43
Protein (%)		16·9	16·4	0·45
Ash (%)		3·3	3·1	0·12
Minerals (mg/100 g)				
Calcium		862	767	61·6
Phosphorus		566	530	20·1
Potassium		235	243	3·8
Sodium		148	149	2·6
Magnesium		32	30	1·4

Values, expressed in the units indicated in column 1, are means for groups of eight pigs and those marked with asterisks are significantly higher than the corresponding value for the other group: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$.

Table 6. *Effect of carbohydrate source on liver composition of miniature pigs*

Component		Liver content (%) in pigs fed diet no. 1	Liver content (%) in pigs fed diet no. 2	Standard error of the mean
	Liver weight			
	(g)	168	150	14·0
	(% of body weight)	4·10	4·19	0·177
	Wet liver			
Water		77·3	78·7	0·41
Fat		5·3	5·2	0·29
Protein		16·1*	14·8	0·38
Ash		1·2	1·2	0·03
	Fat-free wet liver			
Water		81·7	83·0*	0·40
Protein		17·0*	15·6	0·39
Ash		1·3	1·3	0·03

Values are means for groups of eight pigs and those marked with an asterisk are significantly higher than the corresponding value for the other group: $*P < 0{\cdot}05$.

cium did not differ appreciably between the two studies. The potassium content was significantly greater in the wet, but not in the fat-free wet, carcasses of pigs fed the diet containing modified starch. Comparison of the mineral content of carcasses of starch-fed *v.* sow-fed has previously been discussed (Anderson *et al.* 1973).

Liver composition

The protein content of the wet and fat-free wet liver of pigs fed the control starch diet was significantly greater than that of pigs fed the diet containing modified starch (Table 6). This effect appeared to be the result of a lower water content in the livers of the control pigs rather than the result of differences in fat or ash content. The effect of treatment on the water content was similar in fat-free liver and wet carcass. The water content of the liver of pigs in the present study was somewhat greater and that of protein was somewhat less than the levels in the pigs studied in the first starch-feeding experiment.

CONCLUSION

Under the conditions of this study, the body weight of young Pitman–Moore pigs fed a 'thin-boiling' waxy corn starch chemically modified as described in Sec. 121.1031(f) of Title 21 of the Code of Federal Regulations was significantly lower at 14 and 21 days, while the water content of the wet carcass and fat-free wet liver was significantly greater and the carcass-fat and liver-protein contents were significantly lower than the corresponding values for pigs fed a diet containing the control starch as the sole source of dietary carbohydrate. With the exception of these parameters, growth, blood chemistry and the composition of liver and carcass were similar over a 25-day feeding period whether the pigs were fed a 'thin-boiling' waxy corn starch or acetylated distarch glycerol.

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Digestibilité du glycérolé d'amylopectine acétylée. Effet sur la croissance, les caractéristiques biochimiques du sérum et la composition des tissus chez le porc miniature Pitman–Moore

Résumé—On a sevré à l'âge de 3 jours deux groupes de chacun 8 porcs miniatures Pitman–Moore, puis on leur a servi *ad libitum* pendant 25 jours des rations de formules identiques, au type d'hydrate de carbone près. Les formules comportaient 6% de maïs cireux à ébullition fine ou un glycérolé d'amylopectine acétylée. Le poids vif des porcs soumis au régime à l'amidon témoin était significativement plus élevé, aux âges de 14 et de 21 jours, que celui des porcs qui recevaient la formule dans laquelle le glycérolé d'amylopectine acétylée était le seul hydrate de carbone; à l'âge de 28 jours le poids du caecum vide et la teneur en eau des carcasses humides et du foie humide dégraissé étaient significativement plus basse et les teneurs en graisse de la carcasse et en protéines du foie significativement plus élevées chez les porcs du premier groupe que chez ceux de second.

Verdaulichkeit von acetyliertem Distärkeglycerin—Einfluss auf Wachstum, biochemische Serumwerte und Körperzusammensetzung von Pitman–Moore-Zwergschweinen

Zusammenfassung—Zwei Gruppen von je acht Pitman–Moore-Zwergschweinen wurden im Alter von 3 Tagen abgesetzt und hatten dann 25 Tage lang uneingeschränkten Zugang zu einem die Saumilch ersetzenden Futter, das für beide Gruppen gleich mit Ausnahme des Kohlehydrattyps war. Das Futter enthielt 6% dünnkochender Maisstärke oder acetylierten Distärkeglycerin. Am 14. und 21. Lebenstage was das Körpergewicht der Ferkel, welche das Stärkekontrollfutter erhalten hatten, signifikant höher; am 28. Lebenstag waren das Gewicht des leeres Blinddarmes und der Wassergehalt des frischgeschlachteten Tiers und der frischentnommenen fettfreien Leber signifikant geringer und der Körperfett- und der Leberproteingehalt signifikant höher als die entsprechenden Werte bei Ferkeln, welche das acetylierten Distärkeglycerin als das einzige Kohlehydrat enthaltende Futter erhalten hatten.

Fd Cosmet. Toxicol. Vol. 12, pp. 209–218. Pergamon Press 1974. Printed in Great Britain

IN VITRO ANALYSIS OF TRANSPORT OF 2,4,5-TRICHLOROPHENOXYACETIC ACID BY RAT AND DOG KIDNEY

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Abstract—Renal transport of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) was studied quantitatively *in vitro* using renal cortical slices from dogs and rats, in an attempt to explain species differences in the biological half-life of the compound. Addition of 2,4,5-T to slices of rat renal cortex competitively inhibited active transport of *p*-aminohippuric acid without altering transport of the organic cation, *N*-methylnicotinamide. Renal cortical slices from rats and dogs actively accumulated 2,4,5-T. Accumulation was oxygen dependent and saturable, and was reduced in the presence of other anions (*p*-aminohippurate and probenecid). A reduction in the potassium concentration of the medium reduced accumulation of 2,4,5-T by rat tissue but not by dog tissue. Acetate in the medium increased accumulation of the herbicide in dog but not in rat tissue. Finally, the ability of renal tissue from newborn rats to accumulate 2,4,5-T was significantly less than that of adult tissue. It is concluded that the primary route of renal elimination of 2,4,5-T is active secretion of the compound. The greater ability of adult rat tissue to transport PAH explains the shorter biological half-life of 2,4,5-T in this species.

INTRODUCTION

2,4,5-Trichlorophenoxyacetic acid (2,4,5-T) is a plant-growth regulator and herbicide of relatively low toxicity, a property apparently due in part to its rapid excretion via the kidneys. 2,4,5-T is more toxic to dogs than to rats. Piper, Rose, Leng & Gehring (1973) demonstrated that the rate of renal excretion of 2,4,5-T was greater in rats than in dogs and suggested that this difference could account for the differences in toxicity.

2,4,5-T is an organic acid and as such might be excreted both by glomerular filtration and by active secretion into the urine. Dybing & Kolberg (1967) observed that a congener of 2,4,5-T, dichlorophenoxyacetate (2,4-D), interfered with renal tubular transport of *p*-aminohippurate (PAH), which could indicate competition between the compounds for a common transport system. Fang, Fallin, Montgomery & Freed (1973) demonstrated that the biological half-life of 2,4,5-T in newborn rats was markedly greater than that in adults. Inasmuch as renal function in general, and anion transport specifically, is immature in newborn rats (Kim, Hirsch & Hook, 1972), these data also support the presumption that the biological half-life of 2,4,5-T could be related to the ability or inability of an animal to excrete the drug. It was therefore of interest to quantify the renal handling of 2,4,5-T

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by rats and dogs. To minimize possible differences in excretion due to differences in blood flow, volume of distribution and other factors, an *in vitro* technique was employed to estimate differences in intrinsic transport capacity.

EXPERIMENTAL

Preparation and incubation of cortical slices. Dogs were anaesthetized with pentobarbitone (30 mg/kg, iv) and male Sprague-Dawley rats were stunned by a blow on the head. Kidneys were quickly removed and placed in ice-cold saline (0·9% NaCl). Renal cortical slices were prepared free-hand and kept briefly in cold saline until incubated. Slices were incubated in 2·7 ml phosphate buffer (Cross & Taggart, 1950) containing varying concentrations of the compounds being measured. Incubations were carried out in duplicate in a Dubnoff apparatus at 25°C under a gas phase of 100% oxygen at pH 7·4. The duration of incubation was 90 min, except when the rate of uptake was being determined, when incubation times were 2 and 12 min. Following incubation, the slices were quickly removed from the beakers, blotted and weighed. Both the tissue and a 2 ml aliquot of medium were treated as outlined by Cross & Taggart (1950) and Hook & Munro (1968).

Estimations. PAH was estimated by the method of Smith, Finkelstein, Aliminosa, Crawford & Graber (1945), while 1 ml of slice and medium homogenate was added to 10 ml of modified Bray's solution (2·5 g 2,5-diphenyloxazole and 100 g naphthalene/litre dioxane) and the amount of ^{14}C was counted in a Beckman LS-100 liquid scintillation counter. Results were expressed as slice/medium (S/M) ratio where S = mg/g or dpm/g tissue and M = mg/ml or dpm/ml medium.

Effect of 2,4,5-T on PAH transport. The effect of 2,4,5-T on the transport of other organic ions was determined by adding the herbicide to beakers containing rat-kidney cortical slices and $7{\cdot}4 \times 10^{-5}$ M-PAH and $6{\cdot}0 \times 10^{-6}$ M-*N*-methylnicotinamide ([^{14}C]NMN). The latter concentration was equivalent to $2{\cdot}5 \times 10^{-2}$ μCi/ml. Slices were incubated for 90 min in the presence of increasing concentrations of 2,4,5-T. To characterize the type of inhibition of PAH transport produced by 2,4,5-T, the effect of the compound on the rate of uptake of PAH into rat-kidney slices was determined. Kidneys from five rats were pooled, slices were prepared and equally divided into two groups of 12 beakers. Duplicate incubations were conducted at three PAH concentrations (1, 4 and 8×10^{-4} M). The uptake of PAH at each concentration was determined after incubation for 2 and 12 min. The difference in uptake between 2 and 12 min divided by 10 was used as the rate of uptake of PAH/min at that concentration. The rate of uptake at each concentration was determined and the data were plotted on a Hofstee plot. Similar experiments were then conducted in the presence of $2{\cdot}5 \times 10^{-5}$ M-2,4,5-T.

Accumulation of 2,4,5-T by renal cortical slices of the rat and dog. The ability of cortical slices from rats and dogs to accumulate 2,4,5-T[^{14}C] was first determined at a constant incubation-medium concentration of $2{\cdot}5 \times 10^{-5}$ M-2,4,5-T. Tissues were incubated from 5 to 120 min under oxygen or nitrogen. In subsequent experiments the tissues were incubated for 90 min and the concentration of 2,4,5-T was varied from $2{\cdot}6 \times 10^{-6}$M to $1{\cdot}3 \times 10^{-3}$ M. The accumulation process for 2,4,5-T was characterized by determining the effect of adding transport-system inhibitors (PAH and probenecid) to the incubation medium. The effect of lowered potassium in the medium was determined by decreasing the potassium concentration from 20 to 5 mM. In addition, the effect of sodium acetate (final concentration 10^{-2} M) was determined.

Accumulation of 2,4,5-T by renal tissue from newborn rats. The ability of renal cortical slices from 10-day-old rats to accumulate 2,4,5-T[^{14}C] was determined at incubation times varying from 5 to 120 min. In each experiment two or three litters of animals were required for a complete time course of observations.

RESULTS

Addition of 2,4,5-T to incubation beakers containing rat-kidney cortical slices, together with PAH and NMN produced a dose-related depression of PAH accumulation (Fig. 1). NMN accumulation, on the other hand, was not significantly affected by the herbicide. No effect of 2,4,5-T was seen at 1×10^{-5} M but a 2×10^{-5} M concentration reduced PAH accumulation by approximately 40%. At 5×10^{-5} M nearly 75% of the uptake of PAH was inhibited. Inhibition of PAH uptake by 2,4,5-T appeared to be competitive in nature, in that the estimated maximal velocity of uptake of PAH into rat-kidney cortical slices was not influenced by the herbicide (Fig. 2). Only the apparent K_m appeared to be altered by the compound.

Renal cortical slices from both rat and dog appeared actively to accumulate 2,4,5-T (Figs 3 & 4). In rat tissue, the 2,4,5-T S/M ratio increased with incubation time from 5 to 90 min and appeared to plateau between 90 and 120 min (Fig. 3). In the presence of nitrogen the S/M ratio approached 2. A similar pattern was seen in dog tissue (Fig. 4), but there was a temporal and quantitative difference. In dog tissue, a plateau was attained after incubation for 60 min. In rat tissue the peak S/M ratio was about 14, whereas in dog-kidney slices the highest ratio obtained was about 8·6. As in rat tissue, the S/M ratio for 2,4,5-T in dog slices in the presence of nitrogen was around 2.

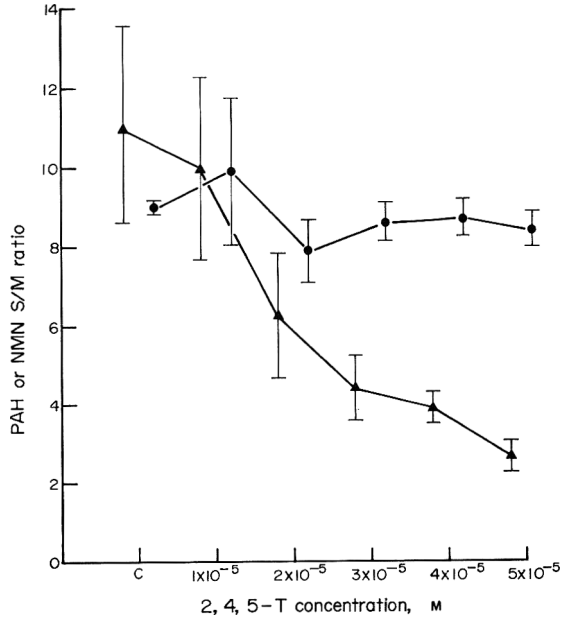

Fig. 1. Effect of 2,4,5-T on accumulation (S/M ratio) of PAH (▲) and NMN (●) by rat-kidney cortical slices. Points represent means ± SEM of three separate experiments.

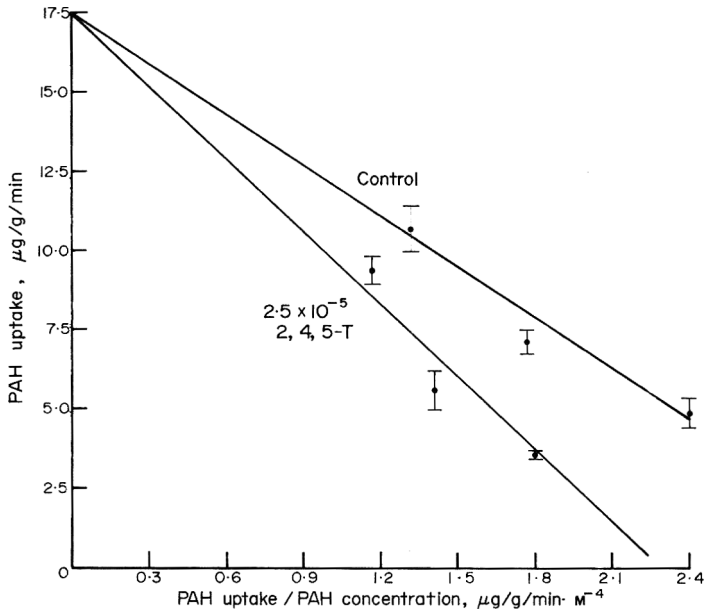

Fig. 2. Hofstee plot representing the effect of $2{\cdot}5 \times 10^{-5}$ M-2,4,5-T on PAH uptake by rat-kidney cortical slices. Slices from five rats were pooled and incubated at three PAH concentrations for 2 and 12 min. Uptake represents rate of uptake/min between 2 and 12 min. Points represent the mean ± SEM of four experiments. Lines were drawn by inspection.

In both rat and dog tissue, the 2,4,5-T S/M ratio declined with increasing concentration of the anion in the medium (Fig. 5). The 2,4,5-T S/M ratio was inhibited by both PAH and probenecid in tissue from both species (Figs 6 & 7). Probenecid produced a dose-related depression of 2,4,5-T accumulation in tissue from both species, whereas only the highest concentration of PAH (10^{-4} M) appeared to inhibit accumulation. In rat tissue

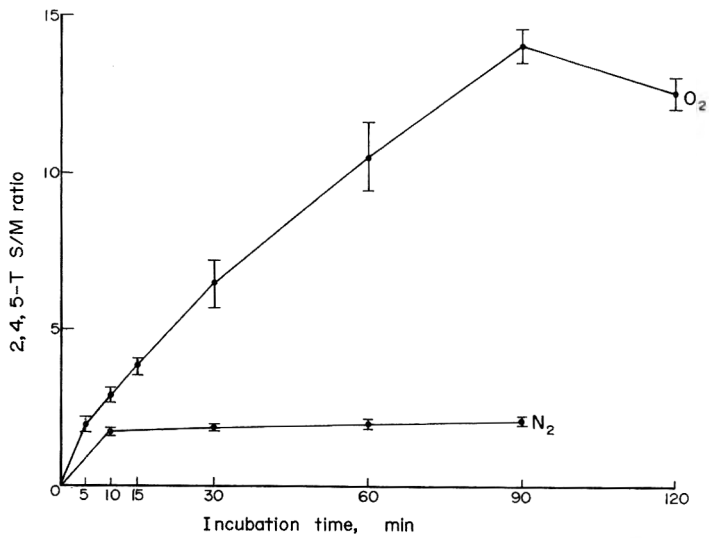

Fig. 3. Accumulation (S/M ratio) of 2,4,5-T[^{14}C] in a concentration of $2{\cdot}5 \times 10^{-5}$ M by rat-kidney cortical slices under oxygen and nitrogen. Points represent means ± SEM of three experiments.

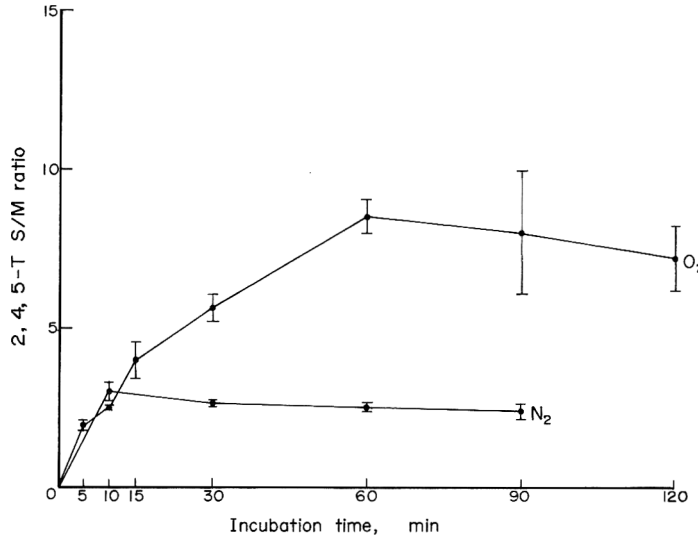

Fig. 4. Accumulation (S/M ratio) of 2,4,5-T[^{14}C] in a concentration of 2·5 × 10^{-5} M by dog-kidney cortical slices under oxygen and nitrogen. Points represent means ± SEM of three experiments.

reduction of the medium concentration of potassium to 5 mM produced a significant depression of the S/M ratio (Fig. 6) whereas in dog tissue no difference was seen (Fig. 7). Addition of 10^{-2} M sodium acetate to the medium had no significant effect on accumulation of 2,4,5-T by rat tissue (Fig. 6) whereas with dog tissue the S/M ratio was markedly enhanced (Fig. 7).

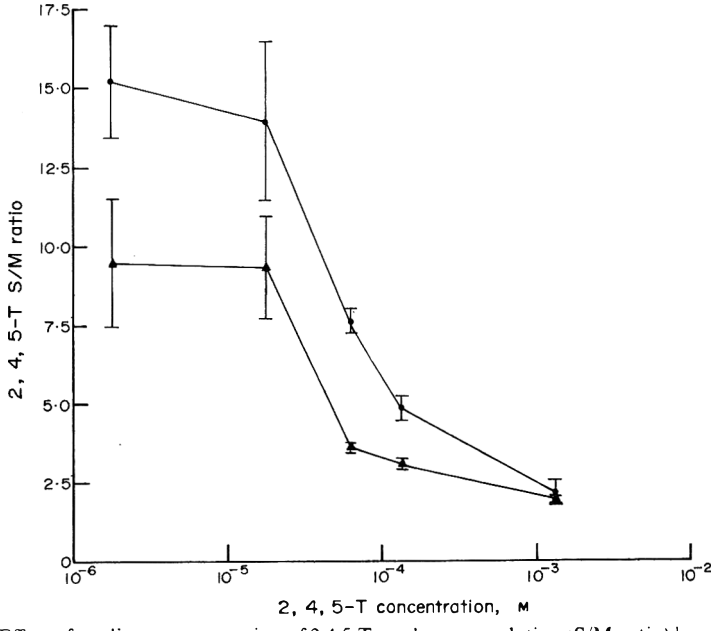

Fig. 5. Effect of medium concentration of 2,4,5-T on the accumulation (S/M ratio) by renal cortical slices of the rat (●) and dog (▲) under oxygen (90 min). Points represent means ± SEM of three experiments.

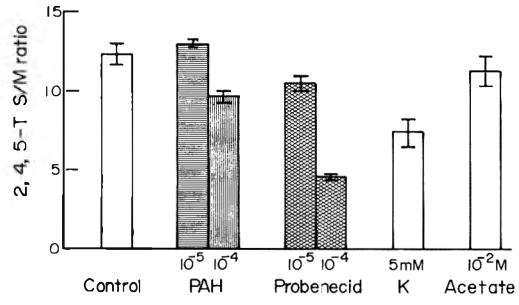

Fig. 6. Effect of alterations in medium composition on the accumulation (S/M ratio) of 2,4,5-T by rat-kidney cortical slices. Bars represent means ± SEM of three experiments.

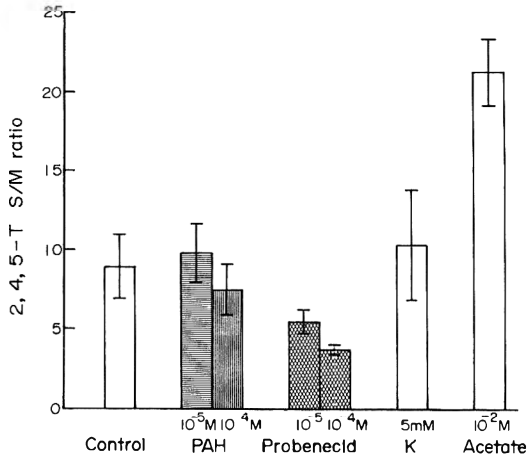

Fig. 7. Effect of alterations in medium composition on the accumulation (S/M ratio) of 2,4,5-T by dog-kidney cortical slices. Bars represent means ± SEM of three experiments.

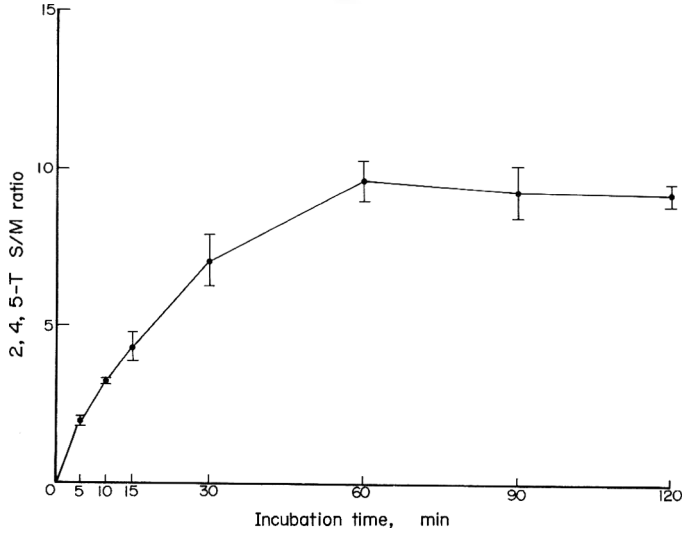

Fig. 8. Accumulation (S/M ratio) of 2,4,5-T by renal cortical slices from 10-day-old rats. Points represent means ± SEM of three experiments.

Accumulation of 2,4,5-T by renal cortical slices from the newborn rat is illustrated in Fig. 8. The 2,4,5-T S/M ratio increased with incubation time to a value of only 9·7 after 60 min.

DISCUSSION

There is a significant species difference in the toxicity of 2,4,5-T. The single oral LD_{50} for dogs is approximately 100 mg/kg (Drill & Hiratzka, 1953) and 300 mg/kg for rats (Rowe & Hymas, 1954). Piper *et al.* (1973) demonstrated that the biological half-life of 2,4,5-T in dogs (77 hr) was considerably longer than that in rats (4·5 hr) and suggested that this difference in half-life could explain the difference in toxicity. In both species, most of the drug was excreted unchanged in the urine. When the dose of 2,4,5-T was increased in rats, the biological half-life was markedly prolonged (25 hr), suggesting that the excretory capacity of the animals could be exceeded. Since most of the material appeared in the urine and since there was a marked difference in the biological half-life, the data suggested that there was a species difference in the ability of the kidneys of the dog and rat to transport 2,4,5-T. One object of this investigation was to quantitate this difference in an attempt to explain the difference between rats and dogs in the biological half-life of this compound.

As the ability of rats to excrete 2,4,5-T appears to be a saturable process and the compound exists as an organic anion, it was not unreasonable to assume active secretion of the compound by the anion secretory system of the kidney. On this assumption, it should be possible to inhibit transport of other organic anions with 2,4,5-T. Therefore as a first step in this investigation the effect of 2,4,5-T on the transport of a standard organic anion, PAH, was determined. Since organic cations are actively transported by the kidney by a system similar to and in parallel with that for organic anions, the inhibition of PAH transport by a drug which does not affect cation transport can be used as an index of selectivity of effect (Hook & Munro, 1968). An actively transported anion added to the incubation mixture would inhibit PAH and not the base, whereas a general metabolic inhibitor would inhibit both acid and base transport (Hook & Hirsch, 1973). As shown in Fig. 1, 2,4,5-T selectively inhibited the *in vitro* uptake of PAH by rat-kidney cortical slices without influencing the uptake of NMN, indicating a selective effect on anion transport.

To determine the nature of the inhibition of PAH transport by 2,4,5-T, a kinetic analysis was performed. Inasmuch as the S/M ratio for PAH measured in renal cortical slices is determined in a steady state system (after 60 min of incubation), it truly reflects the ability of cell membranes to maintain a concentration gradient. Ross & Farah (1966) indicated that short incubation times must be used to measure the rate of PAH transport, since uptake during these short periods of time would not be decreased by intracellularly accumulated PAH. To determine the effect of 2,4,5-T on PAH transport, anion uptake was measured at incubation times of 2 and 12 minutes. Net uptake of PAH between 2 and 12 minutes was used as an estimate of the rate of PAH transport. In the presence of $2{\cdot}5 \times 10^{-5}$ M-2,4,5-T, the pattern of inhibition was that of a classical competitive inhibition, in that the maximal velocity of PAH uptake appeared not to be altered. Rather, the effect of the drug was on the apparent K_m (Fig. 2). Thus it was concluded that 2,4,5-T competitively inhibited the transport of PAH. This was presumptive evidence that the compound itself was actively transported by the same system. In preliminary studies, injection of 2,4,5-T into dogs markedly reduced the clearance of PAH without altering inulin clearance.

To determine the transport characteristics of 2,4,5-T in rat and dog kidneys, the *in vitro* slice technique was used in order to eliminate problems of possible differences in plasma binding, rates of metabolism and other factors. Piper *et al.* (1973) demonstrated that essentially all of the radioactivity excreted following administration of labelled 2,4,5-T was the parent compound. Thus, no attempt was made to characterize or investigate possible metabolism. In both rat and dog tissue, accumulation of 2,4,5-T met the criteria for active transport. Accumulation of radioactivity increased with incubation time and appeared to plateau in both tissues in the presence of oxygen. In the presence of nitrogen, however, there was only slight accumulation of the compound, indicating the necessity for oxygen in the uptake process. In both tissues, the S/M ratio declined with an increasing concentration of the anion in the medium suggesting that uptake was a saturable process. Furthermore, inhibition of uptake by both PAH and another anion, probenecid, reflected the competitive nature of the uptake process.

The uptake of organic anions is sensitive to the concentration of inorganic ions in the medium (Hong & Park, 1971). In rat tissue, reduction of the medium concentration of potassium to 5 mM significantly reduced the 2,4,5-T ratio. In dog tissue no difference was seen when the potassium concentration of the medium was similarly reduced. However, this could have been due merely to the fact that there was sufficient potassium within the tissue itself to allow transport to continue unabated. Quite possibly a further reduction in the potassium concentration would have produced an effect on 2.4.5-T accumulation.

Acetate has been shown to enhance PAH accumulation by renal cortical slices (Cross & Taggart, 1950) and to increase tissue accumulation of PAH when infused *in vivo* (Mudge & Taggart, 1950). It has been suggested that acetate might be involved in a necessary energy step involved in PAH transport (Cross & Taggart, 1950). On the other hand, it has also been suggested that the addition of acetate leads to the removal of endogenous inhibitors of anion secretory system (Schachter, Manis & Taggart, 1955). This latter suggestion is supported by the data reported herein. For instance, the accumulation of 2,4,5-T by rat tissue (which is normally higher than that of the dog) was not influenced by acetate. The lower S/M ratio for 2,4,5-T in dog tissue was markedly enhanced by acetate. This might suggest that in dog tissue there is normally some anion present that acts as an inhibitor of 2,4,5-T transport. This, then, would explain the species difference seen.

That the difference in biological half-life of 2,4,5-T is due to differences in renal transport of 2,4,5-T is further substantiated by the observations in newborn tissue. Fang *et al.* (1973) demonstrated a greatly prolonged half-life of 2,4,5-T in newborn rats. The data illustrated in Fig. 8 show that the ability of 10-day-old rats to transport 2,4,5-T is markedly less than that of adults, a fact which explains the prolonged half-life.

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Analyse *in vitro* du transport rénal de l'acide 2,4,5-trichlorophénoxyacétique chez le rat et le chien

Résumé—Afin d'expliquer les différences de la demi-vie biologique de l'acide 2,4,5-trichlorophénoxyacétique (2,4,5-T) selon les espèces animales, on a étudié quantitativement *in vitro* le transport rénal de ce produit en utilisant des coupes de cortex rénal de chiens et de rats. L'addition de 2,4,5-T aux coupes de cortex rénal de rat inhibe compétitivement le transport de l'acide *p*-aminohippurique sans altérer celui du *N*-méthylnicotinamide, cation organique. Les coupes de cortex rénal de rat ou de chien, accumulent activement le 2,4,5-T. Cette accumulation est dépendante de l'oxygène et est saturable; elle est réduite en présence d'autres anions (*p*-aminohippurate et probénécide). Une diminution du taux de potassium du milieu fait diminuer l'accumulation de 2,4,5-T dans les tissus de rat, mais non dans les tissus de chien. La présence d'acétate dans le milieu fait augmenter l'accumulation de l'herbicide dans les tissus de chien, mais non dans les tissus de rat. Enfin, on constate que les tissus provenant de rats nouveau-nés sont significativement moins aptes à accumuler le 2,4,5-T que les tissus de rats adultes.

On conclut que la route primaire d'élimination rénale du 2,4,5-T réside dans le sécrétion active du produit. La plus grande aptitude à transporter l'acide *p*-aminohippurique, que possède le tissus de rat adulte, explique pourquoi la demi-vie biologique du 2,4,5-T est plus courte chez cette espèce animale.

In-vitro-Analyse des Transports von 2,4,5-Trichlorphenoxyessigsäure in der Ratten- und Hundeniere

Zusammenfassung—Der Transport von 2,4,5-Trichlorphenoxyessigsäure (2,4,5-T) in der Niere wurde *in vitro* quantitativ unter Verwendung von Nierenrindenschnitten von Hunden und Ratten untersucht, um Speziesdifferenzen der biologischen Halbwertszeit der Verbindung erklären zu können. Die Zugabe von 2,4,5-T zu Schnitten der Nierenrinde der Ratte hemmte Kompetitiv den aktiven Transport von *p*-Aminohippursäure ohne Änderung des Transports des organischen Kations *N*-Methylnicotinamid. Nierenrindenschnitte von Ratten und Hunden akkumulierten aktiv 2,4,5-T. Die Akkumulation war sauerstoffabhängig und sättigungsfähig und in Anwesenheit

anderer Anionen (*p*-Aminohippurat und Probenecid) reduziert. Eine Verminderung der Kaliumkonzentration des Mediums setzte die Akkumulation von 2,4,5-T durch Rattengewebe, aber nicht die durch Hundegewebe herab. Acetat im Medium erhöhte die Akkumulation des Herbicids im Hunde-, aber nicht im Rattengewebe. Ausserdem war die Fähigkeit des Nierengewebes von neugeborenen Ratten, 2,4,5-T zu akkumulieren, signifikant geringer als die des Gewebes von erwachsenen Tieren. Es wird daraus geschlossen, dass der primäre Weg der Entfernung von 2,4,5-T aus der Niere die aktive Ausscheidung der Verbindung ist. Die grössere Fähigkeit des Gewebes erwachsener Ratten, *p*-Aminohippursäure zu transportieren, erklärt die kürzere biologische Halbwertszeit von 2,4,5-T in dieser Spezies.

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THE USE OF GRADED CONCENTRATIONS IN STUDYING SKIN SENSITIZERS: EXPERIMENTAL CONTACT SENSITIZATION IN MAN

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Abstract—Human subjects were used to evaluate the skin sensitization potential of several substances used in marketed drugs and cosmetics, as well as other chemical agents. The results show that a modified Draize test is a useful, predictive patch-test procedure. Potentially strong sensitizers show a dose–response relationship when a range of concentrations of test material are used (at induction), whereas weak sensitizers may not; thus a range of concentrations of test materials may be used as a means of disclosing the potential of these compounds to produce delayed skin hypersensitivity.

INTRODUCTION

Contact dermatitis is produced by local skin application of allergenic compounds, as well as of irritant chemicals. The discovery that allergenic effects were involved led investigators to turn their attention to the development of appropriate methods for assessing the capacity of substances to produce delayed skin hypersensitivity in man. Among the methods investigated were *in vitro* tests, such as lymphocyte transformation (Dutton & Eady, 1964; Sarkany, 1967) and macrophage-migration inhibition (David, Al-Askari, Lawrence & Thomas, 1964), animal tests such as those in guinea-pigs (Landsteiner & Jacobs, 1935 & 1936) and swine (Mali, 1966), and tests involving enhanced delivery of the test material across the skin barriers by the use of occlusion (Buehler, 1965), by application of acanthogenic chemicals (Baer, Rosenthal & Sims, 1956), by intradermal injection (Landsteiner & Jacobs, 1935 & 1936), by repeated application (Draize, 1955; Shelanski & Shelanski, 1953) or by skin stripping with pressure-sensitive tape (Marzulli & Maibach, 1970). In tests based on special skin preparation, Kligman (1966a,b,c) used sodium lauryl sulphate and Epstein, Kligman & Senecal (1963) used skin freezing. Other methods were based on the use of dermatological patients known to be sensitive (Marzulli & Maibach, 1973) or on guinea-pigs rendered sensitive with Freund's adjuvant (Maguire, 1973).

In an earlier paper, we discussed the possibility of increasing the amount of material penetrating the skin simply by using different concentrations of the test material (Marzulli, Carson & Maibach, 1968). A wide variety of compounds has now been tested on several thousand subjects and certain aspects of the usefulness and limitations of this approach are reported.

EXPERIMENTAL

These studies were conducted on normal male human paid subjects, aged 21–50 yr, by the use of the well known Draize test (Draize, 1959). The test subjects were approximately 82% Caucasian, 13% Negro and 5% American Indian (Mexican). This test, like other predictive sensitization tests, is derived from Jadassohn's diagnostic patch test introduced around the turn of the century. Basically, each test was divided into three consecutive phases. The initial or induction phase consisted of a 3·5-wk period of repeated chemical insults to the skin aimed at initiating the sensitization process. This was followed by approximately 2 wk of incubation, or so-called period of rest, during which a completion of immunological events is thought to occur. Finally, there was a challenge or elicitation phase in the form of a new contact to determine whether sensitization had in fact taken place.

The test material (0·5 g) was applied to the upper lateral portion of the arm and covered with an occlusive patch (Johnson & Johnson Square Band Aid®, without perforations) for 48 or 72 hr. Usually, 10 epicutaneous applications were administered successively at the same site for induction. Following a rest period of approximately 2 wk, the challenge patch was applied and allowed to remain for 72 hr, after which the reaction was read. Challenge was always done with a non-irritant concentration of the test material.

Skin reactions were assigned four grades of intensity: 1, erythema; 2, erythema and induration; 3, vesiculation; 4, bulla formation. In the sensitization phase, the test substance was generally applied at use concentration as well as at higher and lower concentrations. Except where otherwise noted, petrolatum was used as the test vehicle because of our considerable background information on its use. For convenience in presentation, the grading reported here is given on a positive or negative basis. Generally, those reactions showing erythema and oedema (grade 2 or over) were accepted as positive. To verify reproducibility, positives were retested a week or two later. Most grade 1 (erythema only) subjects were retested approximately weekly to ascertain any increase or decrease in the severity of the reaction. When the latter was the case, the subjects were considered to have had an irritant response.

RESULTS

Induction concentrations

Data given in Table 1 show the effects of using a range of concentrations of test materials at induction, followed by a single concentration at challenge. Positive results obtained at the highest concentrations indicate that these substances are potentially strong skin sensitizers. A statistically greater number of skin responses was found in the group given the highest concentration of mafenide, benzocaine, bronopol (2-bromo-2-nitropropane-1,3-diol) and *p*-phenylenediamine. Thus the sensitization index (the frequency of occurrence expressed as the percentage of individuals in a test population who appear to have been sensitized) of 0 for benzocaine tested at 2% for induction rose to 6 when the compound was tested at 20%, and a sensitization index of 2 for mafenide rose to 16 when the induction concentration was raised from 5 to 20%. Similar results were obtained for bronopol and *p*-phenylenediamine. Formalin and captan might be expected to show a significant reduction in sensitization index if lower concentrations were used for induction, but these tests were not done.

The data in Table 2 represent the results of using, at induction, graded concentrations of nine compounds of low sensitization capability. Although concentrations of up to 20%

Table 1. *Skin sensitization test results (Draize procedure), showing an increase in the incidence of sensitization with higher concentrations of test material at induction*

Compound*	Induction concentration (%)	Challenge concentration (%)	Response Fraction	Response %	Statistical significance (*P* value)
Mafenide	5	10	2/93	2	
	20	10	18/108	16	<0·002 (S)†
Benzocaine	2	2	0/92	0	
	10	10	2/173	1·2	<0·40 (S)‡
	20	10	6/99	6·0	
Bronopol	2	2·5	0/66	0	
	5	2·5	11/93	12	<0·01 (S)†
p-Phenylenediamine	0·01	0·01	7/97	7·2	
	0·10	1·00	11/98	11·2	<0·001 (S)‡
	1·00	1·00	47/88	53·4	
Formalin§	0·1	1	0/45	0·0	
	1	1	4/89	4·5	
	3	1	5/88	5·7	
	5	1	4/52	7·7	0·12 (NS)‖
	10	1	8/102	7·8	
Glutaraldehyde	0·1	0·5	0/102	0·0	
	5·0	0·5	7/30	23·3	<0·001 (S)‡
Captan	1	1	9/205	4·4	

S = Significant NS = Not significant

*Administered in petrolatum except where otherwise indicated.

†Based on fourfold chi-square contingency test adjusted for continuity.

‡Based on fourfold chi-square contingency test of the two experiments with similar challenge levels adjusted for continuity.

§Formalin is 37% aqueous formaldehyde. Aqueous dilutions were tested. Dilutions referred to here are dilutions of formalin.

‖Based on the one degree of freedom chi-square for the two low-level initial concentrations *v.* the two high-level initial concentrations not adjusted for continuity.

were used for induction, there was no evidence of skin sensitization with any of these compounds. These data thus demonstrate an important aspect of the technique, namely, that false positives are not likely to be produced by this method when tests are conducted on weak sensitizers.

Challenge (elicitation) concentrations

Table 3 gives the results of testing two groups of subjects with the lachrymatory materials, α-chloroacetophenone (CN) and *o*-chlorobenzylidine-malononitrile (CS). Each group was tested with a single concentration of CN or CS at induction, but with three different concentrations at challenge. The data show that the challenge concentration is also important in disclosing the proclivity of a strong skin sensitizer to produce positive findings. The mechanisms are obviously different, however, At low challenge concentrations, the zero incidence of skin reactions represents a failure to respond despite the fact that the subjects were indeed sensitized (as evidenced by their response to higher challenge concentrations). The results are similar to those given in Table 1, in that the sensitization index rose sharply when the challenge concentration was increased from 0·01 to 1·0%, indicating that CS and CN are also strong sensitizers. The increase in sensitization index occurred despite the fact that the substances were not used at irritant concentrations.

Table 2. *Skin sensitization test results (Draize procedure), showing no effects of higher concentrations of test material (mainly antimicrobials) at induction*

Compound*	Induction concentration (%)	Challenge concentration (%)	Response (fraction)
Chlorocresol	5†	5	0/98
	10	5	0/88
	20	5	0/66
Chloroxylenol	5	5	0/208
	10	5	0/66
	20	10	0/110
Hexachlorophene	0·5	0·1	0/48
	5·0	1·0	0/51
	20·0	1·0	0/80
Bithionol	5·0	5·0	0/49
	20·0	20·0	0/77
3,4',5-Tribromo-	1	1	0/93
salicylanilide (TBS)	5	1	0/81
Trichlosan	5	5	0/61
(2,4,4'-trichloro-2'-	20	1	0/58
hydroxydiphenylether)	20	1	0/25
Trichlocarban (TCC)	1·5	1·5	0/200
	10·0	1·0	0/88
Propylene glycol	12	12	0/204
	60	12	0/89
Benzalkonium chloride	5† and 1	0·1	0/186

*Administered in petrolatum.
†First four applications.

Results with weak primary irritants

The data in Table 4 illustrate the type of irregular results someimes seen when weak primary irritants are tested. The five test materials used were weak skin sensitizers. The use of graded concentrations at induction failed to produce a graded response. These results supported the usefulness of the technique, as well as the lack of a strong skin-sensitization proclivity for the test materials.

Table 3. *Skin sensitization test results (Draize procedure), showing effects of varied challenge concentrations on the same subjects*

Compound	Induction concentration (%)	Challenge concentration (%)	Response		Statistical significance (*P* value)
			Fraction	%	
α-Chloroacetophenone (CN)	1	1·0	7/10	70	
in acetone	1	0·1	7/10	70	<0·01(S)*
	1	0·01	0/10	0	
o-Chlorobenzylidine-	1	1·0	5/9	55	
malononitrile in	1	0·1	1/9	11	<0·03(S)†
alcohol (CS)	1	0·01	0/9	0	

S = Significant
*Based on the fourfold chi-square test of the 0·01% challenge level vs each of the other challenge levels adjusted for continuity for cells with expected value < 5.
†Based on the fourfold chi-square test of the 0·01% vs the 1% challenge level adjusted for continuity for cells with expected value < 5.

Table 4. *Skin sensitization test results (Draize procedure), showing insignificant effects of higher concentrations of test material at induction (with weak sensitizers)*

Compound*	Induction concentration (%)	Challenge concentration (%)	Response Fraction	Response %	Statistical significance (*P* value)
Furacin	0·2	0·2	1/93	1	
	0·5	0·5	1/99	1	0·91 (NS)†
	5·0	5·0	0/99	0	
Neomycin	0·5	0·5	0/54	0	
	5·0	5·0	3/136	1·6	0·91 (NS)†
	20·0	20·0	0/42	0	
Dichlorophene	5	5	0/208	0	
	20	5	1/110	0·9	0·74 (NS)‡
Sorbic acid	10	5	0/131	0	
	20	5	1/121	0·8	0·83 (NS)‡
Dibromosalicylanilide	1	1	2/103	1·9	
(DBS)	5	1	0/104	0·0	0·24 (NS)§
	20	1	3/104	2·9	

S = Significant NS = Not significant

*Administered in petrolatum.

†Based on two degrees of freedom chi-square for the three levels of challenge concentrations adjusted for continuity where expected cell value is < 5.

‡Based on fourfold chi-square test adjusted for continuity where the expected value is < 5.

§Based on the fourfold chi-square for the three levels of challenge concentrations adjusted for continuity where expected is < 5 for the 5% *v.* 20% induction concentration.

Product use tests

The success of the Draize predictive patch-test procedure in identifying subjects who would react to topically applied allergenic substances in marketed products is shown in Table 5. Between 20 and 50% of subjects who were patch-test positive to 1% formalin (0·4% formaldehyde) by the Draize procedure showed responses to products containing 0·4–0·6% formaldehyde. The patch-test procedure would appear to provide a more severe challenge, as it was applied under cover (occlusion), whereas the commercial product was applied without cover. Similarly, 32% of subjects who were patch-test positive to 5% benzoyl peroxide had skin reactions when commercial acne preparations containing 5% benzoyl peroxide were applied to the face and the area was left uncovered.

Table 5. *Semi-use tests on patch-test positive subjects with formaldehyde-containing cosmetics and an acne preparation containing 5% benzoyl peroxide*

Product description	Application site	Fractional response
Subjects patch-test positive to 1% formalin (0·4% HCHO)		
Dry skin lotion (0·5% HCHO)*	Right face and forearm	5/10
Cream rinse (0·4% HCHO)*	Left face and forearm	4/10
Bubbling bath oil (0·6% HCHO)*	Right shoulder	2/10
Subjects patch-test positive to 5% benzoyl peroxide		
Acne preparation (5% benzoyl peroxide)†	Forehead and jaw	9/28

*Product was applied twice daily for 21 days (uncovered).

†Product was applied once daily for 4 days (uncovered).

DISCUSSION

The use of exaggerated concentrations of test materials at induction appears to be a worthwhile procedure for disclosing the proclivity of a substance to produce contact skin sensitization. A graded response is produced by graded concentrations of strong sensitizers; therefore, by estimating the slope of the response it should be possible to find the safe concentration of even strong sensitizers for use in marketed products. Generally speaking, if use concentration in a marketed product is 1/100 of the concentration producing a 0·1% response (by calculation) in the Draize predictive test procedure and if the slope of the response (at different concentrations) is steep, serious problems of safety would not be anticipated. Each individual situation would, of course, have to take into account factors that could adversely affect this margin of safety, such as frequency of application, contact area, permeability of skin site, occlusion, and so on. Bronopol, a strong sensitizer, has recently been introduced into cosmetic formulations as a preservative. The steep slope response for bronopol, in contrast to the flat slope response for formalin, would suggest that the former substance may be used safely in situations where the use of formalin would be unwise.

The data presented demonstrate that substances that are not strong sensitizers are not likely to give false-positive reactions by this procedure (when high concentrations are used at induction), provided that a non-irritant concentration is used for the challenge.

Griffith (1973) recently reported on the use of exaggerated concentrations of fluorescent whitening agents (FWAs) for predicting which of these materials could safely be used in laundry products. He found cause to reject three test FWAs that gave positive results for skin-sensitizing potential and accepted 28 that gave no positive results; populations of 50–80 subjects were usually used in each test. In these studies, the FWAs were tested at concentrations up to 0·1%, which are somewhat exaggerated with regard to eventual use conditions; however, they are not exaggerated with regard to possible experimental test conditions. The method we have used, by contrast, involves graded concentrations at induction, beginning around 1% and increasing up to the maximum tolerated concentration (possibly as high as 20%), with challenge concentrations lower than those that produce skin irritation.

The need to use a non-irritant but sufficiently high concentration of the test material at challenge was adequately demonstrated by the data in Table 3. This requirement for a challenge concentration high enough to elicit an appropriate response, yet not so high as to produce a false-positive response, cannot be stressed too vigorously.

The data in Table 4 reflect low-frequency sensitization effects which appear to be unrelated to the test concentration used at induction. Indeed, the differences in sensitization index are not statistically significant from one another or from zero, and they are probably due to the variability in response in the human population, since occasional sensitization events can occur at any test concentration of a weak sensitizer when it is tested in a small population. These differences are important in that they show the value of using a large test population to disclose a potential for sensitization in a weak sensitizer.

Baer, Ramsey & Bondi (1973), using diagnostic patch tests on clinical subjects suffering from skin diseases consisting mostly of eczematous eruptions, obtained *diagnostic* (not *predictive*) sensitization indices of 7 and 13·5 (significantly different) in two test populations (229 and 540 subjects) with 2% *p*-phenylenediamine. In addition, the test populations gave sensitization indices of 9 (540) and 12 (229) when tested with 2% aqueous formaldehyde; these values are not significantly different. In both cases, the higher diagnostic sensitization

indices were associated with test panels having contact dermatitis of an aetiology thought to be related to the test material. The results are therefore expected. Their results with 2% aqueous formaldehyde are also not unexpected when compared with the sensitization indices of 6–8 obtained in the present study by using 0·4% formaldehyde at challenge. On the other hand, the data on *p*-phenylenediamine in the present study clearly show that positive findings by predictive tests (consisting of 11 separate insults) of a normal population with this strong sensitizer greatly exceed positive findings seen in clinical patients given patch-tests (diagnostic), possibly for the first time, with this material. In the present study, 1% PPDA produced a sensitization index of 53 in a population of 88.

Kligman (1966a) has reported extensively on a so-called 'maximization test', which involves the application of sodium lauryl sulphate, prior to application of the test material both during induction and challenge, and the use of a high concentration of the test chemical. The functions of sodium lauryl sulphate in this system are not entirely clear but it promotes an inflammatory reaction that may make the system more amenable to an allergic response. It probably also enhances skin penetration of the test material. There is danger, however, that the interaction of sodium lauryl sulphate with the test chemical may interfere with the proper interpretation of findings. By contrast, the present method is a 'maximization test', which is simpler to perform than the Kligman test and does not suffer from its limitations.

In the present studies, assays were performed with occluded patches. It has been adequately documented in man that occlusion increases penetration significantly; for instance, with hydrocortisone, a minimal penetrant, a tenfold increase was produced by occlusion (Feldman & Maibach, 1966). Similar observations with several pesticides have been made in man (R. J. Feldmann and H. I. Maibach, personal observations 1973). Furthermore, Magnusson & Hersle (1966) have shown that the use of patch tests with non-occlusive adhesive tapes in dermatological patients with known clinically relevant allergic contact dermatitis resulted almost routinely in false-negative findings. For these reasons, with the relatively small sized panels of test subjects used in these assays, we favour the occlusive patch as a device for exaggerating the exposure and increasing the reliability of the test.

In these experiments petrolatum was used as an all-purpose vehicle. This was an expedient decision; it is recognized that theoretically it would be preferable to do an exploratory study of the effects of different vehicles and make a final selection on the basis of a rational interpretation of the findings. Petrolatum offers another barrier to the test compound's penetration. In limited studies in man, cream and ointment vehicles permitted less penetration of certain molecules than was seen with quickly evaporating solvents such as acetone (H. I. Maibach and R. J. Feldmann, unpublished observation 1972). There is at least one example (chlorhexidine) where patch testing with petrolatum has given a false-negative result (Ljuggren & Moller, 1972). Yet as a practical matter, the general usefulness of petrolatum has led to its almost exclusive use in the routine patch test for antigens used by the International Contact Dermatitis Research Group (ICDRG) and the North American Contact Dermatitis Research Group (NACDRG).

A major problem is encountered in testing final formulations. Although, as a matter of convenience, it would be a simple task to test a final formulation without testing high concentrations of the component ingredients, this can lead to false-negative findings (Epstein *et al.* 1963) and may account for the often stated claim that the Draize test is of little value (Kligman, 1966b). Only when data on individual components are obtained can one be certain that they do not possess a significant sensitization potential that is hidden by the rela-

tively low concentration in a final formulation. Ranking the proclivity of a chemical to sensitize requires testing and consideration of both the individual ingredients and the final formulation. Testing of individual ingredients allows the use of the enhanced concentrations referred to here; testing of final formulations provides data on the complex results of mixing several chemicals.

The approach that we now suggest is to test individual ingredients at their intended use concentration and at two higher concentrations (at induction), the highest being up to 20% if practical. In all cases one must be certain that challenge is performed with a non-irritant concentration. Although not ideal for testing final formulations containing several ingredients, the best tentative approach is to examine also the final formulation. The authors are of the opinion that in all cases, if the potential of an ingredient or a final formulation to produce skin sensitization is to be approximated, at least 200 subjects are needed.

Nothing presented here should be interpreted as detracting from the value of guinea-pig testing. Properly performed, the guinea-pig test offers a useful screening method to determine the sensitization potential of individual ingredients (Magnusson & Kligman, 1970).

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Examen des sensibiliseurs de la peau à l'aide de concentrations graduelles. Sensibilisation expérimentale par le contact chez l'homme

Résumé—On a évalué sur des sujets humains le potentiel de sensibilisation de différentes substances entrant dans des médicaments et des produits cosmétiques commercialisés, ainsi que d'autres agents chimiques. Les résultats montrent qu'une variante du test de Draize constitue une épreuve épicutanée commode et de bonne valeur pronostique. Avec des sensibiliseurs potentiellement puissants on constate qu'il existe un rapport entre la dose et la réaction quand on emploie une gamme de concentrations du produit essayé (pour l'induction), tandis que ce n'est pas le cas avec des sensibiliseurs faibles. Une gamme de concentration des substances à essayer peut donc servir à déterminer l'aptitude de ces substances à provoquer une hypersensibilité à retardement de la peau.

Die Verwendung abgestufter Konzentrationen bei der Untersuchung hautsensibilisierender Substanzen: Experimentelle Kontaktsensibilisierung beim Menschen

Zusammenfassung—Versuchspersonen wurden zur Prüfung des Hautsensibilisierungspotentials verschiedener im Handel befindlicher Medikamente und Kosmetika und anderer chemischer Substanzen verwendet. Die Ergebnisse zeigen, dass ein modifizierter Draize-Test ein brauchbares, Voraussagen ermöglichendes Patchtestverfahren ist. Potentiell stark sensibilisierende Substanzen zeigen eine Dosis-Wirkungs-Beziehung, wenn abgestupse Konzentrationen des Testmaterials verwendet werden (bei der Induktion), während schwachsensibilisierende Substanzen eine derartige Beziehung nicht zu zeigen brauchen; somit kann eine Abstufung von Konzentrationen von Testsubstanzen als Mittel zur Feststellung der Fähigkeit dieser Verbindungen benutzt werden, eine verzögerte Hautüberempfindlichkeit hervorzurufen.

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SHORT PAPERS

RAPID FORMATION OF CARCINOGENIC *N*-NITROSAMINES BY INTERACTION OF NITRITE WITH FUNGICIDES DERIVED FROM DITHIOCARBAMIC ACID *IN VITRO* UNDER SIMULATED GASTRIC CONDITIONS AND *IN VIVO* IN THE RAT STOMACH

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Summary—The formation of the carcinogen dimethylnitrosamine (DMN) from the fungicide bis-(dimethyldithiocarbamato)zinc (ziram) in the presence of sodium nitrite has been studied *in vitro* under conditions similar to those existing in the stomach and also *in vivo* in the rat. Under the selected conditions, the optimum pH for the formation of DMN was 1·5–2·0. At pH 2·0, more than 1 mg DMN was produced after a 10-min incubation of 10^{-4} moles ziram with a twentyfold molar excess of nitrite. This corresponds to about 8% of the theoretical yield, assuming that two molecules of the carcinogen were formed from one molecule of ziram. Tris(dimethyldithiocarbamato)iron (ferbam) and bis-(1-pyrrolidinylthiocarbonyl)disulphide (dipyrrolidylthiuramdisulphide; DPTD) produced DMN and *N*-nitrosopyrrolidine respectively under the same conditions.

Introduction

The formation of carinogenic *N*-nitroso compounds by interaction of nitrite with substances susceptible to *N*-nitrosation has received much attention recently. Substances forming carcinogenic *N*-nitroso compounds under nitrosating conditions include secondary and tertiary amines (some widely used drugs among them), alkylureas and amino acids (Friedman, 1972; Lijinsky, Conrad & Van de Bogart, 1972; Lijinsky, Keefer, Conrad & Van de Bogart, 1972; Mirvish, 1970 & 1972; Sander, Schweinsberg & Menz, 1968).

Many agricultural chemicals also contain structures that can be *N*-nitrosated. Human food may contain residues of such compounds and the possibility that they can be *N*-nitrosated in the gastro-intestinal tract or during processing when they are in contact with nitrite should be considered, since nitrite is a common constituent of many food commodities.

We have therefore investigated *in vitro* and *in vivo* the interaction of nitrite with bis(dimethyldithiocarbamato)zinc (ziram; $[(CH_3)_2N\text{-}CS\text{-}S]_2Zn$), selected as one of the most widely used representatives of the dithiocarbamate fungicides. Two other fungicides, tris(dimethyldithiocarbamato)iron (ferbam; $[(CH_3)_2N\text{-}CS\text{-}S]_3Fe$) and bis-(1-pyrrolidinylthiocarbonyl) disulphide (dipyrrolidylthiuramdisulphide; DPTD; $C_4H_8N\text{-}CS\text{-}S\text{-}S\text{-}CS\text{-}NC_4H_8$) were examined qualitatively for nitrosamine formation.

Experimental

Materials. Ziram was obtained in 99% purity from Schering AG, Berlin/Bergkamen. Ferbam was synthesized according to published methods (Houben-Weyl, 1955) and DPTD was obtained in technical-grade purity from BASF, Ludwigshafen.

Experiments in vitro. A suspension of the fungicide (10^{-4} moles) in 20 ml of an aqueous solution of sodium nitrite (2×10^{-3} moles) was incubated in a closed titration vessel, maintained at 37°C in a constant-temperature water bath. The reaction was started by adjusting the pH of the suspension to the required value with a few drops of 15% HCl. The pH was then held constant by means of a pH-stat which automatically added required quantities of 1 N-HCl or 2 N-NaOH to the system. At the end of the specified incubation time the reaction was terminated by the addition of 2 ml 2 N-NaOH. The alkaline reaction mixtures were filtered when necessary and worked up further as described below.

Experiments in vivo. Four male Wistar rats, about 200 days old, were used. An aqueous suspension of ziram (10^{-4} moles in 1 ml) and an aqueous solution of sodium nitrite (4×10^{-3} moles in 0·5 ml) were given simultaneously by stomach tube. After 15 min, the animals were killed. The stomach and upper intestine were removed, rapidly comminuted and stored in 5 ml 0·4 N-NaOH for subsequent analysis.

Isolation of nitrosamines and quantitative assay. Reaction mixtures from both *in vitro* and *in vivo* experiments were saturated with sodium chloride and steam-distilled. Each distillate was acidified with 2 ml 2 N-H_2SO_4 and redistilled. After dilution with water to an appropriate volume, the nitrosamine content was determined by ultraviolet spectrometry at the absorption maximum of nitrosamines (227–230 nm). The reaction mixture without nitrite, taken through the same procedure, served as the photometric reference. Ultraviolet spectrometric results were confirmed by gas chromatography (3 m column, 10% FFAP on 100/120 GasChrom Q) using a Coulson electrolytic conductivity detector in the pyrolytic mode (Rhoades & Johnson, 1970) for selective detection of nitrosamines. Recovery experiments with known amounts of dimethylnitrosamine (DMN) gave yields of 95–97% for this isolation procedure. For each value, the mean of at least two separate determinations was calculated. In some cases, especially in the *in vivo* experiments, the identity of nitrosamines was further confirmed by interfaced gas chromatography/mass spectrometry on an LKB 9000 GS/MS system.

Results and Discussion

Nitrosamines are formed when fugicides derived from dithiocarbamic acid are incubated with nitrite under acid conditions. The optimum pH for the formation of DMN from ziram after a 10-min incubation was 1·5–2·0 (Fig. 1). The rate-limiting process was obviously the acid-catalysed decomposition of the dithiocarbamate. This was shown by preincubation of ziram (10^{-4} moles) in hydrochloric acid at pH 1·5 for 20 min. The substance, which in neutral aqueous medium is very poorly soluble, dissolved completely with decomposition within 20 min. Subsequent nitrosation of the mixture at pH 3·4 with a fourfold stoichiometric concentration of nitrite yielded the same amount of DMN as was obtained after nitrosation of the equivalent amount of dimethylamine hydrochloride. Direct incubation of 10^{-4} moles ziram for 20 min at pH 3·4 with the same nitrite concentration yielded only 33% of the value obtained as described above. Under these conditions undissolved ziram was still present in the mixture.

Dialkyldithiocarbamates are known to decompose under acid conditions into the corresponding dialkylamines (Houben-Weyl, 1955). However, since substantial amounts of DMN are formed in strongly acid medium at pH 1 (Fig. 1), the nitrosation is unlikely to proceed via the liberation of dimethylamine. Under these conditions the relatively strong base dimethylamine ($pK_b = 3{\cdot}28$) is nitrosated only to a very minor degree (Mirvish, 1970). The reactive intermediate is therefore more likely to be the unstable free dimethyldithiocarbamic acid.

Table 1 shows the effect of varying the stoichiometric nitrite concentration on the yield of DMN at a constant pH of 2·0. An increase in DMN yield from 1·6 to about 8% of the

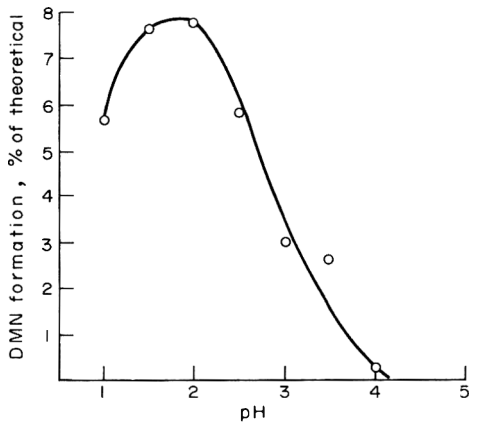

Fig. 1. Dimethylnitrosamine formation from ziram (10^{-4} moles) and sodium nitrite (2×10^{-3} moles) in relation to the pH of the reaction mixture in a 15-min reaction at 37°C.

Table 1. *Relation between the formation of dimethylnitrosamine from ziram and the concentration of nitrite in the reaction mixture*

Concentration of $NaNO_2$ (10^{-4} moles/20 ml)*	DMN formed	
	μg	% of theoretical yield
1	230	1·6
10	450	3·0
20	1150	7·8
40	1260	8·5
100	1850	12·5

*Reaction mixtures containing 10^{-4} moles ziram and various concentrations of $NaNO_2$ in 20 ml were reacted for 10 min at pH 2·0 and 37°C.

theoretical yield was obtained by raising the nitrite concentration from equimolar to a 20-fold molar excess. Further increases in nitrite concentration resulted in a less pronounced increase in DMN yields.

At pH 2·0, the reaction was already complete after 10 min. Higher pH values, however, required longer incubation periods. At pH 4·0 for instance, the DMN yield after nitrosation for 10 min with a 20-fold molar excess of nitrite was 0·5% of the theoretical against 3·5% after a 60-min reaction time.

At a given concentration of nitrite (2×10^{-3} moles/20 ml) the percentage yields of DMN increased with decreasing concentrations of ziram, the values at pH 2 being 0·5,

2·6, 7·8 and 13·9% of the theoretical yield with ziram concentrations of 10^{-3}, 2×10^{-4}, 10^{-4} and 2×10^{-5} moles/20 ml, respectively. This observation is readily explained by the low solubility of the compound in aqueous media. Within the relatively short reaction time of 10 min, only limited amounts of ziram (about 10^{-4} moles) decomposed at pH 2. Higher amounts of the compound required longer incubation times to give comparable percentage yields of DMN.

Ferbam behaves in the same way as ziram, giving rise to similar amounts of DMN under equal nitrosating conditions. DPTD, which was only studied qualitatively, yielded *N*-nitrosopyrrolidine, as expected.

The *in vivo* formation of DMN from ziram and nitrite was investigated in four rats. After incubation for 15 min in the rat stomach with a 40-fold excess of nitrite, the average yield of DMN from 10^{-4} moles ziram was 126 μg, corresponding to about 0·9% of the theoretical value. The individual values were 95 and 136 μg in two fasted rats and 106 and 165 μg in the non-fasted animals.

Conclusion

The results of these studies indicate that fungicides derived from dithiocarbamic acid rapidly form significant amounts of carcinogenic nitrosamines when they come into contact with nitrite under the conditions prevailing in the stomach. Nitrite is commonly used as a food additive, especially in foods of animal origin, but it may also become a contaminant of human food unintentionally (Kolari & Auman, 1970; Phillips, 1971). It is also invariably present in concentrations of 1–10 ppm in normal human saliva (Varady & Szanto, 1940; authors' own unpublished results). Fungicides derived from dithiocarbamic acid are widely used as protective agents in the cultivation of fruits and leafy vegetables and in wine-growing (Wegler, 1970). Residual levels of such fungicides in the human diet therefore represent a potential starting material for the formation of carcinogenic nitrosamines when they are ingested with nitrite.

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TOXICITY OF FATTY ACIDS IN ASSAYS FOR MYCOTOXINS USING THE BRINE SHRIMP (*ARTEMIA SALINA*)

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Summary—An investigation of potential mycotoxins produced by *Aspergillus sydowi* involved an established technique based on the toxicity of mycotoxins towards larvae of the brine shrimp (*Artemia salina*). Fractionation of toxic extracts from the fungus showed that a major part of the observed toxicity resided in the fatty acid fraction, of which linoleic acid was a major component. Further investigations showed that some common naturally occurring fatty acids possess toxicity towards the brine shrimp comparable with that of several known mycotoxins.

Introduction

We have been screening Aspergillus and Penicillium species from broiler litters (Dennis & Gee, 1973) as potential producers of mycotoxins. As one biological test, we have used the brine shrimp method described by Harwig & Scott (1971) and with this it was found that extracts from some cultures of *A. sydowi* were consistently toxic. Fractionation showed that the toxicity exhibited was mainly due to the presence of unsaturated fatty acids. Further investigation of the toxicity of a homologous series of saturated and unsaturated fatty acids showed that some fatty acids were markedly toxic.

Experimental

Identification of the toxic components of extracts from A. sydowi

Isolates of *A. sydowi* obtained from broiler-house litter samples were cultured in a liquid medium (24 ml yeast extract–sucrose medium in 250 ml Erlenmeyer flasks) and after incubation for 1 wk at 25°C the mycelial mats were frozen in liquid nitrogen, disintegrated using a glass rod and extracted with 30 ml boiling chloroform. The chloroform extracts were evaporated to dryness and redissolved in 1 ml chloroform. The brine shrimp screening method of Harwig & Scott (1971) was modified for the use of small glass cups, and chloroform extracts (40 μl aliquots) were evaporated in the cups. Brine shrimp medium (6 drops, $\simeq 0{\cdot}2$ ml) containing 30–40 larvae was added and the cups were incubated for 16 hr at 30°C. Of the 23 isolates screened, 11 caused $>90\%$ mortality.

Large-scale culture of a typical strain of *A. sydowi* provided an extract which was fractionated by preparative thin-layer chromatography on silica plates developed with benzene–methanol (9:1, v/v). The plate area was arbitrarily divided into five bands and eluted with methanol, and the fractions were individually tested for toxicity using the LC_{50} assay method described by Harwig & Scott (1971). The fraction containing toxic material (band centred at R_f 0·4) contained palmitic, stearic, oleic and linoleic acids on analysis by mass spectrometry. The crude chloroform extract was therefore partitioned between 87% ethanol and light petroleum b.p. 60–80°C according to the procedure of Galanos & Kapoulas

(1962), to give a light petroleum fraction of neutral lipids containing free fatty acids. This fraction was methylated with diazomethane in ether and purified by preparative thin-layer chromatography on silica gel using the solvent system hexane–ether (85:15, v/v) and the fatty acid methyl esters were removed with ether. The mixture in iso-octane was analysed by conventional gas chromatography to determine its fatty acid composition. This showed that the original extract contained 7% free fatty acids, composed of linoleic (70%), oleic (10%), stearic (2%) and palmitic (12%) acids.

Determination of the toxicity of fatty acids to brine shrimp larvae

High purity fatty acids (Sigma Chemicals, 99% +) were tested for toxicity using the LC_{50} assay method.

The unsaturated fatty acids were tested for possible contamination with peroxides before use. Each acid was methylated with diazomethane in peroxide-free ether and the resultant esters were examined by thin-layer chromatography (0·25 mm silica gel G) with hexane–ether (7:3, v/v) as the developing solvent. No peroxide could be detected on spraying with ferrous thiocyanate reagent (Stahl, 1969).

Results and Discussion

In the even-numbered series of saturated fatty acids (Table 1) toxicity to the brine shrimp larvae increased sharply from $C_{6:0}$ to a maximum at $C_{12:0}$ and then declined. Neither LC_{50} values nor other comparative toxicity figures could be determined for $C_{14:0}$, $C_{16:0}$ and $C_{18:0}$ acids because of their increasing insolubility in the brine shrimp medium. However, all three of these acids were less toxic than the $C_{12:0}$ acid. For unsaturated fatty acids, toxicity to the brine shrimp increased with the degree of unsaturation; $C_{18:1}$ and $C_{20:1}$ unsaturated acids showed little toxicity but $C_{18:2}$, $C_{18:3}$ and $C_{20:4}$ acids were highly toxic.

Table 1. *Toxicity of fatty acids to brine shrimp larvae*

Acid	Structure*	LC_{50} (μg/ml)
Caproic	6:0	>300
Caprylic	8:0	240
Capric	10:0	36
Lauric	12:0	5
Myristic	14:0	>27†
Palmitic	16:0	>14†
Stearic	18:0	>20†
Oleic	18:1	87
Linoleic	18:2	3·3
Linolenic	18:3	2·4
Eicosenic	20:1	>30†
Arachidonic	20:4	1·5–2·0

*No. of carbon atoms: no. of double bonds.
†These concentrations are near the solubility limit in the brine shrimp medium. Mortalities < 10%.

The relationship found between fatty acid structure and toxicity to the brine shrimp larva parallels that reported for several other organisms, e.g. yellow fever mosquito larvae (*Aedes aegypti*), a species studied by Saxena & Thorsteinson (1971). In this case, saturated fatty acids with a chain length of 10–13 carbon atoms showed the greatest toxicity, and

oleic, linoleic and linolenic were the most toxic of the unsaturated acids. A similar relationship between the constitution of fatty acids and their acute toxicity to the aphid (*Aphis rumicus*) was reported by Tattersfield (1927).

In the use of the brine shrimp as a screen for potential mycotoxins, the high toxicity of linoleic acid now recorded is important, since this is a product of fungal metabolism which may be present in quite high concentration in the chloroform extracts used. In the present case, our crude extracts contained at least 5% linoleic acid and Strong & Peterson (1934) found that the crude lipid extract from *A. sydowi* contained 22% free fatty acids. Other Aspergillus species, including *A. flavus* (Singh, 1957), and *A. terreus* and *A. ochraceus* (Singh & Sood, 1972), produce high yields of fat when grown under suitable conditions in media containing sucrose as a carbon source.

No particular specificity has been claimed for the brine shrimp in assays for mycotoxins, but the high toxicity of certain fatty acids that are likely to occur in fungal extracts suggests that caution is necessary when using the organism for this purpose.

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Review Section

CHEMICALS, THE CONSUMER AND CREDIBILITY*

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(*Received 29 September 1973*)

Your kind invitation to speak before the Association this evening provides an unusual opportunity to discuss elements of our respective roles in contributing to the food supply of the American people. As indicated, I intend to talk about the use of food chemicals and the reactions of the average consumer to the knowledge that his food supply contains food additives and colourings and may be highly processed in an industrial operation. As a point of departure, I believe that the preponderance of evidence points to the fact that food chemicals and food technology are here to stay. The motto "Better Living Through Chemistry" does not exclude consideration of our diet, and I do not reject the concept that leads to the paraphrase: "Better Eating Through Chemistry".

A number of important trends contribute to the increasing use of food chemicals, and one does not have to be a scientist to be aware of these trends. The shortage of red-meat protein, for example, calls for active consideration of other protein sources: fish-protein concentrate, soya protein, peanut flakes and even single-cell protein grown on substrates of petroleum or formaldehyde condensation products. While short-term factors have drawn current attention to the protein supply, this is clearly a problem that will remain with us. When it takes 6–7 acres to produce, from beef, protein that could be derived directly from a single acre of soya beans, and when land values and feed costs are considered, one cannot avoid the conclusion that the direct use of vegetable protein will grow dramatically. Obviously, the conversion of vegetable protein into a product suitable to current consumer preferences requires a certain amount of ingenuity in the application of food technology.

With respect to fats, cholesterol and the arteriosclerotic process, there is a call for meats from animals fed so as to increase the level of unsaturated fat in the meat. Work in Australia has progressed impressively in this direction. A cholesterol-free egg substitute is a similar response to this need. Certainly these products cannot be delivered without significant reliance on food chemicals and technology.

As for carbohydrates, even sucrose is under attack in relation to the aetiology of diabetes, cardiovascular disease and dental caries, and the food manufacturer may be faced with the need to provide not only alternative sweeteners but materials that supply the technical effects now provided by sucrose.

Concurrent with pressures on conventional sources of protein, fat and carbohydrate is a trend toward snack foods and foods manufactured for automated distribution. It is predicted

*Address to the Grocery Manufacturers of America, Inc., on 29 August 1973.

that by 1980 the fraction of meals eaten away from home will have risen to perhaps 70%, almost twice the current level. Because of the cost and scarcity of traditional restaurant personnel, preparation of food is being shifted to an increasing degree to an industrial type of manufacturer, and the distribution system is increasingly based on self-service. These factors require emphasis upon food-processing technology, fabricated foods, measures to control food-borne disease and tight control of quality and costs.

In sum—chemicals in foods will play a greater role than has previously been the case, and for good reason.

Now as to the consumer, we find that he is becoming increasingly aware of his food supply—its quality, its composition, the manner in which it is presented to him and its cost. His heightened awareness derives from a number of factors which characterize our times. The consumer is made sensitive by continued reference in the media to his food supply: bans on non-nutritive sweeteners and growth promotants, public doubts about colour additives, botulinal growth in mushrooms, criticism of over-enthusiastic merchandising schemes. The cost of food has brought about a more critical examination of the relative nutritional properties of different foods, a willingness to try alternative dietary patterns to reduce diet costs, and a vague hostility toward the food supplier. The growth of nutritional labelling and associated educational programmes promoted by the FDA to make labelling more useful will augment the level of awareness.

This increasing awareness on the part of the consumer does not imply that his attitudes are free of bias with respect to chemicals in the food supply. Indeed there is reason to believe that he is increasingly suspicious. The bias has as its background a general disenchantment with science in general and chemicals in particular. I am not sure that anyone can fully explain this trend. Some of the reasons are frankly beyond our direct influence: disillusionment with war in South-East Asia, evidence for unwarranted military-industrial interdependence and malfeasance in high places. Other factors are more specific: mercury at Minamata Bay, DDT in California shore birds, DES and vaginal carcinomas in young women, PCBs, HCBs... the list goes on. At any rate this diffuse negative reaction can become focused upon the food supply when the consumer has unfortunate personal experiences with foods that fail to meet his expectations, when familiar items such as cyclamates or swordfish are removed from commerce, or when advertising attempts the hard-sell of a product with dubious merits.

Now enters the consumer spokesman or consumer activist, who represents the consumer to an unknown degree, an observation acknowledged by activists themselves. Yet anyone who has worked with consumer organizations cannot avoid the conclusion that they do reflect broad consumer apprehensions and raise the awareness level of consumers by focusing criticism on what they regard as bureaucratic inertia and bumbling, industrial double-dealing, and exploitation and deception of the consumer. Their objectivity can be impaired by a lack of financial and scientific resources and by previous unhappy experiences in the marketplace. Even though the FDA is a frequent target, I must conclude that the consumer movement is essentially beneficial, serving to keep the regulators and the regulated on their toes and to counteract the consumer's passivity about his own well-being and shopping habits.

Credibility fits into the picture when we recognize that the consumer is less than convinced that chemical additives in the food supply and food fabrication techniques are in his best interests. As we have noted, there is widespread doubt as to the capacity of science to solve the problems of society. Thalidomide, parathion and DES all seem to coalesce

in the public mind to suggest that chemicals are in the main deleterious, or at least that serious doubt must accompany assertions of benefit.

Recent experience with respect to credibility and additives is related to the FDA's proposal to increase the content of iron in flour and related products. One would not predict that iron supplementation would call forth much criticism, especially since the proposal had been supported by the Council on Foods and Nutrition of the American Medical Association, the American Society for Clinical Nutrition, the American College of Nutrition, the American Public Health Association, the Food and Nutrition Board of the National Academy of Sciences and other scientific bodies. Nevertheless consumers, commenting both as individuals and as representatives of various organizations, opposed by more than six to one the iron supplementation proposal. A large proportion of these comments was stimulated by numerous articles in the press, as evidenced by the enclosure of, or reference to, such articles. Many of those persons commenting unfavourably specifically stated their anxiety with respect to the whole concept of processed foods and the use of additives, whether they were nutrients or had other purposes. Many stated their desire to see a return to the consumption of natural foods and felt that the food industry removed too much nutritional value during processing and that replacement was not an acceptable alternative to leaving in more "natural goodness". That these views are contrary to modern nutritional knowledge and to the realistic capacity of the agricultural and food industry seeking to provide a nutritionally adequate food supply to the Nation emphasizes the existence of a "credibility gap". A balanced view is not promoted when one reads in the news that the Institute of Food Technology is comprised of the "fake food specialists" and that industrial foodmakers push foods that "cause serious health problems for millions of duped citizens". Unfortunately, there is no widely read rebuttal to this presentation. Surely the "natural" or "organic" food industry is a reaction, in part at least, to lack of credibility on the part of the consumer with respect to the safety and wholesomeness of the food supply.

As we recognize the inevitable extension of applications of chemistry throughout wide segments of the food industry and the increasing reliance upon the benefits brought about through food technology, we must examine our interaction with the consumer and our respective roles in providing him with a valid basis for accepting of rejecting a broader application of chemicals and industrial processing in the manufacture of foods. I can assure you that such acceptance will not come from an advertising barrage to the effect that chemicals are good for you; the susceptibility to this approach is approximately nil.

The matter of advertising and merchandising is not irrelevant, however, and although most of you do not have primary responsibilities in this area of corporate management, I would urge, as my first point, examination of the contribution of advertising to the consumers' inventory of information—or misinformation. The increased prominence of labelling, with reference to specific compounds, e.g. stearoyl lactylate, sodium erythorbate, propyl paraben, will make it difficult to conceal additives. At some point the public must understand why these compounds are used and what the food product would be like without them. This calls for an educational programme, factual in character and designed by people who understand that the over-sell will surely backfire. The contribution of the food industry to nutritional education is not a new concept, but one which deserves re-emphasis. And for openers, enlightened self-interest can lead the way.

Words, their meaning and what the public may think they mean are tools of the advertising trade. We are all aware of cases in which the words promise more than the product delivers, but there are inconsistencies in the other direction as well, when words suggest

that a product is inferior when it need not be. I personally find the official designation "meat by-product" to be unnecessarily negative in tone and suggestive of the likelihood that a nutritionally inferior or poorer-quality product is being presented. There is nothing inferior about by-products from a nutritional or safety point of view, and they deserve a more positive presentation. Fortunately many of the so-called "imitation" labels are being replaced with new and distinctive names, so that a product can stand or fall on its own merits without word comparison with some traditional model. Margarine is margarine, not imitation butter. It is quite probable that fabricated foods with no traditional counterpart will be developed. Credibility as to the attributes of such foods should not be impaired by words that promise too much, but neither should a legitimate, synthesized food carry the burden of detrimental labelling or nomenclature. There is ample room for collaborative efforts in this regard.

It has become fashionable and evidently commercially useful, even outside the "natural" or "organic" food industry, to capitalize upon public doubts with respect to additives in the merchandising of certain foods. Cereals are advertised as having a "natural taste" not available in processed cereals. Frankfurters are proclaimed to have "no dried milk—no cereal—no soy concentrate". Reference is made to "natural protein", as if there were some other kind. There is even natural beer. One must question the long-term utility of propagating and reinforcing an attitude that may ultimately have to change.

If the benefits of food additives or synthesized foods are to be realized with a reasonable level of public acceptance, it would be prudent for the marketing of a specific food to be postponed until a quality product had been achieved. In this respect it is reassuring to note a certain reserve in the retail marketing of soya-protein products until such time as the product will attract long-term consumer confidence.

My second point is that our common recognition of the need for chemical additives and the industrial processing of foods in no way reduces the necessity to prove that such additives or technologies are safe. Proof of safety is clearly in your area of responsibility. We are entering a period in which safety evaluation will become increasingly rational in terms of test methodologies and the ethical and legal definitions of safety. A rich armamentarium of biomathematical tools is at hand to correct many of the problems of experimental design and analysis of data. The selection of proper animal models continues to present difficult choices, because we have incomplete knowledge of the extent to which extrapolations to man can be made from data developed in non-human systems. The National Center for Toxicological Research has been organized with the specific purpose of examining the implications of comparative pharmacology and dissimilar metabolic patterns. There is a standing invitation to scientists from industry and academic institutions to participate in this national effort, and there is every reason to believe that the scientific skills reflected in the organizations represented here tonight can contribute substantially. On a reciprocal basis, knowledge developing at the Center will contribute to more realistic assessments of the value of observations made in animals for generalization to human responses.

The recently proposed criteria for requirements of analytical sensitivity are intended to bring about an accommodation to the fact that advances in analytical instrumentation are rapidly bringing us into the parts per trillion range, when the concept of zero tolerance begins to lose meaning. We have yet to hear the last word on this issue, but regardless of the nature of the final outcome, it will represent a move towards the rational interpretation of the significance of residues and contaminants.

Associated with the issue of safety is the concept of risk. Consumer activists have played an important role in directing public attention to the fact that different levels of risk are appropriate to different degrees of benefit and that the presumed benefits derived from the use of certain additives can be very small indeed. The fact that risk–benefit considerations, as applied to food and feed additives and drug or pesticide residues, had never been thoroughly examined led to the formation of the Citizens' Commission on Science, Law, and the Food Supply. It will be instructive to note carefully the deliberations of this group, which hopes to examine rational bases for the dimensions of warranted risk as applied to food constituents. You represent the professional links with the Citizens' Commission and you will find it appropriate to consider their contributions as you determine the composition of your products.

Further, in the field of ethics and law, Congress has directed the FDA to re-examine the bases of the Delaney anti-cancer clause and to develop knowledge that may be used in a consideration of alternatives to or modifications of this clause. This reflects an awareness on the part of the Congress that additives may have important uses and that "zero residue" levels are becoming meaningless.

I doubt whether any one of us can predict what will come of suggestions that all foods be required to be "nutritious", much as drugs are now required to be "effective". Cortez Enloe, writing in *Nutrition Today*, calls attention to the unanimous Supreme Court decision that gave the FDA sweeping authority to take ineffective drugs off the market, to impose stringent rules for assessing product effectiveness and to deny manufacturers hearings on contested actions. He points out that health professionals show signs of becoming similarly aroused about nutrition and that the public's interest in the safety and nutritional qualities of food is pushing Congress toward new initiatives in the food field, possibly paralleling those that now apply to drugs.

The posture of the food industry over the next few years with respect to the utilization of food additives, the credibility of advertising, the adequacy of safety testing and the level of rapport with consumers and health professionals will surely be reflected in actions on the floor of Congress, and in regulations of Federal agencies.

We both have important functions in bringing to the American consumer foods that are nutritious and appealing. Fundamental to the accomplishment of this objective is the use of chemicals in food technology, and yet the use of chemicals will continue to be vigorously challenged in direct proportion to doubts in the public mind as to their safety and efficacy. We are prepared to work with you towards a common goal wherein the attributes of food chemicals are accurately appraised and fairly presented to the consumer. Only in this way can advances in science, so welcome in other fields, contribute in equal measure to the quality and abundance of the food supply.

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REVIEWS OF RECENT PUBLICATIONS

Long-term Programme in Environmental Pollution Control in Europe. The Hazards to Health of Persistent Substances in Water. Annexes to a Report on a Working Group Convened by the Regional Office for Europe of the World Health Organization. Technical Documents on Arsenic, Cadmium, Lead, Manganese and Mercury. Helsinki, 10–14 April 1972. WHO, Copenhagen, 1973.

This volume consists of summaries of data on arsenic, cadmium, lead, manganese and mercury. The summaries were compiled by groups of scientists working under the auspices of WHO, but any implications or judgements expressed do not necessarily represent WHO policies or opinions. The book has been produced as an aid for those working on problems of metal toxicology and related matters. Although it has been reproduced in English, French and Russian, it is not for sale, being available only in a limited number of copies for those with a *bona fide* need of the information it contains.

In spite of the title, the data presented refer to the levels of the various metals in food, air and water, and the text discusses the toxicological impact of all these occurrences. The data for each metal are very clearly presented under specific headings, and the original references are appended to each of the five sections.

Each section ends with a rather brief interpretation of the data presented, in terms of the degree of hazard and the steps that may be taken to contain or reduce it. These are the personal opinions of the small group that compiled the data, and as such should not be regarded as valid for all time. For instance, the stated permissible daily intake of cadmium (about 60 μg/day) is likely to change in the light of current investigations.

This volume will clearly be of great value to those working in the field, and could well become a model on which the presentation of other data on environmental chemicals could be based.

IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man. Vol. 1. International Agency for Research on Cancer, Lyon, 1972. pp. 184. £0·40.

This is the first volume in what promises to be a series of informative publications on a subject of great social importance. The series will be based on a succession of meetings of the IARC Working Group on the Evaluation of the Carcinogenic Risk of Chemicals to Man, the first volume being the outcome of a meeting of the group held in Geneva in December 1971. Launching the series, this volume clearly defines at the outset the principles that guided the choice of compounds for evaluation and the selection of literature considered to be relevant to the topic. Equally clearly established are the criteria that were selected by the experts as a basis for the classification of a compound as carcinogenic to animals and to man. The difficulties encountered in extrapolating animal results to the possible situation in man are recognized and "no attempt has been made to interpret the animal data in the absence of human data in terms of possible human risk".

Despite this understandably cautious attitude, the panel of experts has made some critical assessment of the validity of the animal data. The result will be invaluable to national

and international agencies responsible for deciding whether a particular compound should be permitted or banned.

A particularly important feature of the monographs is the attention paid to chemical structure, physical properties and other attributes by which a compound can be identified. Equally important is the coverage given to the metabolism and pharmacokinetics of the compounds, since these aspects may, in some instances, permit some degree of "extrapolation from animals to man".

The topics selected for this first volume are a mixture of inorganic and organic substances, the latter including both synthetic compounds, some of which are commercially important, and natural products. The inorganic representatives are limited to beryllium and its compounds, haematite and iron oxide and lead salts, but the more numerous materials from the organic side are carbon tetrachloride and chloroform, auramine, 4-aminobiphenyl, benzidine and *o*-tolidine, dimethyl- and diethylnitrosamines, methyl- and ethylnitrosoureas. *N*-methyl-*N*,4-dinitrosoaniline, aflatoxins, cycasin, safrole, isosafrole and dihydrosafrole, sterigmatocystin and *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]acetamide.

The monographs on these compounds measure up to a high standard, being both clear and concise and providing an objective assessment as far as this is possible. Subsequent volumes, which should include a wide variety of important environmental chemicals, are anticipated with enthusiasm.

IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man. Some Inorganic and Organometallic Compounds. Vol. 2. International Agency for Research on Cancer, Lyon, 1973. pp. 181. £1·50.

With a format following the general pattern that proved successful in Volume 1 of the series, this second set of monographs is based on the deliberations of two IARC Working Groups on the Evaluation of the Carcinogenic Risk of Chemicals to Man, recorded at meetings held in Lyon in October and November 1972. The October meeting was concerned solely with asbestos, while the November meeting tackled a number of environmentally important metals and their compounds.

As in the previous volume, each monograph presents a comprehensive and factual review of experimental, clinical and epidemiological data together with some evaluation of these data. In the case of the human data one feels that this evaluation has been made with commendable care, but in the evaluation of animal data, little consideration seems to have been given to the pitfalls inherent in assessing the carcinogenicity of chemical compounds. One is left with the impression that insufficient attention was paid to the extensive experimental evidence pointing not only to the unsuitability of the mouse as a species for carcinogenicity tests but also to the irrelevance of certain experimental results in the rat. This approach renders "extrapolation to man" a most difficult task, but to some extent the Working Parties got round this difficulty by the simple device of making no attempt "to interpret the animal data in the absence of human data in terms of possible human risk", which leaves one questioning the justification for the considerable time and expense spent on carcinogenicity testing in animals.

In this issue, compounds of cadmium, chromium, arsenic and nickel, together with tetraalkylleads and iron–carbohydrate complexes, are considered in detail. For each compound a brief but informative outline of chemical composition and common usage is given,

wherever possible, and the available biological data are laid out logically and clearly. The contributions on arsenic and its compounds and on iron complexes stand out for their thoroughness and balance, having largely avoided the pitfalls mentioned earlier. The case for the carcinogenicity of arsenic to man is very well presented and is convincing despite the absence of positive results in animals. Equally convincing is the case against the carcinogenic hazard presented to man by iron–carbohydrate complexes in spite of their ability to induce tumours in animals.

The question of asbestos is also given prominence in this volume. The summary of data on the crystal structure of the different forms clarifies an area that might otherwise remain obscure to many outside the field of mineralogy, and is of particular importance in view of the growing weight of evidence pointing to the influence of crystal shape on the induction of tumours by asbestos.

On the whole the monographs are written in a clear, simple style which is not unpleasant to read although a mass of scientific detail has necessarily been included.

Fourteenth Progress Report of the Standing Technical Committee on Synthetic Detergents. Department of the Environment. HMSO, London, 1973. pp. 29. £0·30.

Generally encouraging conclusions as to the state of detergent pollution are reached in the latest report of the committee established in 1957 to keep an eye on the detergent levels in the surface waters and sewage works of the UK. The slight but general improvement in the average percentage removal of anionic surfactants at sewage works has continued, and specific surveys at Luton and Stevenage have given overall values of 92–94 and 93–96% respectively. Most rivers showed a slight decline in surfactant residues during 1971, the condition of the rivers Thames and Lee being particularly satisfactory. However, in the Aire and Calder in Yorkshire the surfactant concentration increased, with a particularly marked rise in polyglycol residues, and consequently there was no abatement of the long-standing foaming problem.

The sale of soap products again declined in 1971 and there was no increase in the total consumption of anionics or of domestically used nonionics. Total consumption of ethoxylated nonionics was estimated at 16,000 tons, 12,500 tons of this being used for industrial purposes. Unfortunately, the hoped-for voluntary agreement to phase out the use of hard nonionics, mentioned in the previous Progress Report, did not materialize, because imports could only be restricted if a public health risk were involved.

The internationally acceptable method for assessing biodegradability of anionic surfactants developed by the Organisation for Economic Co-operation and Development was published in 1971, and work on a suitable test for nonionics continued. Difficulties over analytical methods have now been largely resolved, and work on the effect of temperature on the biodegradability of nonionics has been reported. On the insistence of the UK Government, the EEC draft directive on synthetic detergents was revised during 1972 to provide for a minimum average biodegradability of 90%, rather than the 80% originally specified.

The Soap and Detergent Industry Association put forward proposals for voluntary prior notification of changes in the composition of detergent products. Since discussions were also proceeding with the Confederation of British Industry on the subject of a general

agreement for notification of the introduction of new chemicals into the environment, it was felt that this general agreement should take precedence over a more limited one on detergent products, but the detergent proposals are being used as a basis for discussion.

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BOOK REVIEWS

Selective Toxicity. The Physico-chemical Basis of Therapy. 5th ed. By A. Albert. Chapman and Hall, London, 1973. pp. xv + 597. £7.

This new edition of *Selective Toxicity*, a standard and widely acclaimed best-seller on the subject of chemotherapy, preserves the general format of the earlier editions but has been extensively revised and augmented by the inclusion of much new material. As a consequence, additional chapters have been included in each of the two parts into which the book is divided.

The first part, consisting of six chapters, is devoted to topics of general interest. These range from the health and economic benefits and the potential hazards resulting from the judicious use and indiscriminate abuse of pesticides, drugs and antibiotics to the principles underlying and the factors influencing the biological effect of a compound. The role of the hepatic drug-metabolizing enzymes and the gastro-intestinal microflora in mediating the metabolic transformation of compounds and thereby influencing their subsequent pharmacodynamics is discussed, and ample examples are given of agonistic and antagonistic effects resulting from the interaction of drugs administered simultaneously. Two chapters provide adequate, albeit brief, outlines of the morphology, structure and chemistry of intracellular constituents and describe interspecies differences in biochemical pathways relevant to the metabolism of foreign compounds. An excellent yet concise historical account is given of the development of the science of chemotherapy, and the chapter on pharmacodynamics elucidates the factors influencing the tissue- and species-specificity of compounds.

The second section of this book, a logical complement to the first part, provides a physico-chemical rationale for the biological activity of a wide range of compounds, relating this to their chemical structure and conformation. The influence of chemical bonds and steric and electronic configuration on the biological effects of compounds is discussed; and the way in which the interplay between these same factors and the energetics of enzymatic processes can be applied to the design of new chemotherapeutic agents is described, with particular reference to inhibitory or stimulatory effects. The relevance of the lipid solubility and ionization of compounds to their translocation across membranes and the role of metals in the chelation of drugs and their subsequent transport and disposition in the organism form the subjects of other valuable chapters. Other sections of equal merit refer to the influence of covalent bonds, steric factors, free radicals and surface activity on the biological properties of compounds.

Toxicology is an extremely complex and hybrid field, ranging as it must across various disciplines. This book will be of undoubted value in providing a sound framework not only for the novices but also for the High Priests of this esoteric art.

Residue Reviews. Residues of Pesticides and Other Contaminants in the Total Environment. Vol. 44. Edited by F. A. Gunther. Springer-Verlag, Berlin, 1972. pp. vii + 192. DM 52.

The names dichlorvos, Vapona and DDVP (*O,O*-dimethyl-2,2-dichlorovinyl phosphate) are all familiar to Journal readers. A great deal of information has been generated on this

material and reference has been made to many of the studies. A large section of this volume of "Residue Reviews" is taken up with a critical review, by members of a Task Force of the Environmental Health Sciences Center in Oregon, of the available literature relating to the possible use or misuse of DDVP-containing resin strips and to any potential hazard to human health that might result therefrom. The general conclusion is that, other than potential cholinesterase inhibition, DDVP exposure from resin strips does not create a significant hazard; mutagenicity, teratogenicity and carcinogenicity do not occur at sublethal doses. Dermal hypersensitivity appears to be infrequent or minimal. In the authors' opinion, however, combined conditions of high temperature, low humidity and restricted ventilation could conceivably create an environment in which atmospheric concentrations of DDVP could exceed established safety standards.

The problem of persistent residues of the polychlorinated biphenyls (PCBs) is another familiar topic, and one that has received a great deal of publicity in recent years, particularly in the USA where it was the subject of an FDA environmental impact statement at the end of last year. This present contribution is concerned with the levels found in the environment and an evaluation of toxicological and physiological effects. It is concluded that while further work in mammals is required, the indications are that the decision to limit environmental input is a wise one.

Still on the subject of the organochlorines and hard on the heels of the PCBs, follows a chapter on DDT, with its occurrence in marine phytoplankton under review. The significance of DDT residues in marine phytoplankton is related not only to the toxic effects of the chemical in these organisms but also to the transfer of DDT to higher levels of the food chain and the sedimentation of DDT residues with detritus of phytoplanktonic origin. It is suggested that although the measurement of DDT residues in phytoplankton presents technical problems, more such measurements should be made in order to assess the potential of these transfer processes.

A comprehensive coverage of the pesticide regulations in Argentina is included in this volume. The tolerances established for various foods are listed in detail. In addition, sections are included covering problems associated with the surveillance and control of pesticides, steps to be taken in connexion with the import and export of pesticides and methods to be followed for testing non-registered products.

The remainder of this volume comprises chapters dealing with the possible importance of transfer factors in the bacterial degradation of herbicides in natural ecosystems and with guidelines for the environmental study of pesticides, with particular reference to the determination of bioconcentration potential.

As with previous volumes, a wealth of information is included in this slim publication.

Mutagenic Effects of Environmental Contaminants. Fogarty International Center Proceedings no. 10. Edited by H. E. Sutton and Maureen I. Harris. Academic Press, New York, 1972. pp. xiv + 195. £3.50.

In view of impending requirements in Europe and North America and the proposals that have been formulated by WHO for mutagenesis testing, the appearance of this volume is timely. Testing for possible mutagenicity will be of concern to many industries, particularly those directly concerned with drugs and chemicals, and the growing interest in this facet of environmental toxicity will give rise to increasing demands for information on

basic mechanisms, experimental methods, the meaning of results and the present status of genetic hazards.

This volume, which can be read in a few hours, will help to meet this need, and will be particularly valuable for those with some biological training and experience. Although the book is of the multi-author type, the careful editing it has clearly received has largely succeeded in levelling out the variations in relevance, precision and readability frequently associated with compilations of this kind. The spectrum of genetic topics covered includes the biochemical basis of mutations, detection systems in bacterial, mammalian and non-mammalian organisms, the effects of mutagenic changes in man and the problems encountered in the monitoring of human populations for genetic change. In addition, many industrial and other compounds associated with some possibility of mutagenic hazard are reviewed briefly.

Each chapter is accompanied by a short list of references. These could perhaps have been more extensive in view of the Editors' stated objective of providing a comprehensive reference book. However, this is not a serious deficiency, as the subject is rapidly expanding and any future volumes will suffer a similar fate.

To all scientists interested in the chemical environment, and particularly to those working in the field of chemical toxicity, this volume can be unreservedly recommended.

Biochemical Actions of Hormones. Vol. II. Edited by G. Litwack. Academic Press, New York and London, 1972. pp. xiii + 542. £12.95.

Tradition has it that the word "hormone" (from the Greek verb meaning to excite or arouse) was coined by E. H. Starling and first used in his 1905 series of four Croonian Lectures, collectively entitled "The Chemical Correlation of the Functions of the Body". Considered broadly, the hormones are a heterogeneous collection of substances grouped together for operational, rather than chemical, reasons. Recognition that hormones play a key role in the control of biological systems has provided an enormous stimulus for the elucidation of the modes of their action at the biochemical level. Unfortunately, the multiplicity of effects that can be achieved, even by single hormones, has afforded considerable scope for speculation in areas which frequently suffer the defect that they are safe from too rigorous testing against real systems. Moreover, even though a voluminous descriptive literature exists, many views regarding hormone action remain within the area of the empiricist.

Bearing these points in mind, the quoted aims of the two-volume series "The Biochemical Actions of Hormones"—"to provide in one source an up-to-date survey of molecular and biochemical approaches bearing on the problem of hormone mechanism"—are laudable, if somewhat unrealistic in view of the notable slowness encountered in the publication of the glossier type of book. The format of both volumes is the now-familiar multi-author collection of essays devoted to specific areas within the general field of endocrinology. Since, in the reviewer's experience, each chapter of such a book is prepared by the chosen expert(s) working in isolation of one another, it is inevitable that there is more than a modicum of overlap. On the other hand, and with due regard to the fact that Volume I was published in 1970, those acquainted with the excellence of presentation in the first part will be pleased to learn that the Editor, Gerald Litwack, who also features in joint authorship, is again to be complimented on his choice of fellow authors and subjects for

the second part and, indeed, on maintaining the same high standard of contribution, illustration and indexing across both volumes.

No single book reviewer should have the temerity to comment in detail on individual elements of a work of such scope. Like its predecessor, Volume 2 has twelve chapters, eleven of which are devoted to hormones in higher animal systems and the twelfth to insect hormones (Volume I was organized in much the same way, with Chapter 12 devoted to plant hormones). Some contributions seem more timely than others. One remembers welcoming Olga Greengard's "The Developmental Formation of Enzymes in Rat Liver", J. R. Tata's "Regulation of Protein Synthesis by Growth and Developmental Hormones" and K. L. Manchester's "Insulin and Protein Synthesis" when Volume I was published. Undoubtedly motivated by the same self-interest (although now wearing a different professional hat), the reviewer finds particularly meritorious the chapters on "Cyclic AMP and Hormone Action" (R. W. Butcher, G. A. Robison and E. W. Sutherland), "Multiple Hormonal Interactions. The Mammary Gland" (R. W. Turkington) and "Mechanism of Action of Gonadotropine and Prolactin" (R. I. Dorfman). Others will undoubtedly base their opinions on different chapters.

In any event, it is now well recognized that the value of books such as these depends on three major factors. First, the comprehensiveness of original literature coverage, secondly, the quality and criticality of comment on individual contributory works and thirdly, the order and juxtaposition of material, chosen from the plethora of available original papers, designed to reveal the relationships between different studies. Viewed overall, both volumes are to be recommended on all three counts. An added, and particularly refreshing, feature in some instances is the inclusion of experimental observations not previously published elsewhere. Altogether a highly praiseworthy effort in a particularly involved area of scientific endeavour.

BOOKS RECEIVED FOR REVIEW

Enzyme Inhibitors as Substrates. Interactions of Esterases with Esters of Organophosphorus and Carbamic Acids. By W. N. Aldridge and Elsa Reiner. North-Holland Publishing Company, Amsterdam. pp. xvi + 328. Dfl. 65.00.

Soap Photodermatitis. Photosensitivity to Halogenated Salicylanilides. By P. S. Herman and W. M. Sams, Jr. Charles C. Thomas, Springfield, Illinois, 1972. pp. x + 181. $14.75.

Inborn Errors of Metabolism. Report of a Symposium on the Relation Between Developmental Biochemistry and Inborn Errors of Metabolism. Edited by F. A. Hommes and C. J. Van Den Berg. Academic Press, London, 1973. pp. xv + 375. £7.

Residue Reviews. Residues of Pesticides and Other Contaminants in the Total Environment. Vol. 46. The Problem of Residues in Meat of Edible Domestic Animals After Application or Intake of Organophosphate Esters. Edited by F. A. Gunther. Springer-Verlag, Berlin, 1973. pp. ix + 250. DM 70.20.

Fd Cosmet. Toxicol. Vol. 12, pp. 251–268. Pergamon Press 1974. Printed in Great Britain

Information Section

ARTICLES OF GENERAL INTEREST

NITROSAMINES IN SEARCH OF TUMOURS

Compounds of the nitrosamine and nitrosamide groups induce tumours in a wide variety of vertebrate species. The rat is particularly susceptible to their carcinogenic activity and in this species more than one organ can be affected by a single compound. Work carried out in the last 2 years has served to confirm the versatile carcinogenic activity of many nitrosamines and nitrosamides.

Carcinogenic activity

N-Nitrosopiperidine produces cancer in both the oesophagus and the liver of rats. Ito *et al.* (*Gann* 1971, **62,** 445) investigated the histogenesis of the oesophageal tumours by light and electron microscopy. The feeding of a basal semi-synthetic diet containing 0·03% of this nitrosamine for periods of 12–20 weeks produced in the oesophagus multiple growths varying from hyperplastic areas to frank carcinomas. Under the light microscope, the cells appeared squamous in shape. They were usually regular and arranged in an orderly manner in the benign lesions but were irregular in size and shape in carcinomas. 'Pearl' formation and invasion of subjacent muscle were also seen in the malignant lesions. Ultrastructurally, the cells of the benign lesions differed little from the normal squamous cells, whereas the carcinoma cells contained vacuolated mitochondria, free ribosomes and spiral intranuclear inclusions. The appearance of these cells was similar to that of the cells in urinary-bladder tumours induced by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (Ito *et al. ibid* 1969, **60,** 401). At week 20 after the start of treatment, the incidence of oesophageal cancer varied from 50 to 82% in rats fed the test diet for 12–20 weeks. The incidence of liver carcinomas in these same rats was 85–100% at week 20.

The carcinogenic activity of nitrosopyrrolidine, another cyclic nitrosamine, was investigated afresh by Greenblatt & Lijinsky (*J. natn. Cancer Inst.* 1972, **48,** 1687). They administered the carcinogen in the drinking-water for 5 days/week for 67 weeks. The calculated daily intake of the nitrosamine was 16 mg/kg and a total dose of 1340 mg was estimated to have been consumed by each rat. All of the 25 rats that survived to the end of the 105-week observation period developed hepatocellular carcinoma. Of the 12 male rats surviving, seven also developed testicular tumours, four of which were mesotheliomas of the tunica vaginalis, a relatively rare tumour in this species. Interstitial cell tumours accounted for the remainder.

The carcinogenic activity of butyl-(4-hydroxybutyl)nitrosamine, known to be a potent bladder carcinogen, appears to be influenced by the sex hormones. When it was administered continuously in the drinking-water at a level of 0·022% to 50 male and 50 female mice from 11–13 weeks after birth until death, all the mice that could be autopsied (82

in all) were found to have developed bladder carcinoma, without the appearance of other toxic effects (Bertram & Craig, *Eur. J. Cancer* 1972, **8**, 587). Metastatic spread occurred in 13 of the mice. Lethal tumours developed much earlier in the males than they did in the females, the mean times from start of treatment until death being 190 and 253 days, respectively. This difference was abolished, however, if the males were castrated or, conversely, if the females were treated with testosterone. This hormone also restored to control values the tumour-induction time in castrated males.

Unlike butyl-(4-hydroxybutyl)nitrosamine, diethylnitrosamine (DENA) causes hepatocellular carcinomas in mice and rats when administered by the oral route. In hamsters, carcinomas in the nasal cavities and squamous-cell papillomas in the larynx, trachea and stem bronchi resulted when DENA was administered sc in a weekly dose of 20 mg/kg body weight (Mohr *et al. J. natn. Cancer Inst.* 1972, **49,** 595). In a group of ten male hamsters treated in this way, all the animals developed tumours in the respiratory system and died after treatment for 16–25 weeks. A bronchogenic squamous-cell carcinoma was found in each of the two animals that survived longest. The same range of tumours was observed in hamsters whose mothers were treated with DENA in sc doses of 5, 10 or 20 mg/kg daily from day 1 to day 30 after delivery, indicating the transfer of DENA or its active metabolites to the offspring in the mothers' milk (*idem, Z. Krebsforsch.* 1972, **78,** 73). Dibutylnitrosamine (DBNA), given orally in olive oil in doses of 300, 600 or 1200 mg/kg during lactation also induced this range of tumours in the hamster offspring, indicating that this, like the diethyl analogue, was excreted in the milk either unchanged or in the form of active intermediates. In these experiments the mothers also developed tumours, although in both generations the effects of DBNA were less marked than those of DENA. The first tumour appeared in the parent generation 13 weeks after the beginning of treatment with DBNA and 21 weeks after treatment with DENA began. The induction periods were much longer in the offspring.

At the bottom of this homologous series, dimethylnitrosamine (DMNA) has also been shown to affect a range of tissues. Kuwahara *et al.* (*Gann* 1972, **63,** 499) found that weekly sc or ip injections of 0·15 mg DMNA for periods of up to 25 weeks produced in three strains of mice a high incidence of haemangioma in the retroperitoneal and abdominal regions. Haemangiomatous lung and liver tumours were also seen in mice given sc injections of DMNA, but there were few tumours of this kind in the liver and none in the lung after DMNA was given by ip injection or at a level of 100 ppm in the diet. The work of Ingram (*Br. J. Cancer* 1972, **26,** 206) indicates that the versatility of DMNA as a carcinogen relates not only to tissues but also to species. Six or seven ip injections of 16 mg DMNA/g body weight administered to newts (*Triturus helveticus*) over a period of 3–4 weeks produced hepatocellular carcinomas in the two newts (out of 19) that survived for more than 3 months after treatment. Liver tumours were diagnosed at autopsy 7 and 13 months after treatment.

The ease with which the 'species barrier' can be crossed is shared by other nitrosamines. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine, for instance, produces gastric carcinoma not only in rats but also in dogs. When given orally to dogs in concentrations of 167 μg/ml in the drinking-water for 1 month and 83 μg/ml for the following 13 months, this compound produced multiple tumours which histologically presented the appearance of gastric adenocarcinoma (Shimosato *et al. J. natn. Cancer Inst.* 1971, **47,** 1053). The tumours appeared with greater frequency in the antrum along the lesser curvature and in the cardiac portion along the greater curvature.

Targets in the nervous system

The nitrosamines, despite their versatility as carcinogens, have not been reported to produce tumours of the central nervous system even when administered to newborn animals. In contrast, the alkylnitrosamides appear to produce tumours of this type not only in the newly born but also in adult animals. Furthermore, when administered to the mother, these compounds induce brain tumours in the offspring. Thus ethylnitrosourea (ENU) administered to BD IX rats in a single iv injection of 80 mg/kg on day 15 of pregnancy induced transplantable tumours in both the central and peripheral nervous system of the offspring (Wechsler & Ramadan, *Naturwissenschaften* 1971, **11,** 577). Intracerebral or sc injection of a single dose of 50 mg ENU/kg in newborn Long-Evans rats was equally effective, with 81–85% of the rats treated in this way developing tumours in the brain and spinal cord (Grossi Paoletti *et al. Pharmac. Res. Commun.* 1972, **4,** 201). These were classified histologically as neurinomas, oligodendrogliomas, mixed polymorphic gliomas, gliosarcomas and sarcomas. Some 70% of the affected animals had multiple tumours.

A comparable result was obtained in mice by Searle & Jones (*Nature, Lond.* 1972, **240,** 559). A single dose of ENU in the 10–160 mg/kg range injected sc into day-old mice resulted in the production of equal numbers of neural tumours in the mice of the IF strain and in those of the DBA strain, but although comparable numbers of mice from each strain were given identical treatment, no tumours of the central nervous system were observed in those of the A and C57BL strains.

A study of the comparative potency of ENU and methylnitrosourea (MNU) in the production of brain tumours revealed that ENU was considerably more potent (Jänisch *et al. Arch. Geschwulstforsch.* 1972, **39,** 99). MNU administered to rats in a single iv dose during the last third of pregnancy induced tumours of the central and peripheral nervous systems in 39·7% of the offspring, whereas ENU induced such tumours in 97·4%.

MNU has also been shown to be capable of inducing brain tumours in adult rats (Swenberg *et al. Lab. Invest.* 1972, **26,** 74). Repeated iv administration of 5 mg MNU/kg weekly for 36 weeks resulted in the production of tumours of the nervous tissue in 97% of the experimental rats, which were of two strains, Sprague–Dawley and Fischer. Male Sprague–Dawley rats had the highest incidence (100%) of grossly detectable brain tumours and the shortest median survival time (265 days). No extraneural tumours were observed in the males of either strain. Fewer Sprague–Dawley females developed neurogenic tumours, 89% having grossly detectable brain tumours, but 22% of this group had extraneural malignancies. Fischer rats of either sex developed more peripheral nerve tumours and fewer brain tumours than did Sprague–Dawley rats, although the total incidence of neurogenic tumours was comparable. The female rats of this strain showed a 55% incidence of extraneural tumours.

The histology of brain tumours induced by MNU and ENU was studied in detail by Schiffer *et al.* (*Tumori* 1971, **57,** 333) in a series of 300 tumours induced experimentally, mostly in Long-Evans rats. MNU was administered iv at 50 mg/kg once a month, while ENU was given once only to neonatal rats sc or intracerebrally in a dose of 50 mg/kg or to pregnant rats in a dose of 10 mg/kg for transplacental transmission to the foetus. The tumours were classified into isomorphic oligodendrogliomas, micro-oligodendrogliomas, mixed and enteromorphic gliomas, polymorphic gliomas and gliosarcomas.

Other nitrosamides, apart from MNU and ENU, induce tumours of the nervous system. A single oral dose of *n*-propylnitrosourea (120 mg/kg) administered to four BD II rats on day 19 of pregnancy resulted in the production of tumours of the brain and peripheral

nervous system in seven of the 19 offspring (Ivankovic & Zeller, *Arch. Geschwulstforsch.* 1972, **40,** 99). Two of the animals had brain tumours, and four tumours of the trigeminal nerve were found. Two additional animals had kidney tumours. The mean induction time of all these tumours was 330 days.

These authors (Zeller & Ivancovic, *Z. Neurol.* 1972, **202,** 121) also showed that *N*-butylnitrosourea (BNU) produced brain tumours when injected in a single sc dose of 120 mg/kg into Sprague–Dawley rats on their first day of life. Multiple neurogenic malignancies were observed in 17 of the 25 treated rats, the median induction time being about 290 days. The total of 29 tumours observed included five in the brain, three in the cerebellum and five in the spinal cord. The eight surviving rats appeared to be completely resistant, however, having failed to develop any tumours by the time the study was reported.

The peripheral nerves seem to be the major target for the oncogenic activity of BNU injected sc into hamsters, according to Matsuyama & Suzuki (*Experientia* 1971, **27,** 1459). When a dose of 20 mg/kg body weight was given sc to week-old hamsters and repeated five times at weekly intervals, about half the treated animals developed tumours in the subcutaneous, parathymic, mesenteric and retroperitoneal regions. Histologically and ultrastructurally some of the tumours displayed features characteristic of neoplastic growths originating from the terminal parts of the peripheral nerves.

Another nitrosourea, *N,N'*-dimethylnitrosourea failed to produce tumours in the nervous system of the hamster, however. This nitrosamide has been shown to affect injection-site connective tissue, the central nervous system and the thymus in rats or mice (Druckrey *et al. Z. Krebsforsch.* 1967, **69,** 103; Hiraki, *Gann* 1971, **62,** 135) but no such tumours were found in adult hamsters given dimethylnitrosourea in weekly sc injections of 40 mg/kg for 17–31 weeks (*idem, ibid* 1971, **62,** 321). Instead, some 75% of these animals developed tumours of the forestomach, breast and uterus.

Alkylation, immunosuppression and cancer

The rat has been employed extensively in the past not only to study the carcinogenic properties of nitrosamines and nitrosamides but also to investigate the mechanism by which tumours are produced. An early and striking finding in this respect was the demonstration of alkylation of the nucleic acids of all organs, and particularly of those of the liver, and it was widely believed that this interaction was of fundamental importance in the induction of cancer. Evidence has now been presented (Garcia & Lijinsky, *Z. Krebsforsch.* 1972, **77,** 257) indicating that the two processes may not be as closely connected as was at first thought. These authors gave five cyclic nitrosamines—nitroso-3-pyrroline, nitrosopiperidine, nitrosomorpholine, nitrosoheptamethyleneimine and dinitrosopiperazine—in the drinking-water to groups of 30 rats, each rat receiving approximately 2 mg of one of the nitrosamines daily on 5 days/week for 50 weeks. A substantial proportion of the animals treated with nitrosopiperidine or nitrosoheptamethyleneimine developed carcinomas of the oesophagus. In addition the latter induced tumours in the trachea and lung. Tumours of the nasal cavity predominated with dinitrosopiperazine and the main targets for nitrosomorpholine were the nasal cavity and liver. Despite this evidence of unequivocal and versatile carcinogenic activity, no alkylation of nucleic acids was detected in the various target organs.

Similarly, Goodall *et al.* (*Cancer Res.* 1968, **28,** 1217) failed to demonstrate any alkylation of rat-liver nucleic acids by *N*-nitrosohexamethyleneimine, which was, nevertheless,

shown to be a powerful hepatocarcinogen when given orally to this species. It has also been shown to be active in hamsters, but the target organ is different. A single sc dose (64 mg/kg) of this compound administered to hamsters induced more tumours in the trachea than in any other single organ, the incidence of tracheal tumours showing some indication of a rough dose-response relationship (Althoff *et al. Z. Krebsforsch.* 1972, **78,** 78). Mice given the same treatment showed virtually no increase in tumours of the respiratory tract. However, when tumours at other sites were taken into account, more mice than hamsters were affected.

Immunosuppression has been claimed to be a characteristic of chemical carcinogens (Ball, *J. natn. Cancer Inst.* 1970, **44,** 1). This claim was not substantiated, however, when the immunosuppressive activity of four carcinogenic nitroso compounds was investigated in male Sprague–Dawley rats (Scherf, *Z. Krebsforsch.* 1972, **77,** 189). The compounds tested were DMNA, ethyl-*n*-butylnitrosamine, *N*-nitrosomorpholine and MNU, administered by stomach tube for 24 weeks in weekly doses corresponding to 5, 2·5, 1·25 and 0·625% of the LD_{50} of each compound. The immunological status of the animals was assayed at weeks 12, 18 and 24 by the plaque test of Jerne *et al.* (In *Cell-bound Antibodies*, edited by B. Amos and H. Koprowski, p. 109, Wistar Institute Press, Philadelphia, 1963). It was found that DMNA and MNU were potent immunosuppressive agents, while ethyl-*n*-butylnitrosamine was slightly less potent and the highly carcinogenic *N*-nitrosomorpholine possessed only a relatively weak immunosuppressive capacity.

The publications mentioned in this article add one more chapter to the continuing story of the potent carcinogenic properties of the nitrosamine and nitrosamide compounds. They provide ample confirmation of the ability of these compounds to affect a variety of species, to spare very few organs in their selection of sites for activity and to produce tumours after a "one-shot" treatment. The especial susceptibility of the very young animal to their oncogenic action is also underlined. No doubt further publications will add more episodes to this sombre story.

EASY WITH THE RAPESEED OIL

The feeding of rapeseed oil (RSO) as a major source of calories has been reported to lead in several animal species to excessive deposition of fat in the heart muscle, with subsequent myocardial damage. This effect has been attributed to the relatively high content of erucic acid in the oil, and it has been suggested that the mechanism of RSO toxicity involves interference with the metabolic function of mitochondria, since some disturbance of oxygen uptake and an uncoupling of oxidative phosphorylation has been demonstrated. It seems that this disturbance of mitochondrial function may combine with the relatively slow rate of metabolism of erucic acid and its restricted deposition in depot fat in some species to lead to its accumulation in the heart when high levels are fed.

Further studies on lipid deposition

Walker (*Nutr. Metabol.* 1972, **14,** 8) has reported that rats fed a diet containing RSO or a 1 : 5 mixture of corn oil and ethyl erucate showed the highest deposition of erucic acid in their adrenals, followed in descending order by accumulation in the plasma, heart, spleen, kidney, liver, erythrocytes, testis and brain. Of the total fatty acids, erucic acid accounted for about 15% in the adrenals and about 6% in the heart of rats given the corn oil–erucate mixture. The RSO diet produced less deposition of erucic acid and a relatively

greater accumulation of eicosenoic acid in the tissues, than did the erucate-fortified diet. Further work by Walker *et al.* (*Comp. Biochem. Physiol.* 1972, **42B,** 619) showed that erucic acid accounted for 23–30% of the total fatty acids incorporated into the cholesteryl ester fraction of the adrenal and ovarian lipids of female rats fed for 10 weeks on a 1 : 5 mixture of corn oil and ethyl erucate.

An increase in the myocardial concentration of fatty acids, including erucic acid, has been reported in rats within 1 week of the feeding of a diet containing 20% fat as RSO (Beare-Rogers & Nera, *ibid* 1972, **41B**, 793). Erucic acid deposition and histological changes in the heart were more marked in weanlings than in older rats, but when RSO feeding was continued for 10 weeks from weaning there was some falling off in the myocardial concentration of erucic acid. Similar results were recorded in gerbils fed 20% RSO. The later reduction in myocardial lipid deposits in rats may be explained on the basis of some experimental results reported by Struijk *et al.* (*Biochim. biophys. Acta* 1973, **296,** 253). In rats fed for 6 or 11 days on a diet in which 50% of calories was provided by RSO containing 48% erucic acid, a mean increase of 45% in post-heparin lipoprotein lipase activity was found, compared with that in controls given comparable dietary levels of olive oil or sunflower-seed oil. Determinations carried out in rats fed the same RSO diet for 1, 2, 3, 6, 10 and 14 days showed a marked rise in post-heparin lipoprotein lipase activity after day 3 with a peak at day 6, after which the level remained high. This high level of enzyme activity thus correlated in time with the decrease in accumulated triglyceride in the heart. Rocquelin (*Annls Biol. Anim. Biochim. Biophys.* 1973, **13,** 151) showed that comparable effects on erucic acid deposition were obtained with refined and with crude RSO, each containing about 50% erucic acid. When either oil was given to rats at a dietary level of 15%, the total fatty acids in the myocardium contained 33–34% erucic and 8% gadoleic acids, while the corresponding levels in the liver were about 2% of each. When an equivalent level of interesterified RSO was fed, the proportions of these two acids in the myocardial fatty acids rose to 37·5 and 9·4% respectively.

In comparison with their findings in rats, Beare-Rogers & Nera (*loc. cit.*) found that erucic acid accumulation was relatively delayed in the myocardium of the squirrel monkey, but it did show a threefold increase between weeks 1 and 10 of feeding with the 20% RSO diet. In contrast, miniature pigs and commercial piglets fed 10 or 20% RSO showed little increase in the cardiac level of fatty acids, although small fat droplets, absent from the control hearts, were seen in the myocardium (Beare-Rogers & Nera, *loc. cit.*). Erucic acid accounted for 1–2% of the myocardial fatty acids. In pigs given a corn–soya diet containing 10% RSO, Walker (*Can. J. Anim. Sci.* 1972, **52,** 713) found that erucic acid accounted for 1·3–4·6% of the total fatty acids in the spleen, erythrocytes, heart, liver, kidney and testis, a range that compared closely with the 1·4–4·9% he found in these organs in his study in rats (Walker, *Nutr. Metabol.* 1972, **14,** 8). Unlike the situation in the rat, however, erucic acid concentration in the pig was greatest in the plasma and adipose tissue (more than 7% of total fatty acids), but very low in the adrenals and ovaries. The deposition of this acid in the adipose tissue of pigs raises the practical question of its possible incorporation into the human diet via this route.

Erucic acid concentrations in the rat adrenal have been further studied by Carney *et al.* (*Biochim. biophys. Acta* 1972, **280,** 211). Comparing results in rats fed 10% RSO (containing 34% erucic acid) or 10% corn oil in the diet for 12 weeks, these authors found that the cholesteryl ester of erucic acid (and of eicosanoic acid) appeared to be deposited in addition to, rather than at the expense of, the other cholesteryl esters, such as the arachidonate

and adrenate. The fact that stimulation of prostaglandin production by adrenocorticotrophin is depressed in adrenal homogenates from RSO-fed animals, compared with that in controls fed corn oil, suggests that there may be impairment of ATP production in this organ, as has been indicated in the heart.

Metabolic disturbances and tissue lesions

Food consumption and growth were significantly lower in rats fed a diet containing 31·5% (w/w) RSO (60% on a calorie basis) for 64 weeks than in those fed sunflower-seed oil (Hornstra, *Nutr. Metabol.* 1972, **14,** 282). The poorer digestibility of the RSO diet was shown to be due to the high content (about 50% of the RSO) of erucic acid, which was less readily digested because of its long chain length. Once absorbed, however, erucic acid was almost completely metabolized, but in the process there was an increase in oxygen consumption indicative of an uncoupling of oxidative phosphorylation. Overall, the digested calories from the RSO diet were utilized less efficiently than those from the control diet.

Leclercq (*Nutr. Rep. Int.* 1972, **6,** 259) has reported that the digestibility of erucic acid is similarly poor in the domestic hen. The metabolizable energy derived from peanut oil at a level of 20% in the fowls' diet was 200 kcal/kg of diet greater than that from a 20% RSO diet. Apparent utilization of erucic acid was lower than that of oleic acid. Only small traces of erucic acid were found in the lipids of blood, liver, fatty tissues and eggs of the test fowls, and the feeding of [14-^{14}C]erucic acid showed it to be rapidly converted into oleic acid. In fact, apart from its slower rate of absorption from the gut, the metabolism of erucic acid closely resembled that of oleic acid. This could explain the higher level of unsaturated fatty acids found in the tissues of animals given a dietary excess of either oleic or erucic acid, but makes it difficult to explain why yolk production should be markedly reduced in fowls fed erucic acid in the form of RSO. The reduction in egg weight and yolk weight in fowls fed 5% RSO has been reported by Leclercq (*Annls Biol. Anim. Biochim. Biophys.* 1972, **12,** 505) to be the same as that produced by 2% trierucine in the diet, indicating that it is erucic acid itself which is responsible for the deleterious effect of RSO on reproductive function in fowls.

The feeding of RSO has also been associated with growth retardation in ducklings (Abdellatif *et al. Nutr. Metabol.* 1972, **14,** 17). In this study, ducklings were fed for 3 weeks on diets in which 60% of the total calories were provided by a mixture of RSO and soya-bean oil (the RSO/SBO mixture), a mixture of RSO and hardened palm oil (RSO/HPO) or a mixture of soya-bean oil and hardened palm oil (SBO/HPO). Growth retardation was pronounced in the birds given the RSO/SBO mixture and was not markedly improved by increasing the protein content of the diet from 23% of the total calories (26% w/w casein) to 32% of total calories as casein or soya protein. With the RSO/HPO diet, the growth retardation was less severe and was largely offset by either type of protein supplementation of the diet. The RSO/SBO diet also caused increased haematocrit readings, hydropericardium, vacuolation in the heart and skeletal muscles, liver cirrhosis and adverse haemopoietic changes, but only the skeletal and cardiac changes were seen to any marked degree in birds given the RSO/HPO mixture. Although RSO/SBO feeding did not alter the total serum-protein level, serum globulin rose at the expense of serum albumin except when the high soya-protein supplement was given. With the RSO/HPO diet, the serum-albumin level was normal, but the globulin level remained high. These findings lend some support to the possibility that RSO fed to ducklings impairs albumin synthesis in the liver.

an event which may in turn predispose to the development of hydropericardium in these birds (Abdellatif *et al. loc. cit.*).

The experimental data and the speculations on rapeseed oil and its important constituent, erucic acid, are thus accumulating, but we have so far found no further leads on the human consumption and metabolism of these substances. Studies on these aspects of the problem demand attention and it would be of great interest to have something to report on this ere long.

STUDIES ON SOYA-BEAN PRODUCTS

Soya-bean products have become an important source of dietary protein for livestock and they are also finding increasing use in human nutrition. In this context, various toxic factors known to be present in the raw soya bean are obviously of interest, although most if not all of these are destroyed during processing. One such factor is a heat-labile trypsin inhibitor. This has been held responsible for the growth inhibition, reduced feed efficiency and pancreatic hypertrophy seen in rats fed raw soya-bean diets (*Cited in F.C.T.* 1967, **5,** 93), though not all species are equally susceptible and some other factor also appears to be associated with the growth depression that occurs in animals fed raw soya bean (*ibid* 1971, **9,** 924). The goitrogenic activity of the raw soya bean has been attributed to another heat-labile factor which leads to a reduction in enterohepatic circulation and a consequent increase in the faecal excretion of endogenous thyroxine (*ibid* 1967, **5,** 93).

Studies on these factors continue but, at least in ruminants, the issue of growth depression is likely to be complicated by the fact that the protein in soya-bean meal is readily soluble in rumen fluid. This tends to increase the extent of microbial hydrolysis and depress the amount of dietary protein available for postruminal degradation and utilization by the animal. Like the others already mentioned, this effect is counteracted to some extent by heat-treatment of the meal. Moreover, this is not the only suggested means of depressing the solubility of the soya-bean protein, treatment with formaldehyde or tannic acid being other possibilities.

Nishimuta *et al.* (*J. Nutr.* 1973, **103,** 49) studied diet digestibility and nitrogen balance in lambs fed daily on 800 g of a diet containing 17–19% of a normal solvent-extracted soya meal and compared the findings with those in lambs fed the same quantity of a soya meal that had been heat-treated or treated with formaldehyde (1 g/100 g dried meal) or tannic acid (9 g/100 g dried meal). Although cellulose digestibility was significantly reduced by heat-treatment, nitrogen retention was highest in the lambs consuming this diet. Formalin treatment reduced crude-protein digestibility, but not cellulose digestibility, and increased resistance to postruminal enzymatic degradation, so that nitrogen utilization was markedly depressed. Tannic-acid treatment had little effect on either digestibility or nitrogen retention. The various soya treatments had no notable effect on the plasma levels of total free amino acids, but the proportions of individual amino acids varied between the groups. The control group, for example, had higher levels of alanine and glycine and lower levels of leucine, lysine, phenylalanine and tyrosine than lambs on the treated diets, while comparatively high levels of isoleucine, valine and lysine were associated with the heat-treated diet.

Free amino acids in the plasma, as well as in the intestinal contents, have also been compared in chicks fed raw or heated soya-bean meal (Bielorai *et al. ibid* 1972, **102,** 1377). Amino acid levels in the intestine were much lower with the former than with the latter diet, particularly in respect of valine, cystine, methionine, isoleucine, leucine, tyrosine and phenylalanine. For these essential amino acids, the differences were proportionately much

greater with diets containing 22% soya-bean protein than with those containing 15%. These differences were reproduced much more clearly in plasma from the hepatic portal vein than in that from the heart. The results of this study, which included the demonstration of an undigested protein containing essential amino acids in the intestine of chicks fed raw soya bean, again pointed to an overall reduction in amino acid utilization from this protein source.

Returning to the problem of the goitrogenic activity of unheated soya-bean flour, we have two complementary contributions on the possible identity of the factor responsible. Konijn *et al.* (*J. Sci. Fd Agric.* 1972, **23,** 549) extracted and fractionated unheated soya-bean flour and subjected each fraction to various physico-chemical studies and to assays of its effect on thyroid function. As a result, a thyroid-depressing agent was isolated and shown to be destroyed by autoclaving (commercial heat-processing) and to be of relatively small molecular weight, possibly in the 4000–5000 range. Given orally, this agent decreased iodine uptake by the rat thyroid and increased the tri-iodothyronine-binding capacity of rat serum. Further studies by this group (*idem, J. Nutr.* 1973, **103,** 378) suggested that the compound might be an oligopeptide or a glycopeptide composed of one or two amino acids and one sugar molecule. Support was also obtained for their earlier findings on the mechanism of the agent's goitrogenicity, findings which seem to be in some conflict with previous reports of excessive faecal excretion of thyroxine in laboratory animals and occasionally in infants fed soya-bean diets (*Cited in F.C.T.* 1966, **4,** 123).

Another question has arisen in connexion with the development of an industrial soya protein, which is prepared by alkaline modification and is used in the preparation of soya-protein textured foods. de Groot & Slump (*J. Nutr.* 1969, **98,** 45) reported a net decrease in protein utilization, but no clinical or histological abnormalities, in rats fed alkali-treated soya protein. Woodward & Alvarez (*Archs Path.* 1967, **84,** 1153), had found, however, that the feeding of a similar material with a comparable amino acid composition caused renal lesions in weanling rats. These lesions were characterized by cytomegaly in the cells of the straight portion of the proximal tubule. In an attempt to identify this nephrotoxic action with either a natural agent in the soya bean or a product of the industrial modification, Woodard & Short (*J. Nutr.* 1973, **103,** 569) fed groups of Sprague–Dawley rats for 8 weeks on diets containing 20% soya-bean proteins, which had been treated in several different ways. The industrial soya protein used was Alpha Protein, in the preparation of which sulphur dioxide rather than hydrochloric acid is used to precipitate the globulin fraction and the precipitated protein is modified by alkaline hydrolysis. Feeding of Alpha Protein itself again caused cytomegalic changes in the renal tubular cells of all the treated rats and this toxicity was not affected by heat-treatment or extraction of the protein with polar or nonpolar solvents. Feeding of various intermediates in the manufacture of Alpha Protein and feeding of edible protein before and after treatment with alkali demonstrated conclusively that this toxicity was induced by the alkali-treatment. Such treatment was also shown to lead to the formation of an unusual amino acid, lysinoalanine, which was present at levels of 0·6% in Alpha Protein and 2·6% in alkali-treated edible soya protein, although it could not be detected in the untreated edible protein.

With the heavy world-wide demands for protein to meet current requirements for both human and animal nutrition, it is obviously essential that full use should be made of all potential protein sources. It is equally obvious, however, that the full use of the soya bean is not entirely without its problems.

FACTORS IN CARBON DISULPHIDE TOXICITY

Industrial exposure to carbon disulphide (CS_2) carries a recognized hazard. Workers in the viscose industry who have been exposed to this compound have been found to suffer from an increase in mean systolic and diastolic blood pressure, and have shown an increased liability to coronary artery disease (*Cited in F.C.T.* 1971, **9,** 599).

Increased sensitivity of the myocardium to endogenous and exogenous catecholamines in rats exposed to CS_2 has been reported by Chandra *et al.* (*Expl. mol. Path.* 1972, **17,** 249). Fasting rats were given two ip injections of phenobarbitone (in doses of 80 and 50 mg/kg, respectively, with a 6-hour interval) and, after a further overnight fast, were exposed to an atmosphere of 4 mg CS_2/litre for 4 hours. This exposure was repeated on the following day, each of the CS_2 exposures being immediately preceded by an injection of noradrenaline in an ip dose of 1·5 mg/kg. Other phenobarbitone-treated rats were given a single dose of noradrenaline with or without a single exposure to CS_2, or were exposed to CS_2 after exposure to cold without receiving noradrenaline.

Myocardial lesions induced by these subtoxic doses of noradrenaline were slight unless the dose was followed by CS_2 exposure, when they became extensive. Animals exposed to CS_2 after stress (exposure to cold) in place of the noradrenaline injection suffered myocardial lesions similar to those produced by two doses of noradrenaline, but production of endogenous catecholamines by cold stress in the phenobarbitone-treated animals had no adverse effect on the myocardium in the absence of CS_2 exposure. Necrotic lesions were preceded by a loss of cytochrome-oxidase and phosphorylase activity in the myocardial tissue, and later by a reduction in succinic-dehydrogenase activity. While this study does not explain the mechanism by which CS_2 increases the myocardial toxicity of endogenous or exogenous catecholamines, it does tie in with earlier suggestions that disorders of catecholamine metabolism may be a factor in the development of ischaemic heart disease in people occupationally exposed to CS_2 (Magos & Jarvis, *Br. J. Pharmac. Chemother.* 1970, **39,** 26; *idem, J. Pharm. Pharmac.* 1970, **22,** 936).

We have seen that exposure to CS_2 increases serum-lipid levels in rats and rabbits, and in rats increases the rate of cholesterol synthesis in the liver (*Cited in F.C.T.* 1973, **11,** 516). El-Gazzar *et al.* (*Br. J. ind. Med.* 1973, **30,** 284) have carried out a survey of 82 workers in the Egyptian rayon industry with a view to assessing the effects of CS_2 exposure on serum levels of zinc and protein. At the time of the examination, 40 of the workers were exposed to CS_2, while nine had suffered exposure for a year or more in the past. In comparison with 33 workers who had never been actually exposed to CS_2, the currently exposed men had abnormally low serum-zinc concentrations (mean value 69 μg compared with 94·7 μg/100 ml). The group with previous exposure showed levels (87·8 μg/100 ml) that were lower, but not significantly lower, than the control values. Urinary zinc concentrations were higher in exposed men than in controls (with mean values of 525 μg compared with 354 μg/g creatinine). Total serum proteins were significantly higher in exposed workers than in controls (9·42 g compared with 7·48 g/100 ml). The difference was apparent in all the protein fractions, but in subjects whose exposure to CS_2 had terminated, only the γ-globulins still showed an increase, the other changes apparently being more transient.

Wrońska-Nofer *et al.* (*Biochem. Pharmac.* 1972, **21,** 2945) have studied the effect of prolonged CS_2 exposure on the turnover rate of nicotinamide–adenine dinucleotides (NAD) in the livers of rats. Female rats were exposed to concentrations of 1·7 (1·45–2·05) mg CS_2/litre for 5 hours/day on 6 days/week for 6 months. After the last exposure period, each rat was given an ip injection of [7-^{14}C]nicotinamide or [7-^{14}C]nicotinic acid. Nico-

tinic acid was the more rapidly and effectively incorporated into liver NAD, with maximum incorporation occurring within 30 minutes of the injection. With nicotinamide, on the other hand, maximum incorporation of the label in the liver NAD occurred 2–4 hours after injection of the precursor, and the maximum level of activity was lower. With both precursors, however, incorporation of labelled material into liver NAD was higher in rats exposed to CS_2. It was also evident that the increased rate of NAD synthesis in the livers of exposed rats was accompanied by an acceleration in NAD degradation. A relatively high excretion of nicotinamide metabolites by rats exposed to CS_2 has already been reported (Wrońska-Nofer & Tarkowski, *Medycyna Pr.* 1965, **16,** 77).

One member of this group (Sokal, *Biochem. Pharmac.* 1973, **22,** 129) has reported experiments in rats to determine the effect of exposure to CS_2 on some components of the electron-transport system in hepatic microsomes. In a first experiment, male rats were exposed to 1·2 mg CS_2/litre for 6 hours/day on 6 days/week; in a second, exposure was to 1·5 mg CS_2/litre for 5 hours/day on 6 days/week. Exposure to these concentrations for 5–7 weeks led to an increase of about 14% in liver weight compared with that in control animals, and total liver microsomal proteins increased in parallel. These exposures reduced by 47% the cytochrome *P*-450 levels calculated on the basis of liver microsomal proteins, while cytochrome b_5 and NADPH cytochrome *c* reductase showed some tendency to increase and lipid-dependent NADH cytochrome *c* reductase rose by about 60%. NADPH and NADH ferricyanide reductases remained unchanged. Magos *et al.* (*ibid* 1973, **22,** 992) demonstrated, by a series of studies on CS_2 exposure combined with pre-exposure treatments with phenobarbitone and/or starvation, that the level of cytochrome *P*-450 was not by itself a measure of sensitivity to the hepatotoxic effect of CS_2. Sokal (*loc. cit.*) observed that rats showed no overt toxic symptoms at the exposure levels he used, and body weights were unaffected. He found, moreover, that the changes observed were most marked in rats killed immediately after termination of the daily exposure. In rats killed 3 days after termination, the values were found to have returned almost to control levels.

The Polish group referred to above also studied the muscular atrophy in CS_2-exposed animals in connexion with the content of NAD in the skeletal muscles (Wrońska-Nofer *et al. Int. Arch. Arbeitsmed.* 1973, **31,** 123). Male rats were exposed to CS_2 in atmospheric concentrations of 1·5 (1·43–1·57) mg/litre for 5 hours/day on 6 days/week for up to 14 months. Muscular weakness of the hind limbs, accompanied by a failure to gain weight or by weight loss, was first seen between months 5 and 7, and from month 9 onwards the incidence of hind-limb paresis and paralysis increased and there was an increasing fall in muscle weight. Histologically, progressive atrophy of the striated muscle fibres was apparent, with no evidence of inflammation or dystrophy, although the two latter conditions have apparently been reported in man. The muscular atrophy was the result of a progressive degeneration of myelinated nerve fibres in the spinal cord and major posterior nerves. While no significant changes were seen in the earlier stages of the test, the NAD content of the affected muscles showed a marked and rapid decrease from month 9 onwards and appeared to be a consequence of the structural and metabolic impairment of the muscle resulting from the loss of the normal nerve supply.

STARCH POWDER FOR SURGEONS' GLOVES

Surgeons have attributed postoperative granulomas to the use of modified corn starch for lubricating gloves. The use of starch for this purpose was introduced as an improve-

ment on talc, which is not absorbable from tissue deposits and therefore must be recognized as a potential foreign-body irritant. Now that starch itself is being incriminated in an increasing number of postoperative complications, a number of questions must be answered. What evidence have we that starch is safer than talc as a surgical-glove lubricant? What biological processes occur to remove starch deposits from tissues? Which types of modified starch are most readily biodegradable? Is it possible that the magnesium oxide added to modified corn starch powder to improve its flow properties may play a role in producing granulomas?

More reports of granulomas attributable to starch powder are coming to light. Cohen & Safaie-Shirazi (*Am. J. Roentg.* 1973, **117,** 334) have described three patients undergoing abdominal operations who complained of pain around the surgical incision and malaise commencing about 2 weeks after the operation. Peritoneal masses containing giant cells which held refractive particles showing the characteristics of starch grains were seen in two patients, one of whom died from a postulated sensitivity reaction to corn starch. In the third patient, 4 weeks elapsed before histological examination was carried out, and no starch particles were discernible. The absorption of starch particles within a period of 4 weeks was not, however, unexpected.

A study of 52 patients who developed peritonitis after the introduction of corn starch into the abdominal cavity (Dutra & Jensen, *Calif. Med.* 1972, **117,** 8) revealed two different patterns of illness. In 34 patients, the development of abdominal pain, tenderness, fever (often with leucocytosis) and nausea and vomiting, sometimes accompanied by diarrhoea, prompted a second operation within 2 months, at which an ip exudate (often fibrinous) and miliary granulomas were seen. In the other 18 patients, who were examined histologically 2 months or longer after the first operation, firm white nodules and dense fibrous adhesions were present, together with miliary nodules in nine of the 18 cases. Starch was identified in these nodules by examination of sections in polarized light, and by staining with iodine and periodic acid–Schiff reagent.

Five cases of peritonitis induced by corn starch after clean and uncomplicated operations have been reported by Soderberg *et al.* (*Am. J. Surg.* 1973, **125,** 455). Adverse symptoms appeared within 13–42 days of operation, the most frequent complaint being of persistent cramping or sharp abdominal pain followed by fever, leucocytosis and positive X-ray findings (principally paralytic ileus). This led to preliminary diagnoses of intra-abdominal abscess, but re-operation revealed severe peritoneal inflammation, ascites, miliary nodules and a greatly thickened omentum. Further examination again confirmed the presence of starch granules. Re-operation on three other patients in whom biopsies had revealed starch granules within foreign-body giant cells (Coder & Olander, *Archs Surg. Chicago* 1972, **105,** 83) showed typical starch granules in the peritoneal exudate. Recovery was hastened in two patients by administration of hydrocortisone or prednisolone. However, an experimental study in mice by Hartmann & Ignatius (*Ann. Surg.* 1972, **175,** 398) failed to confirm the efficacy of corticosteroids against starch granuloma. A starch slurry made by washing a pair of surgeon's gloves with sterile saline was administered by ip injection to the mice, which were subsequently killed in groups of three between days 1 and 28 after treatment. The starch was not completely absorbed from the abdominal cavity, and during the first 24 hours it provoked a marked foreign-body reaction. The inflammatory lesion persisted and starch was still present in the nodules at day 28. The reaction was not prevented by an sc injection of 5 mg hydrocortisone given at the same time as the starch injection.

Aeberhard & Pedrinis (*Schweiz. med. Wschr.* 1973, **103,** 333) consider that the risk accompanying a second operation to remove starch granulomas is high. They describe a fatality that occurred after re-operation for granulomatous peritonitis arising from starch contamination of an incision for gall-bladder surgery. The importance of avoiding unnecessary surgery in cases of starch-induced granulomatous peritonitis and the value of biopsies in confirming the diagnosis of this condition have also been stressed by other authors, including Soderberg *et al.* (*loc. cit.*).

Some cases of peritonitis attributable to starch granulomatosis have been accompanied by skin reactions. Two such cases of dermal or sc starch granulomas have been described by Leonard (*Archs Derm.* 1973, **107,** 101), who also reported on a third patient, in whom the excision of a ganglion cyst was followed in about 6 weeks by induration and tenderness and the appearance of discrete sarcoidal granulomas containing starch material. These developments seem to be relatively rare and may represent a minor problem, but Leonard (*loc. cit.*) stresses the necessity of making a specific search for starch granules to establish their role in granulomatous skin lesions.

Corn-starch contamination may prove to be a problem in kidney-transplant operations, according to Min *et al.* (*Kidney Internat.* 1972, **2,** 291), who discovered starch granules in kidney-biopsy tissue obtained immediately after transplantation of four cadaver kidneys. They postulate that the starch reached the kidneys during harvesting and perfusion procedures. In two transplants, which had to be removed 2 weeks later because of rejection, the starch particles could no longer be seen. This was in keeping with other indications that, in time, starch particles may be dissolved or absorbed, but the authors do not entirely discount the possibility that starch may have some long-term adverse effect on kidney function, although they consider it unlikely.

The prolonged persistence of talc, however, and its ability to remain dormant for years before provoking a reaction is well established. This was recently demonstrated in a case of epididymitis and periorchitis due to talc granulomatosis (Pugh & Stringer, *Br. J. Surg.* 1973, **60,** 240). In this case, glove-powder introduced during an operation for hydrocele performed 11 years earlier was thought to be the source of talc found in the testis. At the later operation, the head of the epididymis was found to have been replaced by a hard white nodule, 2 cm in diameter, consisting of dense fibrous tissue with giant cells closely associated with talc crystals.

The iv injection of suspensions of crushed tablets and of the contents of capsules by drug addicts constitutes another tissue hazard. Lamb & Roberts (*J. clin. Path.* 1972, **25,** 876) found on examining the lungs of eight drug addicts, who died as a consequence of their habit, that all contained small talc emboli and five of them also contained starch emboli. There was a marked foreign-body reaction adjacent to the talc deposits, but only relatively insignificant reactions were associated with starch. Experiments in rats showed that 90% of maize-starch emboli were removed within 24 hours. A similarly rapid removal in man would explain the lack of inflammatory reaction to starch in human veins. However, in spite of the expected lack of accumulation, large amounts of starch (about 1·5 and 5 g in two of the cases) were present in the lungs of these addicts, with only relatively small quantities of talc, and the authors consider that such large deposits of starch may well have contributed to the addicts' sudden deaths.

Katsilabros (*Lancet* 1973, **i,** 379) has found that while infusions of talc or starch introduced into the peritoneal cavity of guinea-pigs produced severe granulomatous reactions, infusion of liquid paraffin with the particulate irritant led to absorption of the solid with

the paraffin within 7–10 days and prevented the development of the usual reaction. The death rate in guinea-pigs given talc or starch infusions was halved when the paraffin was infused 24 or even 72 hours after the suspension was administered. On the other hand, Palmer (*ibid* 1973, **ii,** 150) has countered this suggestion for overcoming glove-powder granulomatosis by reporting the case of a patient who developed widespread peritoneal and serosal adhesions, with multiple fibrocalcific nodules containing oil globules, many years after an emergency appendicectomy, during which mineral oil had probably been used to lubricate the surgeon's gloves. There appears to be some evidence that ip introduction of oleaginous substances can itself provoke the development of granulomas after a long latent period. The length of the latent period could perhaps have been the reason why no such reaction was seen during the experiments of Katsilabros (*loc. cit.*).

Other suggestions for overcoming this problem have included one from Guatemala, advocating the replacement of standard starch powder by starch from the locally-grown "yuquilla" or arrowroot (Herrera-Llerandi, *Br. med. J.* 1973, **3,** 411), and another proposing the use of fine boric acid powder (Wallace, *ibid* 1973, **1,** 746). The latter suggestion is surprising in view of the toxic hazards associated with the application of boric acid to wounds or abraded skin (*Cited in F.C.T.* 1971, **9,** 914) and it seems that we must take care lest we leap from the frying pan ....

There are perhaps better alternatives. One Leading Article (*Lancet* 1972, **ii,** 74) has asked pertinently whether surgical gloves sterilized by X-irradiation really need powdering anyway. Another (*British Medical Journal* 1973, **2,** 502) points out that although peritoneal contamination with starch usually occurs through an abdominal incision, cases have been recorded where it entered the body by way of the Fallopian tubes. Clearly, surgeons should use as little powder as possible, but need they use it at all? Nash (*ibid* 1973, **1,** 485) contends that the only adequate way of counteracting the glove-powder menace is to eliminate the use of powder in the processes used to manufacture surgical gloves. This, however, might not entirely solve the problem. In a case reported by Pemberton & Johnson (*ibid* 1973, **3,** 235), the contamination of an operation wound with corn starch apparently resulted from the use of the starch as a major constituent of an antibiotic dusting powder. Similarly, Michaels & Shah (*ibid* 1973, **2,** 714) have attributed a foreign-body reaction in the middle ear to the use of an insufflation of an antibacterial powder which contained a large proportion of starch. Comments on this aspect are scanty, but they suggest that the question of tissue reactions to starch may involve some consideration of factors other than surgical gloves.

While this latest crop of reports provides ample confirmation of the earlier claims that starch may produce peritoneal granulomas, it contributes little in the way of answers to the questions posed in our introductory paragraph. For such answers we shall have to depend more on experimental studies than case histories. Moreover, the actual incidence of this post-operative complication in relation to the number of patients who appear to 'get away with it' is still unknown. Is it not possible that perhaps those who develop starch granuloma constitute a small minority unusually responsive to foreign bodies, including starch particles?

BENZPYRENE HYDROXYLASE—INNATE DETERMINANT OF PAH TOXICITY?

Introduction

One of the primary characteristics of living organisms is their ability to maintain a state of homeostasis in environments which are often antagonistic to their survival. Today, this

probably represents more of a challenge to nature's resources than ever before, since man and other animals are exposed, not only to natural toxicants, but to increasing quantities of man-made pollutants, such as pesticides and industrial wastes. Fortunately, mammalian species are endowed with efficient mechanisms for coping with at least some of these foreign chemicals.

Perhaps the most important detoxication system in mammals is the elaborate complex of microsomal enzymes, which deals with the majority of foreign organic molecules. On the other hand, these enzymes have a less favourable effect when they give rise to metabolites that are more rather than less toxic than the original compound. The aryl hydrocarbon hydroxylase (AHH) complex, for example, may enhance the carcinogenic potential of certain polycyclic aromatic hydrocarbons (PAH) through the formation of activated metabolites. The AHH complex has been detected in the liver and a variety of non-hepatic tissues of the rat, mouse, hamster and monkey, though tissue concentrations vary considerably with the age, sex, species, strain and environment of the animal (Nebert & Gelboin, *Archs Biochem. Biophys.* 1969, **134,** 76). In an attempt to clarify the role(s) of this hydroxylase system, this article describes some current developments in the continuing study of the biotransformation of PAH.

Kinetics of AHH induction

The response of the hepatic microsomal enzymes to PAH differs widely in various species. This was evident in the experiments of Hansen & Fouts (*Biochem. Pharmac.* 1971, **20,** 3125), who examined the changes in hepatic microsomal oxidases in liver homogenates prepared from rats and mice killed 64–72 hours after the ip injection of benzo[*a*]pyrene (BP) in a dose of 45 mg/kg. Effects on aniline *p*-hydroxylation in rats were particularly striking and involved an upward shift in the optimum pH and pH-dependent increases in both the apparent V_{max} and apparent K_m of the reaction. Changes in the apparent Michaelis-Menton kinetics of (+)-benzphetamine metabolism were also evident, both the rate of this reaction and the magnitude of the (+)-benzphetamine-induced microsomal difference spectrum being decreased. Treatment of rats with BP also increased the apparent V_{max} and decreased the apparent K_m of its own hydroxylation reaction to a marked extent.

Identical experiments were performed on mice, but in this species the above-mentioned parameters were not affected to any significant degree by BP treatment, except in the case of the (+)-benzphetamine-induced microsomal difference spectrum, the magnitude of which was diminished. Treatment with 3-methylcholanthrene (MC), however, did enhance the BP-hydroxylase activity of mouse-liver microsomes, the apparent V_{max} for this reaction being at least doubled.

The most likely explanation for the demonstrated effects of BP on hepatic aniline and benzphetamine metabolism in the rat liver is in terms of a differential induction of the enzymes of the AHH complex and the microsomal mixed-function oxidase system. To test this hypothesis, Williams *et al.* (*ibid* 1971, **20,** 2130) examined the affinity of various PAH for these enzyme systems *in vitro*. Rats were injected ip with 6·8 mg MC in 0·5 ml corn oil, or the same volume of corn oil alone, and were killed 16 hours later. AHH assays were carried out on hepatic microsomal preparations from these MC-treated or untreated rats incubated with one of several PAH. The addition of naphthalene, dibenz[*a,h*]- or [*a,c*]anthracenes, perylene or MC, in concentrations equimolar to the BP substrate had no significant effect on BP hydroxylation in preparations from untreated rats, but marked inhibition of the enzyme was observed with 7,12-dimethylbenz[*a*]anthracene (DMBA)

and phenanthrene. Of the eight PAH used, only DMBA, dibenz[*a,c*]anthracene and MC induced a pronounced (32, 40 and 51%) inhibition of BP hydroxylation in microsomes from rats previously treated with MC. It is also significant that, in control rats, only two hydrocarbons produced more than 25% inhibition of the hydroxylase (31 and 38%), suggesting that none of these compounds has as great an affinity for the enzyme as BP itself. The data indicating that control and induced enzymes differ in their affinities for the various hydrocarbons confirm a differential enzyme-induction effect.

Role of AHH induction in PAH carcinogenicity

The role of the AHH complex in the realization of the carcinogenic potential of some PAH is still largely undefined, some workers having failed to observe any consistent correlation between the known tumorigenicity of PAH and their ability to affect BP hydroxylation. Williams *et al.* (*loc. cit.*) found that *in vitro* the potent carcinogens MC and DMBA had a strong inhibitory effect on the microsomal BP hydroxylase from MC-treated rats, while the non-carcinogens, naphthalene, perylene, phenanthrene and anthracene evoked little or no inhibition. To confuse the issue, however, the carcinogenic isomer of dibenzanthracene (dibenz[*a,h*]anthracene) proved to be a weaker inhibitor than the non-carcinogenic dibenz[*a,c*]anthracene.

Some insight into the genetic basis underlying the carcinogenicity of PAH might be expected to be furnished by an investigation of the susceptibility of AHH to induction by these compounds. This was the aim of recent work by Nebert *et al.* (*Nature New Biology* 1972, **236,** 107) on various tissues of the offspring of crosses between inbred and hybrid C57BL/6N and DBA/2N mice. In C57BL/6N mice, AHH activity was induced more than fivefold by MC-treatment, while the level of activity in MC-treated DBA/2N mice was no greater than the constitutive level. In the F_1 hybrid, hydroxylase activity was always induced in response to MC, but MC-treated progeny from the DBA/2N × F_1 backcross showed a bimodal distribution of AHH activity, the enzyme being inducible to C57BL/6N-type levels in half of the population while in the other half its activity remained comparable with control levels. A similar set of responses was evoked by other PAH. Thus, the presence or absence of hydroxylase induction was concluded to be an all-or-none phenomenon, occurring in the mouse in all tissues that normally contain AHH activity. The capacity for AHH induction by PAH was therefore thought to be attributable to genes at a single locus and inherited as a simple autosomal dominant trait. Subsequent studies by the same group, using clones of mouse 3T3 fibroblasts, have apparently confirmed that AHH induction by PAH may be expressed on a single chromosome, but published details of this work have not yet been traced.

The suggestion of Nebert *et al.* (*loc. cit.*) that AHH inducibility is an "all-or-none" phenomenon has recently been disputed by Wiebel *et al.* (*Archs Biochem. Biophys.* 1973, **154,** 292). Using seven different mouse strains, in which hepatic AHH was known to be either highly inducible or uninducible, these authors studied in a range of extrahepatic tissues the level of AHH induction effected by one of several PAH. They found that the degree of inducibility varied considerably in any given strain between tissues such as the lung, kidney, small intestine and skin, and did not necessarily parallel the inducibility of the enzyme in the liver. In certain strains, a lack of AHH inducibility was, in fact, specific to the liver. Moreover, in a strain in which administration of MC, benz[*a*]anthracene (BA) and 5,6-benzoflavone each induced hepatic AHH to a similar degree, MC proved a far less effective inducer than the other two in extrahepatic tissues. The route of

administration was also a factor, the lack of AHH induction in the skin of DBA mice after ip injection of BA contrasting with a fourfold induction in this tissue after topical application. It seems that although inferior in quantity to that located in the liver, the AHH systems in the lung, intestine and skin may be of particular importance as inactivators (or activators) of potentially harmful substances at their site of entry.

In order to probe more deeply into the reaction of carcinogenic AHH inducers with subcellular components, Gelboin *et al.* (*Fedn Proc. Fedn Am. Socs exp. Biol.* 1972, **31,** 1298) developed a sophisticated tissue-culture system based on cells from hamster and mouse embryos. The enzyme in this system was highly inducible. Using various combinations of inhibitors of RNA and protein synthesis, these authors found that the induction process required continuous protein synthesis, whereas RNA synthesis was required only during the first hour although it could occur even when protein synthesis was blocked. This clearly implied that AHH inducers could affect the expression of genetic information. These workers also described an *in vitro* reaction between BP and DNA catalysed by the microsomal-enzyme system and involving the formation of covalent bonds.

The binding of PAH to both proteins and DNA has long been implicated in carcinogenesis, on the basis of the known ability of PAH to act as electron donors (*Cited in F.C.T.* 1971, **9,** 735). Recently Johnson & Calvin (*Nature, Lond.* 1973, **241,** 271) have produced some support for the hypothesis that electrophilic oxygen, produced in the hydroxylase system, attacks the 6-position of the hydrocarbon to form a carbonium ion, primarily localized at the 1 and 3 positions, which can then react with nucleophilic cellular components. Their evidence was obtained from a study of the behaviour of a model iodonium ion in its reaction with BP. Equimolar quantities of BP and iodine dipyridine nitrate in chloroform reacted rapidly to give 6-iodoBP, while formation of the 6-pyridinium derivative required a 2:1 molar ratio of iodonium reagent to BP. The authors suggest that whereas 6-iodoBP may arise from electrophilic (I^-) attack at position 6, steric considerations may change the position of attack, so that displacement of the equilibrium would result in attack by the bulkier reagent at position 1 or 3 to form the 6-pyridinium derivative. A further possibility is that the reaction proceeds by way of the formation of the radical cation of BP. Evidence has been put forward that such a cation, produced *in vitro* by oxidation with iodine, does react with pyridine to give the 6-pyridinium derivative (Rochlitz, *Tetrahedron* 1967, **23,** 3048), but no radical was detected in the experiments with the model iodonium system.

AHH induction in man

In view of the ubiquitous exposure of the human population to PAH (*Cited in F.C.T.* 1967, **5,** 712), reports that the human foetal liver may lack AHH activity during early gestation merited some attention. Such allegations have, however, been refuted in a short paper by Juchau *et al.* (*Biochem. Pharmac.* 1972, **21,** 2269) describing the occurrence of BP-hydroxylase activity in several organs of the human foetus and in the placenta. Tissue homogenates from four human foetuses and one monkey foetus were analysed for PB-hydroxylase activity, which was detected in the liver, kidneys and adrenal glands. The latter tissue showed the highest levels of activity, ranging from 1077 to 4896 nmol BP hydroxylated/hour/g protein. The higher levels were found in foetuses from smoking mothers, in keeping with the reported ability of cigarette-smoking to enhance the rate of placental BP hydroxylation (Juchau, *Toxic. appl. Pharmac.* 1971, **18,** 665; Welch *et al. Clin. Pharmac. Ther.* 1969, **10,**

100), but there was no indication of a similar trend with the other tissues and, in any event, the study was too limited for any definite conclusion to be drawn on this point.

Analysis of the AHH activity of human placentae immediately after birth has revealed marked differences between individuals whose exposure to PAH might have been expected to be comparable. Among subjects who smoked 15–20 cigarettes daily, Welch *et al.* (*loc. cit.*) found that placental BP-hydroxylase activity varied more than seventyfold. It was suggested that this variation in induction potential could have a genetic basis, emphasizing the need for some simple means of assessing differences in the capacities of individuals to metabolize environmental carcinogens. In a search for a possibly appropriate method, Alvares *et al.* (*ibid* 1973, **14,** 30) studied BP hydrolase activity in cultures of human foreskin obtained at circumcision from 13 neonates. Incubation of the skin in a growth medium containing BA for 16–24 hours caused two- to fivefold increases in BP-hydroxylase activity, such increases being greater than those observed in neonatal rodent skin. Two- to threefold variations were found in both the basal levels and the inducibility of the hydroxylase in neonatal foreskin from the 13 subjects, but no correlation could be made between inducibility and maternal age or race. These findings indicate that the ability of human skin to metabolize BP may prove to be a useful means of assessing the capacity of different individuals to metabolize foreign compounds, including carcinogens.

The important role of AHH in the metabolism of many polycyclic hydrocarbons is beyond dispute. Whether this process leads to detoxication or to lethal synthesis is another question.

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TOXICOLOGY: ABSTRACTS AND COMMENTS

FLAVOURINGS, SOLVENTS AND SWEETENERS

2663. Induction of foetal enzymes by eucalyptol

Jori, A. & Briatico, G. (1973). Effect of eucalyptol on microsomal enzyme activity of foetal and newborn rats. *Biochem. Pharmac.* **22,** 543.

Eucalyptol is known to stimulate liver-enzyme activity in several animal species. In adult rats, pretreatment with this essential oil (injected ip or sc or inhaled in aerosol form) has been shown to reduce pentobarbitone sleeping time and accelerate removal of the drug from the brain (*Cited in F.C.T.* 1970, **8,** 325), while liver homogenates from these eucalyptol-treated rats metabolized *p*-nitroanisole, aminopyrine and aniline more rapidly than did control preparations. The activity of enzymes comprising the microsomal mixed-function oxidase systems is relatively low in the livers of foetal and newborn animals, but may be stimulated by treating the mother at certain stages of pregnancy with various inducing agents capable of crossing the placental barrier. The authors cited above therefore investigated the possibility that treatment of pregnant or lactating rats with eucalyptol might induce these enzymes in the foetus and neonate.

The eucalyptol was given sc in four doses of 500 mg/kg/day either between days 10 and 14 of pregnancy, on the last 4 days of pregnancy or between days 2 and 6 after delivery. In a fourth test group, suckling rats from control mothers were injected directly with four sc doses of 500 mg eucalyptol/kg/day. Adult rats and litters were killed 18 hr after the final treatment and newborn rats between the first 8 and 18 hr after birth. Enzyme activity was measured on the 9000 **g** supernatant fraction of liver homogenates, using aniline or *p*-nitroanisole as the substrate.

Liver microsomal-enzyme activity was greatly enhanced in adult rats treated with eucalyptol both during and after pregnancy and was also increased in the foetal and newborn offspring of such rats. In these offspring, a more marked stimulation of the generally poor drug-metabolizing capacity was demonstrated in connexion with the *O*-demethylation of *p*-nitroanisole than with the *p*-hydroxylation of aniline. Suckling rats treated directly with eucalyptol also showed an increase in liver-enzyme activity, but administration of the oil to lactating mothers did not lead to any enzyme induction in the suckling rats.

It thus appears that while eucalyptol is able to penetrate the placental barrier and reach a concentration in the foetal blood high enough to stimulate hepatic enzyme activity, it is unable to cross the blood–milk barrier to any effective extent. Its placental mobility is compatible with its high lipid solubility, a property reported to have a direct bearing on placental penetration (Dixon & Willson, *Archs int. Pharmacodyn. Ther.* 1968, **172,** 453).

EMULSIFIERS AND STABILIZERS

2664. Effects of sucrose acetate isobutyrate

Krasavage, W. J., DiVincenzo, G. D., Astill, B. D., Roudabush, R. L. & Terhaar, C. J. (1973). Biological effects of sucrose acetate isobutyrate in rodents and dogs. *J. agric. Fd Chem.* **21,** 473.

Dogs fed the soft-drinks stabilizer, sucrose acetate isobutyrate (SAIB) at dietary levels of 0·5–4·0% showed evidence of a mild cholestasis, accompanied by increases in bromsulphthalein retention and in serum alkaline phosphatase (AlkPase). Liver hypertrophy, which was considered indicative of microsomal-enzyme stimulation, was also seen in the males (*Cited in F.C.T.* 1973, **11,** 1140). Rats, however, were apparently unaffected by dietary levels of up to 10% SAIB. Similar, although slightly different, results are reported in the present paper, which has probed more deeply into the reasons for SAIB's observed effects.

Attempts to define oral and ip LD_{50} values for SAIB in rats and mice were unsuccessful, far less than half the animals dying from a dose level as high as 25·6 g/kg. When held in contact with the depilated skin of guinea-pigs, SAIB caused only a slight transient irritation, and a standard test for skin sensitization in this species produced negative results. Rats fed a dietary level of 1% SAIB for 95 days showed no gross or microscopic effects, but 5% SAIB given for the same period led to a slight decrease in male body weights and an increase in female liver weights. However, dietary levels of up to 4% fed for up to 56 days did not affect these parameters or the serum levels of AlkPase, ornithine carbamoyltransferase, triglycerides, cholesterol and urea nitrogen. Also unaffected were the hepatic clearance rate of indocyanine green (ICG) and the activity of liver enzymes indicative of oxidation (*p*-nitroanisole demethylase; *p*-NDase) and conjugation (bilirubin-β-D-glucuronyl transferase; BGTase), although there was a slight depression in glucose-6-phosphatase (G6Pase) activity.

In dogs, there were no adverse effects from a dietary level of 0·2% SAIB for 12 wk, but levels of 0·6% and above increased liver weights, and with 2% or more there was an increase in serum AlkPase, which was reversible on cessation of treatment. Disc electrophoresis, isoenzyme inactivation studies and analysis of tissue AlkPase from liver, kidney, bone, intestinal mucosa and bile indicated that this increase originated from the liver, rather than from the bile as had previously been suggested. A dietary level of 5% induced increases in the ICG clearance rate (a finding reversed within 2 wk of the cessation of treatment) and in liver glycogen and phospholipids. This last effect was attributed to the increase in smooth endoplasmic reticulum previously reported in SAIB-fed dogs (Procter *et al. Toxic. appl. Pharmac.* 1972, **22,** 328). Slight decreases in liver protein and in G6Pase and BGTase activities were observed in dogs given the 5% dietary level, while *p*-NDase activity was unaffected, and it was concluded that microsomal-enzyme stimulation could not be held responsible for the increased liver weight seen in dogs at levels of 0·6% and above. The differences between the rat and dog were attributed to the differing metabolic pathways already demonstrated in these species (Reynolds *et al.* 164th Natn. ACS Mtg, New York, 1972, abstr. no. 40). Since man metabolizes SAIB like the rat, it was concluded that this should be the preferred species for future studies.

PRESERVATIVES

2665. Sorbic acid instead of benzoic acid for cats

Bedford, P. G. C. & Clarke, E. G. C. (1973). A preliminary study of the suitability of sorbic acid for use as a preservative in cat food preparations. *Vet. Rec.* **92,** 55.

Readers may remember the incident reported last year, in which the consumption of meat preserved with an excessively high level of benzoic acid resulted in the poisoning, sometimes fatal, of a number of cats (*Cited in F.C.T.* 1972, **10,** 588). Subsequent studies

(*ibid* 1973, **11**, 146) confirmed the particular susceptibility of the cat to the effects of benzoic acid and strengthened suggestions that some alternative preservative should be used in meat preparations intended for consumption by this species.

In their search for a more appropriate preservative, the authors of the earlier communications on this subject investigated the suitability of sorbic acid. This compound has been shown to be of low toxicity in several experimental animal species and is used in many countries in food for human consumption, but in view of the atypical response of the cat to certain organic compounds, a further small feeding trial was carried out in this species. Pairs of cats were fed a meat-based diet containing sorbic acid at levels between 0·1 and 1% for 28 days. A further pair received a dietary level of 2% for 1 wk.

None of the treated animals showed any changes in clinical behaviour or appearance, and the absence of obvious toxic effects at any of the dose levels tested suggested that the use of sorbic acid at the level of 0·1–0·2% considered to be effective for the preservation of cat-meat preparations would have no adverse effects on the animals. It should be remembered, however, that this preliminary and numerically limited test was only concerned with a short-term response, and no attempt was apparently made to detect possible structural or functional changes in the treated animals. Nevertheless it does indicate that the problem of a severe response of the cat to accidental overdosing, such as occurred with benzoic acid, would not arise with sorbic acid.

AGRICULTURAL CHEMICALS

2666. Mechanisms of parathion metabolism

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

The cholinergic insecticide parathion is of special interest to the toxicologist, since its biotransformation (*Cited in F.C.T.* 1968, **6**, 797) can result in the production of a toxic metabolite, paraoxon, or a nontoxic metabolite, diethyl phosphorothioic acid (DEPT). Experiments in both rats and rabbits have indicated that this dual mode of metabolism is accounted for by the evidence of two mixed-function oxidase systems, and it has also been suggested that this may be related to the known ability of cytochrome *P*-450, the terminal enzyme of the mixed-function oxidase systems, to exist in at least two biologically-active forms, in which the iron in the haem moiety exists in a high-spin and a low-spin state. Since the ratio of these forms can be altered by pretreatment of the animal with 3-methylcholanthrene (MC), the authors cited above examined the effect of MC pretreatment on the rate of formation of paraoxon and DEPT in hepatic microsomal preparations.

Administration of either sodium phenobarbitone (PB) in a daily ip dose of 50 mg/kg for 5 days or of MC in a single dose of 20 mg/kg to adult rabbits, which were killed 18 hr or 3 days, respectively, after the termination of treatment, led to an enhanced rate of parathion metabolism (per unit of protein) by preparations of whole microsomes and of the rough endoplasmic reticulum (RER) isolated from the livers of the treated animals. The same effect was not seen, however, with the fraction consisting of smooth endoplasmic reticulum (SER). In addition, pretreatment with either PB or MC increased the cytochrome *P*-450 content of all three microsomal fractions, but no difference could be discerned between the rates of parathion metabolism, expressed in terms of the *P*-450 concentration,

by RER and SER from untreated, MC-treated and PB-treated animals. Nevertheless, again on the basis of cytochrome *P*-450 concentration, the rate of parathion metabolism by whole microsomes from MC- and PB-treated rabbits was less than that by whole microsomes from untreated animals.

While these workers confirmed that treatment with MC or PB caused changes in the high-spin/low-spin ratios in the *P*-450 of the various microsomal fractions, they were unable to demonstrate any correlation between the concentration of high- or low-spin forms in any microsomal fraction and the relative rates of parathion metabolism to paraoxon or DEPT. It seemed therefore that the enzyme systems catalysing the formation of these two metabolites were not selectively concentrated in any particular microsomal sub-fraction.

2667. DDT and reproduction again

Hart, M. M., Whang-Peng, Jacqueline, Sieber, Susan M., Fabro, S. & Adamson, R. H. (1972). Distribution and effects of DDT in the pregnant rabbit. *Xenobiotica* **2,** 567.

Arscott, G. H., Robson, W. A. & Tinsley, I. J. (1972). Effect of DDE and DDT on reproductive performance of adult White Leghorn male chickens. *Nutr. Rep. Int.* **6,** 307.

Lillie, R. J., Cecil, H. C., Bitman, J. & Fries, G. F. (1973). Dietary calcium, DDT source and age of hen on the reproductive performance of caged White Leghorns fed DDT. *Poult. Sci.* **52,** 636.

DDT and its metabolites pass the placental barrier into the foetus (*Cited in F.C.T.* 1965, **3,** 656; *ibid* 1971, **9,** 152), and have been found in significant concentrations in the kidney, heart muscle and adipose tissue of human neonates (*ibid* 1971, **9,** 152). Their distribution in the rabbit foetus and in the maternal organs that regulate the foetal environment is the subject of the first paper cited above.

Pregnant rabbits were given *p,p'*-DDT in doses of 10 or 50 mg/kg intragastrically on days 7, 8 and 9 of gestation. On day 26, the foetus, the maternal liver, uterus, ovaries and perirenal fat and, to a lesser extent, the placenta, were found to contain substantial quantities of *p,p'*-DDT, *p,p'*-DDD and *p,p'*-DDE. The concentration of *p,p'*-DDT was higher in foetal tissue than in maternal plasma. A higher incidence of prematurity, an increase in the number of foetal resorptions, and a decrease in foetal weight were associated with this treatment, but the same dose levels given on days 21–23 of pregnancy did not have these effects. There was no increase in the incidence of chromosomal aberrations either in maternal or in foetal tissue, nor was there any evidence of teratogenic activity. Furthermore, the addition of *p,p'*-DDT at a level of 100 μg/ml to cultures of human and rabbit peripheral blood failed to induce any increase in the number of cellular aberrations.

We have recently considered the effects of DDT on the reproductive performance of female rats (*ibid* 1973, **11,** 912). The second and third papers cited above are concerned with its effects on reproduction in chicks. Long-term feeding studies were carried out with DDT and DDE in adult male White Leghorn chicks. No significant change in reproductive function, as attested by semen volume, packed sperm volume, fertility and body and testes weights as well as egg hatchability and fertility, followed the addition of DDE at a level of 100 ppm to the diet for 16 wk and 200 ppm for a further 16 wk. One of the three chicks in this group died at wk 31 after developing tremors. Feeding with 100 ppm DDT had no effect on reproductive function, but caused a marked reduction in growth

and, in one chick, a substantial reduction in testes weight. One chick in this group died at wk 15 with characteristic tremors.

The other paper reports the effects of feeding DDT to female White Leghorns in a much more extensive study (using 20 pullets/group), in which dietary calcium and the age of the birds were also considered as factors in reproductive performance. The inclusion in the diet of 10 or 50 ppm DDT, either as the technical-grade material or as a mixture of pure DDT isomers, had no significant effect on body-weight gains, feed intake, egg production, viability, fertility or hatchability, irrespective of the age of the hens or the level of calcium in their diet.

These studies confirm earlier findings on the lack of effect of DDT on reproductive processes in poultry (*Cited in F.C.T.* 1969, **7**, 535).

2668. Effect of pyrethrum on rat liver

Springfield, Angela C., Carlson, G. P. & DeFeo, J. J. (1973). Liver enlargement and modification of hepatic microsomal drug metabolism in rats by pyrethrum. *Toxic. appl. Pharmac.* **24,** 298.

Pyrethrum, a natural pesticide extracted from the flowers of *Chrysanthemum cinarariaefolium*, consists basically of several esters, principally those known as pyrethrins I and II and cinerins I and II, all of which demonstrate a relatively low mammalian toxicity. Recently, however, it was shown that pyrethrum caused liver enlargement, similar to that caused by DDT, and slight cytological changes (*Cited in F.C.T.* 1969, **7,** 535). Since this particular study was concerned only with morphological changes, the present feeding trial was designed to investigate any concomitant changes in hepatic drug metabolism after subacute administration of the insecticide to rats.

Oral administration of 200 mg pyrethrum/kg to male rats for 23 days increased their liver-to-body weight ratios by almost 25%, compared with controls, and decreased hepatic concentrations of DNA. Increases in total lipid concentrations could not alone account for the liver enlargement, and protein concentrations of whole liver homogenates and of microsomal fractions were not significantly different from those of the controls. Water levels in the liver were not affected.

After 6 days of pyrethrum administration, marked decreases were observed in hexobarbitone-induced narcosis, but no concomitant changes could be detected in barbitone-induced sleeping-time, suggesting that the pyrethrum interfered with hepatic drug metabolism. This was confirmed by a demonstration that the activities of the microsomal enzymes responsible for *O*-ethyl-*O*-(4-nitrophenyl)phenyl phosphonothioate (EPN) detoxication, *p*-nitroanisole *O*-demethylation and hexobarbitone oxidation were increased by some 150, 175 and 265% respectively of the control values, by a pyrethrum dose of 200 mg/kg/day given for 13 or 23 days. These effects were shown to be dose-related. NADPH-cytochrome *c* reductase activity and cytochrome *P*-450 levels were also increased. These changes were clearly not just a part of a generalized increase in cellular metabolism, since the oxidation of tryptophan, monitored by following tryptophan-pyrrolase activity in liver supernatants, was not affected in rats given 500 mg pyrethrum/kg orally for 7 days. At this level of pyrethrum administration, liver weights and microsomal-enzyme activities were enhanced from day 4 to at least day 17 of treatment, returning to control levels within 7 days of cessation of treatment.

2669. Chloracne from dioxin

May, G. (1973). Chloracne from the accidental production of tetrachlorodibenzodioxin. *Br. J. ind. Med.* **30,** 276.

An explosion in 1968 at a Derbyshire plant manufacturing 2,4,5-trichlorophenol led to the formation of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin ('dioxin'), a compound established as a teratogen (*Cited in F.C.T.* 1972, **10,** 110). Dioxin is also capable of producing severe liver damage and chloracne. The present paper is concerned with the aftermath of this explosion as it affected the health of exposed workers.

Of 14 men in the building during or immediately after the explosion, 11 showed abnormality in one or more liver-function tests (increased zinc sulphate or thymol turbidity or serum transaminase), but all were within normal limits 10 days later. It was therefore decided that the unit should be reopened after obviously contaminated areas had been sealed off and the rest had been thoroughly cleaned. At first all went well, but about 5 days later some maintenance men working with bare hands developed slight erythema and vesiculation of the ear lobes and cheeks, occasionally accompanied by mild conjunctivitis. The condition was rapidly diagnosed as chloracne, and within the next 7 months 79 cases were recorded. Typically the condition consisted of inclusion-type cysts extending in a characteristic pattern over the face and neck and sometimes also affecting the back of the arms, the sides of the legs, the back and the sternal area. In five cases the antecubital fossae were involved, a condition not previously described. In some men the face bore a liberal smattering of comedones rather than cysts, producing a dusky-grey appearance.

The chloracne was controlled by means of oral oxytetracycline and/or dermal cetrimide and zinc sulphide lotions, with steam-bathing and ultraviolet irradiation in severe cases, and a rigorous campaign of factory and personal cleanliness was introduced. The entire building was again thoroughly cleaned and resurfaced, during which time no-one was allowed to enter without full protective clothing. Only when tests in rabbits demonstrated that dioxin was no longer present on exposed surfaces was the plant reopened. The majority of the chloracne cases made an almost complete recovery within 4–6 months, although in seven of the workers the condition could just be diagnosed 4 yr later. No other exposure-related ailment developed, and continuous monitoring of liver function and urine analyses gave satisfactory results.

The only subsequent cases of chloracne involved two men, not company employees, who 3 yr later came into contact with a large metal vessel, which had been decontaminated at the same time as the rest of the equipment. Although they were exposed for only 1–2 days, their condition was very persistent and was passed on to their immediate family, in contrast to all the other cases. This latter development remains something of a mystery. On the whole, the effects of this explosion and the resulting contamination, although serious, were far less severe than in a similar German incident, in which chloracne affected not only the workers but also a large number of their wives, children and pets, and several instances of fatal liver disorder and one intestinal sarcoma occurred among the workers and were attributed to the dioxin exposure.

As a postscript to the UK incident, it is recorded that an entirely new trichlorophenol production plant, with automated temperature control and multiple safety features, has now been in operation in Derbyshire for 3 yr. All batches of the product are checked for dioxin by gas–liquid chromatography, the method being sensitive to 0·2 ppm, and no cases of chloracne have occurred at the new plant.

FEED ADDITIVES

2670. Trace metals in fish meals

Lunde, G. (1973). Trace metal contents of fish meal and of the lipid phase extracted from fish meal. *J. Sci. Fd Agric.* **24,** 413.

In a recent comment in connexion with a study on fish-protein concentrate (*Cited in F.C.T.* 1973, **11,** 1143), we drew attention to the potential problem that a relatively high level of undesirable residues, such as certain trace metals, might be built up during the manufacture of a concentrate. To a less marked degree, the problem is also likely to occur in the production of fish meals, as the paper cited above indicates.

Trace-metal analyses were carried out on samples of meal produced industrially from a variety of fish and obtained from a range of manufacturers and localities. Samples of fish were also converted in the laboratory into meals which were similarly analysed. As well as analysis of ashed samples, trace-metal and other elemental analyses were carried out on the lipids extracted from the various meals by a chloroform–methanol (2 : 1, v/v) mixture.

Levels of zinc and iron, and to a less marked extent of lead and cadmium, were found to be higher in the commercial fish meals than in those prepared in the laboratory, differences that may have been due to contamination during production or to geographical variations in the levels in the fish used. Selenium, the importance of which as an animal-feed component has recently received a great deal of attention, was present mainly in the protein, probably in the form of seleno-amino acids, at levels of 1–4 ppm. However, the finding of 0·3–3 ppm selenium in the lipid phase showed that lipid-soluble organoselenium compounds did not decompose during the industrial process for fish-meal manufacture. Mackerel and anchovy meals had somewhat higher levels of selenium and of cadmium (up to 0·9 ppm) than other species. Assuming a natural zinc/cadmium ratio in seawater of about 100 : 1, there was evidence of the building up of a higher proportion of zinc in relation to the cadmium present, both in the meals and apparently in the fish. Lead and copper levels suggested no significant increase during meal production.

The phosphorus content of the lipid extracts varied widely and indicated a considerable degree of phospholipid decomposition during storage or processing of the raw material or storage of the prepared meal. Organically-bound arsenic (5–23 ppm) and bromine (8–21 ppm) were also found in the lipid phase, together with zinc, iron and cobalt, which were probably complexed by the phospholipids.

PROCESSING AND PACKAGING CONTAMINANTS

2671. 2-Mercaptoethanol, a potential problem in EO sterilization

White, K., Bruckner, J. V. & Guess, W. L. (1973). Toxicological studies of 2-mercaptoethanol. *J. pharm. Sci.* **62,** 237.

It is well recognized that residual ethylene oxide in rubber or plastics devices sterilized with the gas has an important bearing on the toxicity of such products. Attention has previously been drawn to the possible formation of chlorohydrins by the reaction of this sterilant with chloride ions (*Cited in F.C.T.* 1971, **9,** 740), and the toxicity not only of this compound but of chloroacetaldehyde, one of its metabolites (*ibid* 1973, **11,** 916) has been studied. It has now been suggested that sulphides, including hydrogen sulphide, produced

during the vulcanization of rubber with sulphur may react with residual ethylene oxide resulting from sterilization procedures to form 2-mercaptoethanol (ME) by the mechanism proposed by Bronsted *et al.* (*J. Am. chem. Soc.* 1929, **51,** 428). The toxicity of this potential reaction product has therefore been investigated.

The ip and oral LD_{50} values of ME in mice were 322 and 344·8 mg/kg, respectively. Doses of 480 mg/kg and above were fatal within 1–2 hr, and death apparently resulted from convulsive seizures and subsequent respiratory failure. With lower doses, signs characteristic of CNS depression were observed, the mice slowly entering a state of depression, terminating in coma and death within 1–3 days. Sodium pentobarbitone blocked the rapidly fatal convulsive effect but not the delayed lethal action of ME. The survival rate following the ip administration of an LD_{50} dose was significantly increased by the administration of ethanol, but no beneficial effect was derived from pre-treatment with either atropine sulphate or a combination of metal ions. These results suggested that the toxic effects of ME could not be explained on the basis of a cholinergic reaction or the complexing of ME with metal ions vital to some enzyme systems, but they indicated that ethanol might competitively inhibit the oxidation of ME to some toxic metabolite(s).

All mice receiving an ip dose equivalent to 25 or 50% of the LD_{50} on 5 days/wk died within 4 wk. The animals exhibited progressive weakness and depression, with a diminished response to pain and loss of body weight. Gross and microscopic examination revealed no significant changes, except the presence of small scattered foci of hepatic necrosis.

The irritant effect of undiluted ME was transient on normal rabbit skin but prolonged on abraded skin. Dilutions of 1 : 5 and 1 : 10 elicited a moderate though transient irritation in abraded skin but little reaction on normal skin. Intracutaneous injection of undiluted ME produced a severe reaction and even a dilution of 1 : 50 caused marked inflammation with subsequent necrosis. A concentration of 1 : 100 produced a well-defined irritation only after exposure for 24 hr, and 72 hr after the injection the reaction was essentially negative. There was no inflammation with higher dilutions. No systemic toxicity was detected although the test animals received total doses of up to 800 mg/kg. Undiluted ME was markedly toxic to the rabbit eye and the surrounding mucosa, the resulting corneal opacity being only moderately severe but prolonged. On the penile mucosa of the rabbit, the undiluted material produced severe erythema, moderate oedema and eschar formation 24 hr after a 1-min exposure. Necrotic lesions developed on the scrotum where normal evaporation of the residual material remaining after the exposure was hindered. As in the eye region, dilutions of ME had a relatively slight effect on the penile mucosa and scrotal epithelium.

2672. Favourable findings on phosphine fumigation

Hackenberg, U. (1972). Chronic ingestion by rats of standard diet treated with aluminium phosphide. *Toxic. appl. Pharmac.* **23,** 147.

The fumigation of cereal grains with phosphine (PH_3) by means of aluminium phosphide/ammonium carbamate tablets (Phostoxin) raises the question of whether any hazard arises when diets made from grains fumigated in this way are fed to animals. Previous feeding tests in rats have shown a lack of toxic effect, and it has been suggested that a balance is struck between the intake of small doses of PH_3 and its detoxication and excretion (*Cited in F.C.T.* 1970, **8,** 234).

Albino rats fed for 2 yr on a diet containing, after fumigation and aeration, PH_3 residues varying from 0·167–7·5 mg/kg (average at 3–10 days after treatment about 0·9 mg/kg) showed no evidence of toxicity. Survival figures for the treated animals were similar to those of controls. Haematological and blood chemistry values remained within normal limits and organ weights did not differ significantly from control values. No distinctive pathological changes attributable to the diet were observed, and spontaneous tumours showed a similar incidence in both test and control rats. The results confirm previous findings that PH_3-treatment of food grains involves no discernible hazard.

THE CHEMICAL ENVIRONMENT

2673. Borax with and without honey

Gordon, A. S., Prichard, J. S. & Freedman, M. H. (1973). Seizure disorders and anemia associated with chronic borax intoxication. *Can. med. Ass. J.* **108,** 719.

Weir, R. J., Jr. & Fisher, R. S. (1972). Toxicologic studies on borax and boric acid. *Toxic. appl. Pharmac.* **23,** 351.

There have been several reports of acute toxic effects resulting from the misuse of boric acid in the treatment and care of young infants (*Cited in F.C.T.* 1971, **9,** 914), but the first paper cited above describes the less familiar effects of chronic borate intoxication. The two infants involved were presented over a period of several weeks with pacifiers dipped in a borax and honey preparation. Both developed generalized recurrent convulsions, but only one showed the red erythematous rash and neither the acute vomiting, diarrhoea, oliguria, hyperpyrexia nor circulatory disturbances usually associated with acute boron toxicity. The association of convulsions with boric acid poisoning is not new (*ibid* 1963, **1,** 249), but the severe anaemia, reflecting erythrocyte hypoplasia, may be the first reported incidence of bone-marrow depression associated with borate intoxication. Like the other symptoms recorded, this anaemia receded when administration of the borax and honey preparation ceased and apart from some delay in development, recovery was complete in both children, although their estimated intake of borax was 10 g/wk for 12 wk in one case and 2 g/wk for 5 wk in the other.

The work described in the second paper cited was designed to provide a more complete picture of the toxicity of boric acid and borax in experimental animals. LD_{50} values determined in Sprague–Dawley rats were 4·5–5 g/kg for borax and 3·5–4 g/kg for boric acid. A dose-related emetic response was evoked in dogs, but no deaths occurred with doses up to 6·5 g borax/kg or 4 g boric acid/kg.

The subacute oral toxicity of each compound was tested by feeding dietary levels equivalent to 52·5, 175, 525, 1750 and 5250 ppm boron to rats, and 17·5, 175 and 1750 ppm boron to dogs for 90 days. In rats, there were no apparent effects up to 525 ppm, but at higher levels of either compound the animals showed growth reduction, rapid respiration, inflamed eyes, desquamated skin on tails and paws, and excitation on handling, and gonadal degeneration developed. At the 5250 ppm level both borax and boric acid killed all rats within 3–6 wk. Autopsies on rats that died in this and other groups showed congestion of the liver and kidneys, bright red lungs and a scattered incidence of a swollen appearance of the brain, small gonads and thickening of the pancreas. At a level of 1750 ppm boron, both compounds produced severe testicular atrophy in dogs, but there were no adverse effects at the lower levels.

In rats and dogs, 2-yr feeding studies of borax and boric acid showed similar results. The no-effect level in both species for each compound was 350 ppm boron, but at the higher level (1170 ppm) testicular atrophy was again found. Some indication was obtained that this effect was reversible. A three-generation reproduction study in rats showed no adverse effects on the offspring when the parents were fed borax or boric acid at dietary levels equivalent to 350 ppm boron for 14 wk before mating, and in fact the 350 ppm groups showed increased fertility and lactation. At 1170 ppm, however, the animals were sterile, showing evidence of testicular atrophy and reduced ovulation.

The authors concluded that in terms of the dietary boron equivalent, 350 ppm borax or boric acid had no adverse effects and could be regarded as the overall no-effect level.

2674. Further news of the trace elements

Schroeder, H. A., Tipton, Isabel H. & Nason, A. P. (1972). Trace metals in man: Strontium and barium. *J. chron. Dis.* **25**, 491.

Rosenthal, H. L., Cochran, Olive A. & Eves, Maura M. (1972). Strontium content of mammalian bone, diet and excreta. *Envir. Res.* **5**, 182.

The latest chapter of the trace elements saga pursued by Schroeder and his associates describes the emission-spectrographic determination of strontium (Sr) and barium (Ba) in tissues from 400 human subjects, collected from widespread localities throughout the world. Sr was found in all the samples of human tissues, but the bone content accounted for 99% of body stores. The highest soft-tissue concentrations, very low in comparison with bone, occurred in the aorta, larynx, trachea and lower gastro-intestinal tract. Ba occurs in all human tissues, with 93% of the body burden in bone, but it was not found in all the samples analysed. Americans tended to have less Ba in most tissues and less Sr in the liver and kidneys than other populations. Adults and children from the Far East showed relatively high bone concentrations of Sr. Some tissues appeared to accumulate or lose Ba in the course of ageing, while any marked accumulation of Sr was limited to the lungs and aorta. Ba concentrations in soft tissues showed some correlation with most essential trace metals, whereas the only high correlation of Sr was with calcium (Ca). Newborn infants showed relatively high body concentrations of both Ba and Sr.

Analyses of the diets and drinking-waters from which Ba and Sr might be expected to be derived revealed the highest Sr concentrations in spices, sea-foods, cereal grains, root and leafy vegetables and legumes. It was clear that major losses of both elements occurred during wheat refining and that Sr was lost during sugar refining. An average of 10% of ingested Sr and 6% of ingested Ba is derived from natural waters. Urinary excretion deals with 11% of ingested Sr and 3% of ingested Ba, and some Sr appears in sweat. Soils, most rocks and some coals, contain more Ba than Sr. Marine organisms are capable of concentrating both elements up to 1000-fold. There is no evidence associating excess Sr with any chronic disease and it behaves to some extent as an essential element. Sr deficiency has not been implicated in any specific bone or tooth disease, although it seems possible that it may serve to harden calcified tissues. Ba on the other hand offers no evidence of playing any essential role in mammalian biology, and is more toxic than Sr.

From another survey of Sr comes a report (second paper cited above) that human bone and tooth samples collected in the St. Louis area contained 0·31–0·61 mg Sr/g Ca. The Sr content of four commercial milk products and four composite human diet preparations averaged 1·07 and 1·51 mg Sr/g Ca, respectively. Bones from laboratory rabbits contained

0·85 mg Sr/g Ca in association with a diet containing 4 mg Sr/g Ca, while the levels in the bone and diet of howler monkeys (*Alouatta caraya*) were 1·27 and 5·5 mg Sr/g Ca respectively. The urine and faeces of the rabbits showed an average of 1·8 and 6·0 mg Sr/g Ca, respectively. These levels, compared with the level in bone, indicated some discrimination against Sr, in relation to Ca, by the excretory systems.

2675. Renal damage from gold therapy

Katz, A. & Little, A. H. (1973). Gold nephropathy. An immunopathologic study. *Archs Path.* **96,** 133.

Gold therapy is of value in the treatment of rheumatoid arthritis but its use has been accompanied by reports of adverse reactions. Of these, blood dyscrasia is the most serious, but rashes, gastro-intestinal disturbances and renal intolerance also occur. In the latter connexion, a recent leading article (*British Medical Journal* 1971, **1,** 471) cited a report on 21 cases of proteinuria and four of irreversible toxic nephropathy among 125 patients treated parenterally with gold salts (Handlova, *Unitřní Lékarství*, 1968, **14,** 115). The mechanism by which gold produces renal damage in man is not known, but the relative infrequency of proteinuria or the nephrotic syndrome and the lack of correlation with dosage or blood and urine levels has led to suggestions that hypersensitivity is involved (van den Broek & Maung, *New Engl. J. Med.* 1966, **274,** 210).

This view is echoed by the authors cited above, who present a case of a 54-yr-old woman who had developed the nephrotic syndrome while on gold therapy. Results of a renal biopsy, with subsequent studies by light, immunofluorescent and electron microscopy, indicated that of the 20 glomeruli present in the specimen, five were completely hyalinized. Amyloid could not be detected in the tissue. The epithelial cells revealed extensive foot-process fusion, while the capillary basement membranes of the glomeruli were for the most part of normal width. Gold inclusions, approximately 60 nm in diameter on average and consisting of membrane-bound electron-dense granules were present within the proximal tubular and mesangial cells. Strong positive staining for IgG and IgM was seen along the glomerular capillary walls.

The possibility that the patient's condition was membranous nephritis due to disseminated lupus was excluded on various grounds, and the demonstration of glomerular deposits of immunoglobulins and β1C, together with a decreased level of β1C in the serum, was thought by the authors to be indicative of an immune-complex disease.

2676. More comment on steel prostheses and nickel sensitization

Samitz, M. H. & Klein, A. (1973). Nickel dermatitis hazards from prostheses. *J. Am. med. Ass.* **223,** 1159.

Speculation and argument continue on the question of possible nickel sensitization in patients fitted with stainless-steel prostheses (*Cited in F.C.T.* 1973, **11,** 921). This latest letter, like one that appeared somewhat earlier (Cohen, *J. Am. med. Ass.* 1972, **222,** 585), draws particular attention to the possibility that the stainless-steel screw eventually removed from the nickel-sensitive patient (*ibid* 1973, **11,** 699) may have become corroded during use, so that the nickel could have been more readily released.

The condition of the removed screw was not clear from the original report, but corrosion of stainless-steel orthopaedic prostheses has been described elsewhere (Cohen & Hammond, *J. Bone Jt Surg.* 1959, **41**A, 524; Scales *et al. Br. med. J.* 1961, **ii,** 478). While

the steels now specified for orthopaedic implants may be less prone to corrosion by tissue fluids than those used in the past, it is not known whether the steel used in this particular case conformed to the later specifications. Even if the possibility of corrosion is accepted, however, there remains the question of whether the quantity of metallic nickel, or nickel ion, released in this way would have been sufficient to serve as a sensitizing dose.

2677. Titanium dioxide as a mild lung irritant

Elo, R., Määttä, K., Uksila, E. & Arstila, A. U. (1972). Pulmonary deposits of titanium dioxide in man. *Archs Path.* **94,** 417.

The increasing use of titanium dioxide (TiO_2) in industry demands continuing assessment of its possible hazards. Long-term animal feeding experiments on titanium compounds have indicated a relatively mild degree of toxicity (*Cited in F.C.T.* 1965, **3,** 536), although some interference with reproductive processes in rats and mice has been reported (*ibid* 1972, **10,** 596) and the possible presence of antimony in TiO_2 has in the past given rise to some misgivings in the food-additive sphere (*ibid* 1965, **3,** 127). Most of Ti's potential as an industrial hazard must necessarily stem from its possible effects when inhaled as TiO_2 dust.

Some retention of TiO_2 dust has been demonstrated in the lungs of exposed rats (*ibid* 1964, **2,** 115) but no specific lesions were reported. In the more recent study cited above, lung tissues from three workmen engaged in processing TiO_2 pigments were examined. Two men, who had worked for about 9 and 10 yr respectively in a factory handling TiO_2, experienced recurrent bronchitis with dyspnoea, which was aggravated by the dusty environment and improved during holidays. A biopsy specimen from one of them revealed some bronchiolar hyperplasia. In the lungs of both men, and of a third who had also worked for 9 yr in TiO_2 dust and on whom an autopsy report was obtained after a drowning accident, extensive patches of subpleural pigment aggregation were seen by light microscopy. Some cell destruction and fibrosis was associated with TiO_2 deposits in the interstitium, and there were deposits in the lymphatics, which indicated some degree of clearance of the lung deposits by the lymphatic system. Electron microscopy showed TiO_2 particles in the lysosomes of alveolar macrophages. It seems, therefore, that prolonged industrial exposure to TiO_2 dust, alone or in conjunction with silica or other dusts, may provoke mild pulmonary irritation.

2678. Methylmercury and the rat kidney

Klein, R., Herman, S. P., Bullock, B. C. & Talley, F. A. (1973). Methyl mercury intoxication in rat kidneys. Functional and pathological changes. *Archs Path.* **96,** 83.

Poisoning with methylmercury (MeHg) has been shown to exert its primary effect on the central nervous system in man (Eyl, *Clin. Toxicol.* 1971, **4,** 291) and experimental animals (*Cited in F.C.T.* 1967, **5,** 101), but renal involvement is another important aspect (*ibid* 1973, **11,** 701).

A detailed study of the type of kidney damage produced by MeHg in the rat was undertaken to clarify the functional and structural changes involved. MeHg, as the hydroxide, was administered sc in aqueous solution in daily doses of 2 or 10 mg Hg/kg for 5 days and groups of the treated rats were killed 24 hr or 6 days after the final injection (on days 6 and 11 of the experiment). The rats on the higher dose lost weight rapidly between days 4 and 8 of the experiment, and from day 4 onwards the body weight of rats given the lower

dose level was markedly lower than that of controls. There was some kidney enlargement in the treated animals, but the difference from controls was statistically significant only in the rats killed 6 days after the last 10 mg/kg dose. Levels of total mercury in the kidneys were 60 and 133 μg/g wet weight on day 6 and 62 and 153 μg/g on day 11 in the groups given the lower and higher doses, respectively. An increase in the renal content of inorganic mercury between days 6 and 11 apparently reflected a breakdown of MeHg, since there was a concurrent fall in the level of the latter form. Values for urinary and serum levels of sodium and potassium, for blood urea-nitrogen and for creatinine clearance did not differ significantly in treated and control animals, and a small but significant rise in serum creatinine in the group given 10 mg Hg/kg was apparent only on day 6.

Histologically, focal vacuolar degeneration, with some necrosis, was apparent in the mid-cortex by day 6 in rats given the higher and by day 11 in those given the lower dose level. The most striking early ultrastructural change, apparent by day 6 in rats given 10 mg Hg/kg was mitochondrial swelling with a decrease in both matrix density and the number of granules. There was also an apparent increase in cytosomes (lysosomes). A similar ultrastructural picture was seen on day 11 in the rats given 2 mg Hg/kg.

The findings reported in this study are similar to those associated with relatively small doses of mercuric chloride and indicate that any appraisal of the hazard proffered by MeHg must take into account the possibility of kidney damage resulting from the biotransformation of the organomercurial to the inorganic form.

2679. Modification of acrylamide neuropathy by microsomal-enzyme inducers

Kaplan, M. L., Murphy, S. D. & Gilles, F. H. (1973). Modification of acrylamide neuropathy in rats by selected factors. *Toxic. appl. Pharmac.* **24,** 564.

The ability of acrylamide to cause peripheral neuropathy in the rat (*Cited in F.C.T.* 1967, **5,** 267) and man (*ibid* 1971, **9,** 912) has been well established and described in some detail. The study cited above was concerned with the possible effects of adrenalectomy, a deficiency of certain vitamins and pretreatment with microsomal-enzyme inducers on the progress of the functional neuropathy resulting from repeated ip injections of acrylamide (40 or 50 mg/kg/day) to rats. Impairment of neurological function, and subsequent recovery after cessation of treatment, were measured by a modified 'rotarod' apparatus, consisting basically of a rotating rod on which rats had been trained to maintain their balance for a specific time.

Neither a dietary deficiency of pyridoxine or thiamine nor daily sc injections of hydrocortisone affected the total number of daily doses of acrylamide required to produce a detectable functional impairment. Bilateral adrenalectomy increased the susceptibility of rats to acrylamide; 100% mortality resulted from a total cumulative dose of 300 mg/kg in these rats, whereas none of the sham-operated rats had died after receiving 400 mg/kg. However, the effect was thought to be a non-specific one, due to the weakness and debility of the adrenalectomized animals.

In rats pretreated with DDT or phenobarbitone, the total doses required to cause neurological impairment were 520 and 600 mg/kg respectively, compared with 360 mg/kg in untreated controls. Recovery after cessation of treatment was also delayed in the treated rats. *In vitro* metabolic studies, using the 9000 **g** supernatant fraction of liver homogenates from rats pretreated with phenobarbitone, indicated that the acrylamide-detoxifying capacity of the liver was increased in such rats, and, in fact, in the *in vivo* study the times for the onset of and recovery from neurological impairment were the same both for rats given

daily acrylamide doses of 40 mg/kg alone and for those given acrylamide in doses of 60 mg/kg/day after phenobarbitone pretreatment. Histological studies of the peripheral nerves indicated, however, that these reactions involved more than a simple acceleration of acrylamide detoxification following enzyme induction. Whereas the onset of functional neurological impairment was not associated with any detectable peripheral nerve damage in the rats treated only with acrylamide, severe nerve injury was found in phenobarbitone-treated rats that were killed as soon as a positive result was obtained in the rotarod test.

2680. Getting used to dichloromethane

Weinstein, R. S., Boyd, D. D. & Back, K. C. (1972). Effects of continuous inhalation of dichloromethane in the mouse: Morphologic and functional observations. *Toxic. appl. Pharmac.* **23,** 660.

Female mice exposed continuously to 5000 ppm dichloromethane (CH_2Cl_2) showed increased physical activity and a decrease in food and water consumption during the first few hours. After exposure for 24 hr spontaneous activity decreased again, and the animals became lethargic and dehydrated. Posture became hunched and the hair roughened. During the next 48 hr these effects were intensified and the hair assumed a yellowed and greasy appearance. However, by 96 hr, many mice had resumed their normal activity and were eating and drinking more. Despite a continued state of dehydration, postural and hair changes grew less marked. After 1 wk of exposure the animals, though emaciated and dehydrated, had almost regained their customary activity.

Livers of the treated animals showed increases in relative weight between days 1 and 4, and various changes were detectable from 12 hr onwards. There was early dissociation of polyribosomes and swelling of the rough endoplasmic reticulum of the hepatocytes ("balloon degeneration"), which reached a peak at 48 hr and thereafter partially reversed. Severe but partially reversible fatty degeneration accompanied some inhibition of leucine incorporation into liver proteins. After an exposure period of 3 days, liver triglycerides had increased 12-fold over control values, but this level fell again after 1 wk to a two- or three-fold increase only. Necrosis was evident in a few isolated hepatocytes.

Liver lesions in mice exposed to CH_2Cl_2 thus resembled those provoked by CCl_4, though they were less severe (*Cited in F.C.T.* 1974, **12,** 161). The development of some tolerance to the effects of CH_2Cl_2 was also reminiscent of that observed with CCl_4 (*ibid* 1973, **11,** 901).

2681. The teratogenicity of ethylene thiourea

Khera, K. S. (1973). Ethylenethiourea: Teratogenicity study in rats and rabbits. *Teratology* **7,** 243.

Ethylene thiourea (ETU), a breakdown product of the ethylenebis(dithiocarbamate) fungicides (*Cited in F.C.T.* 1973, **11,** 1144), has been a source of concern in recent years because of its toxic and tumorigenic effects on the thyroid (*ibid* 1973, **11,** 702). The parent fungicides have been found to cause malformations in the nervous system of the rat foetus (Petrova-Vergieva, *Teratology* 1971, **4,** 497), so it is not altogether surprising that ETU has now been shown to possess similar properties.

ETU was given orally in daily doses of 5, 10, 20, 40 or 80 mg/kg to rats and rabbits. The rats were treated from 21–42 days before conception to day 15 of pregnancy or on days 6–15 or 7–20 of pregnancy, whereas the rabbits received ETU only on days 7–20 of

pregnancy. There were no overt signs of toxicity in the mothers of any test group with the exception of the rats given 80 mg/kg/day, 82% of which died after treatment for 7 or 8 days. In this species the numbers of corpora lutea and live foetuses were unaffected by the treatments, but foetal weight was reduced at 40 and 80 mg/kg. Irrespective of the time at which ETU was administered to the rats, lesions were produced in the central nervous and skeletal systems, their severity depending on the dose. The severe effects seen at the two highest dose levels included meningoencephalocele, meningorrhagia, meningorrhoea, hydrocephalus, obliterated neural canal, abnormal pelvic limb posture with equinovarus, micrognathia, oligodactyly and absent, short or kinky tail. Less serious defects were induced by 20 mg/kg, and at 10 mg/kg there was only a retardation of parietal ossification and of cerebellar Purkinje-cell migration. Retarded parietal ossification was the sole abnormality seen at 5 mg/kg, its incidence being limited to small areas and to a few large litters. Rabbits were far less affected by ETU treatment, showing only an increase in resorption sites and a reduction in brain weight at the highest dose level. Renal lesions, characterized by degeneration of the proximal convoluted tubules, were noted microscopically, but there were no skeletal abnormalities that could be attributed to ETU.

2682. Hexachlorobenzene and the liver

Medline, A., Bain, Edith, Menon, A. I. & Haberman, H. F. (1973). Hexachlorobenzene and rat liver. *Archs Path.* **96,** 61.

The consumption of grain treated with hexachlorobenzene (HCB) was responsible for an epidemic of porphyria in Turkey in 1957 (*Cited in F.C.T.* 1967, **5,** 429). In rats, increased porphyrin excretion, liver necrosis and phototoxicity have been induced by a dietary level of 0·2% HCB (*ibid* 1963, **1,** 126; *ibid* 1966, **4,** 224) while in quails a similar syndrome resulted from a dietary level of 80 ppm HCB (*ibid* 1972, **10,** 263). The hepatic changes caused by HCB in rats are described in detail by Medline *et al.* (cited above).

Adult male rats were fed a dietary level of 0·2% HCB for up to 9 wk. An apparent increase in smooth endoplasmic reticulum was seen in the liver after 2 wk, together with large lipid droplets, sometimes surrounded by "rosettes" of glycogen. At the end of a further week there was a marked enlargement of hepatocytes, many of which contained one or more discrete eosinophilic laminated cytoplasmic bodies. These were 2–20 μm in diameter, and by electron microscopy were shown to be of either a myelin-like or "fingerprint" configuration. The former type consisted of a central core of lipid without organelles, surrounded by up to 20 paired, smooth-surfaced membranes, and frequently communicating with smooth and rough endoplasmic reticulum. The fingerprint configurations consisted of up to 50 similar membranes, and sometimes contained a core of lipid droplets accompanied by smooth endoplasmic reticulum and mitochondria. Cytoplasm protruded through the space of Disse into the sinusoids, in which membrane-bound vacuoles containing organelles and glycogen were also seen. The possibility that this protrusion of cytoplasm into the sinusoids represented some artefact was not altogether dismissed.

It is suggested that the cytoplasmic bodies may represent hypertrophy of the smooth endoplasmic reticulum developing in response to the need for an increase in the enzymes responsible for HCB metabolism. Similar changes induced by a variety of other chemicals have been reported elsewhere, and have in some cases been shown to be reversible when treatment was discontinued.

2683. Support for a stilboestrol hypothesis

Greenwald, P., Nasca, P. C., Burnett, W. S. & Polan, Adele (1973). Prenatal stilbestrol experience of mothers of young cancer patients. *Cancer* **31,** 568.

The reported development of vaginal cancer in young women who had been exposed *in utero* to high levels of stilboestrol and other synthetic oestrogens given to the mother (*British Medical Journal* 1971, **3,** 593) has prompted an investigation into the possibility that tumours in other tissues may also have been induced by maternal ingestion of stilboestrol. The survey was based on data in the New York State Cancer Registry, which records all malignant neoplasms diagnosed among residents of New York State, excluding New York City, and thus covers an area with a population of over 10 million. The incidence of malignant neoplasms at several anatomical sites, including the endocrine-dependent organs, in both males and females born after 1947, when synthetic oestrogens first became widely used therapeutically, was compared with the incidence of the same tumours in people born during the previous 9 yr and was related to the use of drugs by the mother during pregnancy. Time trends in age-specific and sex-specific incidence rates of the major types of malignant neoplasm were analysed statistically for the two groups born before and after 1947.

This study produced no evidence of any increase in tumour incidence that could be related to the introduction of synthetic-oestrogen therapy. In fact, stilboestrol had been taken during pregnancy by the mother of only one of the 48 young males and females reported as having tumours of the breast or urogenital organs (other than vaginal adenocarcinomas, which were excluded from the study because they had already been investigated). The one patient who had been exposed to stilboestrol *in utero* had an adenocarcinoma involving both the cervix and vagina and the primary site could not be determined with certainty.

2684. Methyl toluenesulphonate a sensitizer

Nally, F. F. & Storrs, J. (1973). Hypersensitivity to a dental impression material: A case report. *Br. dent. J.* **134,** 244.

A young woman developed a rash on her face and neck and a burning sensation in the mouth, after contact with a polyether dental-impression material. The polyether was basically a tetramethylene glycol with terminal aziridine groups. Patch tests with the polyether base and the thinner were negative, but a violent reaction to the catalyst developed after 24 hr, involving pruritus, erythema and blistering. The patient's reaction was therefore attributed to methyl *p*-toluenesulphonate, the catalyst in the impression compound. When this catalyst was tested undiluted on three volunteers it provoked mild reactions in two of them. The indications were that the patient had been sensitized to methyl *p*-toluenesulphonate when the same impression compound had been used several months before.

2685. More observations on a terphenyl coolant

Adamson, I. Y. R. (1973). Inhaled irradiated terphenyls: Reaction of murine lung and liver. *Archs envir. Hlth* **26,** 192.

It has been reported (*Cited in F.C.T.* 1973, **11,** 1153) that a hydrogenated terphenyl reactor-coolant was devoid of carcinogenic properties when fresh, and when heated and irradiated acquired only feeble carcinogenicity in comparison with tar. A further study of a

terphenyl mixture supports the earlier indication that the risk associated with occupational exposure to these materials, even after their irradiation, is relatively slight.

Albino mice were exposed to irradiated HB40 (a terphenyl mixture) in an aerosol concentration of 2 mg/litre for 4 hr daily, 5 days/wk for up to 8 wk. The experiment was repeated with black mice of a strain particularly resistant to pulmonary disease. Animals were weighed twice weekly and killed at various periods up to 6 wk after cessation of exposure.

During exposure, the mice appeared healthy and showed no difference in weight from controls. In 5% of the treated albino mice (compared with 3% of the controls) extensive bronchiolitis with squamous metaplasia of the epithelial cells of the bronchioles developed, but this change was not seen in the black mice. Ultrastructural lung damage was evident immediately after exposure to the terphenyl, but the membrane debris in the alveoli cleared rapidly. Vacuolation of some mitochondria of the type 2 alveolar epithelial cells was apparent after a minimum exposure period of 2 wk, but did not persist for more than 4–6 wk after termination of exposure. Diffuse cytoplasmic changes developed in the liver more than 2 wk after a 3-wk exposure, with an increase in smooth endoplasmic reticulum, which was interpreted as an adaptive reaction. As in the lung, these liver changes were reversed within a few weeks of cessation of exposure.

The findings indicate that cell renewal in the lung and adaptation in the liver can keep pace with any effects of inhaled terphenyls likely to be encountered at a reactor site, where atmospheric concentrations would be expected to be not more than 20% of those used in this study.

NATURAL PRODUCTS

2686. Further studies on the bracken carcinogen

Hirono, I., Fushimi, K., Mori, H., Miwa, T. & Haga, M. (1973). Comparative study of carcinogenic activity in each part of bracken. *J. natn. Cancer Inst.* **50,** 1367.

Ever since the demonstration, nearly 10 yr ago, that the ingestion of bracken (*Pteridium aquilinum*) induced intestinal tumours in rats (*Cited in F.C.T.* 1966, **4,** 358) efforts have been made to establish not only the identity of the carcinogenic factor (*ibid* 1972, **10,** 603) but also the influence of other dietary constituents on its activity (*ibid* 1971, **9,** 920; *ibid* 1972, **10,** 603) and the possible hazard associated with the use of variously processed brackens for human food (*ibid* 1973, **11,** 705). The situation is, however, still far from clear, and the authors cited above have studied the location of the carcinogenic factor in the bracken plant partly as an aid to the isolation of the carcinogen and partly to give some lead on the question of whether one component is responsible both for the tumour induction and for the other toxic effects that may be seen in livestock grazed on bracken.

Groups of rats were fed a basal diet containing comparable concentrations of pellets prepared from the curled tops or the stalks of bracken fronds or from the whole fronds or the bracken rhizomes. The two former types of pellet were fed for a period of 2 months and the others for 4 months. Each part of the bracken plant produced tumours, most frequently in the ileum, and multiple tumours were common. The incidence of intestinal tumours and the numbers of tumours in individual rats were higher in the groups given pellets made from the curled tops of the fronds than in those fed comparable dietary levels of pellets made from the stalks, but tumour induction was significant with both types of

pellet even when the proportion of bracken material to basal diet was as low as 1 : 8, w/w. In the 4-month feeding study, the latent period of tumour induction was shorter in the rats given bracken rhizome than in those given the fronds and the number of tumours in each rat was greater in the rhizome-fed group. However, there was no significant difference in the total incidence of intestinal tumours at the end of the study, 13/18 rats being affected in the group fed frond pellets and 13/13 in that given rhizome pellets. In all the bracken-fed groups, ileal adenoma was the most frequently encountered tumour and ileal adenocarcinoma was also common. Some, but relatively few, of the treated animals also had bladder tumours, and ileal sarcomas and caecal adenocarcinomas occurred in some of the rats fed a high dietary level of the curled tops of the fronds.

It was evident from this study that while all the bracken plant was carcinogenic, the greatest activity was located in the rhizome. This is the part of the plant that has also been shown to bear the highest concentration of the toxic factor causing bovine bracken poisoning (Evans *et al. Vet. Rec.* 1961, **73,** 852), so it is not impossible that the same agent is responsible for both effects. No tumours were induced in an additional group of rats fed for 4 months on bracken starch, prepared from the rhizomes in the way it is commonly prepared for human consumption in Japan. This suggests that the carcinogenic agent is water-soluble and was washed out during the preparation of the starch.

2687. Bracken and bright blindness

Watson, W. A., Barnett, K. C. & Terlecki, S. (1972). Progressive retinal degeneration (bright blindness) in sheep: A review. *Vet. Rec.* **91,** 665.

One of the toxic effects already attributed to bracken is a pathological change in the sheep retina involving degeneration of the rods and cones and a reduction in the width of the outer nuclear layer, particularly in the tapetal region (*Cited in F.C.T.* 1971, **9,** 453). This progressive retinal degeneration, commonly known as 'bright blindness', occurs in hill flocks in Yorkshire and the Lake District, and by 1971 it had been confirmed in 253 flocks in Northern England. All the affected flocks have been found to have access to bracken-infested grazing. The blindness occurs in a range of breeds and most commonly affects 3- and 4-yr-old ewes.

Affected sheep become permanently blind in both eyes, but there is no conjunctivitis, keratitis or lens opacity. The earliest detectable change is an increased reflection from the tapetum lucidum. Histopathological changes are confined to the retina, the layer of rods and cones and the outer nuclear layers being completely destroyed in advanced cases. At an earlier stage the rods and cones become fragmented, appearing in a fine granular form. Total lactic dehydrogenase activity has been found to be lower in the retinae of the blind ewes than in normal adults, and alterations found in the isoenzyme pattern appear to be an extension of the changes seen in normal development.

Highly significant changes in the lactic dehydrogenase isoenzymes were also found in cases of bright blindness induced experimentally in sheep by the feeding of a concentrate containing 50% dried bracken. Of 22 ewes fed about 1 kg of this concentrate daily for 63 wk, 15 developed clinical signs of bright blindness, the first only 28 wk after the start of the feeding. Autopsy examination confirmed 20 cases of the disease. As in previous experimental and field studies, platelet and leucocyte counts were found to be lower than normal in the bracken-fed sheep, whether or not they showed any sign of blindness.

2688. Another fusarium toxin

Cole, R. J., Kirksey, J. W., Cutler, H. G., Doupnik, B. L. & Peckham, J. C. (1973). Toxin from Fusarium moniliforme: Effects on plants and animals. *Science, N. Y.* **179,** 1324.

Mycotoxins from Fusarium species such as *F. tricinctum* and *F. javanicum* are no strangers to these pages (*Cited in F.C.T.* 1971, **9,** 604; *ibid* 1972, **10,** 884). It is thus no surprise that a new toxin from a strain of *F. moniliforme* has been isolated during the routine screening of corn seed infected with southern leaf blight in the United States.

The main toxic metabolite proved to be a water-soluble compound, to which the trivial name moniliformin was assigned. Physico-chemical analysis suggested a compound of empirical formula $C_6H_{3-6}O_{6-8}$, bearing several hydroxyl groups and probably existing in the form of a salt. In cockerels, oral LD_{50} and LD_{100} values of 4·0 and 6·25 mg/kg, respectively, were determined. Gross and histological lesions were seen only in birds that survived for more than 2 hr after receiving a fatal dose and consisted of ascites with oedema of the mesenteries and haemorrhages of the proventriculus, gizzard, small and large intestines and skin.

The toxin also inhibited the growth of wheat coleoptiles *in vitro* and produced necrosis, interveinal chlorosis, distortion of leaf shape and internodal shortening when sprayed on to intact corn and tobacco seedlings. In tobacco plants, a phenomenon of "rosetting" was attributed to destruction of apical dominance. The phytotoxic effects persisted for about 30 days, and 6 wk after treatment there was little difference between treated and control plants.

2689. Ochratoxin production on ham

Escher, F. E., Koehler, P. E. & Ayres, J. C. (1973). Production of ochratoxins A and B on country cured ham. *Appl. Microbiol.* **26,** 27.

We have recently reviewed literature on the toxicity of the ochratoxins and their biosynthesis by a variety of fungal species, including *Aspergillus ochraceus* and *Penicillium viridicatum* (*Cited in F.C.T.* 1973, **11,** 903). Ochratoxins have been identified in mouldy corn (*ibid* 1970, **8,** 237) and have been shown to be formed in a soil culture of *P. viridicatum* isolated from cottage-roll ham (*ibid* 1970, **8,** 610).

Escher *et al.* (cited above) isolated two strains of *A. ochraceus* and six of *P. viridicatum* from country-cured hams, and cultured each strain on rice, defatted peanut meal or corn for 14 days. Another strain of *P. viridicatum*, not derived from ham but a known producer of ochratoxin A, was included for reference purposes. No ochratoxin (down to a limit of detection of 15 μg/kg) was produced by any of the cultures of the ham-derived strains of *P. viridicatum*, although 80 μg ochratoxin A/kg was detected in rice incubated with the reference strain. On the other hand, both isolates of *A. ochraceus* produced ochratoxins A and B in all three media, and also on country-cured ham. After incubation of *A. ochraceus* on ham for 21 days, at 25°C and 75% relative humidity, one-third of the total ochratoxin A produced was found in the mycelial mat on the ham surface and two-thirds in the outermost 0·5 cm of the ham, with only traces in the next 0·5 cm. The toxin's capacity for diffusion was apparently poor, since it occurred only at depths to which the mycelium had also penetrated.

2690. Rubratoxin B and the foetus

Koshakji, R. P., Wilson, B. J. & Harbison, R. D. (1973). Effect of rubratoxin B on prenatal growth and development in mice. *Res. Commun. chem. Path. Pharmac.* **5,** 584.

Studies on the rubratoxins, which are produced by *Penicillium rubrum* and *P. purpurogenum*, have tended to concentrate on hepatotoxicity and other acute toxic effects (*Cited in F.C.T.* 1972, **10,** 277). In the paper cited above, rubratoxin B has been shown to be both foetotoxic and teratogenic in mice.

Rubratoxin B was injected ip into pregnant mice in doses of 300–500 μg/kg given once or twice daily on days 8–10 or 12–14 of gestation. A dose-dependent increase in the number of resorptions was observed and the period of organogenesis (days 8–10) proved to be the more sensitive of the two periods studied, there being a 90% incidence of resorptions from a single daily injection of 500 μg/kg during this period compared with a 22% incidence when the same dose was given on days 12–14. The decrease in foetal weight among survivors was also both dose-dependent and greatest during the period of organogenesis, being as much as 40% of the control value when 1 mg/kg was administered on day 8. A number of abnormalities that were not found in control foetuses were found in the soft tissues, but not the skeleton, of foetuses from treated dams. Head, eye, ear and kidney abnormalities predominated after treatment on day 8, whereas oro-facial, brain, lung, liver and kidney anomalies were predominant after treatment on days 12–14.

None of the pregnant rats died as a result of rubratoxin B administration, but a decrease in weight occurred with high dose levels and was attributed to the high incidence of foetal resorption.

2691. Contaminants in Indian edible oils

Ranadive, K. J., Gothoskar, S. V. & Tezabwala, B. U. (1972). Carcinogenicity of contaminants in indigenous edible oils. *Int. J. Cancer* **10,** 652.

In some countries, the possible contamination of edible oils with unsuitable solvent residues or with argemone oil raises the question of potential carcinogenicity. This may become highly important when, as is the case in India, edible oils form an essential part of the diet. As part of a wider programme on the safety of edible oils prepared in India, samples of such oils were tested by application to the shaved skin of mice (with or without croton oil as a co-carcinogen), by sc injection into mice in a single dose or in multiple doses at intervals of 2–4 wk, and by administration to mice by gavage in daily doses of 0·1 ml on 5 days/wk for the entire lifespan of the animal.

Five of the six samples of solvent-extracted groundnut oil tested in 1966 showed significant carcinogenic activity, one sample producing tumours by all routes, including cutaneous application without a co-carcinogen. Five samples tested 3 yr later, when food-grade *n*-hexane had replaced the crude solvents used earlier, showed little tumorigenic activity, the only positive finding being a weak response with two of the samples following cutaneous application in conjunction with croton oil. A similar lack of activity was found with most samples of machine-expressed oils and with imported soya-bean oils.

Argemone oil, a possible adulterant of Indian edible oils, was not in itself found to show any evidence of carcinogenicity and had no significant effect when administered with imported soya-bean oil or a laboratory-expressed mustard oil. However, one popular brand of tinned mustard oil produced tumours by all three routes of administration and the addition of argemone oil to this mustard oil apparently increased its carcinogenic potential.

While stricter use of food-grade solvents is obviously reducing the possibility of contamination of edible oils with known carcinogens, the elimination of argemone oil is a more difficult problem, since the plant from which it is derived grows wild in the mustard fields and the argemone seeds closely resemble mustard seeds.

2692. The villain in the wood dust?

Schoental, R. & Gibbard, S. (1972). Nasal and other tumours in rats given 3,4,5-trimethoxycinnamaldehyde, a derivative of sinapaldehyde and of other α,β-unsaturated aldehydic wood lignin constituents. *Br. J. Cancer* **26,** 504.

It has been suggested that α,β-unsaturated aldehydes present in wood lignins may be responsible for the high incidence of nasal adenocarcinomas found in woodworkers in the European furniture industry (*Cited in F.C.T.* 1971, **9,** 899). Some support for this theory has been provided by a small study in which six male rats were injected with 3,4,5-trimethoxycinnamaldehyde (TMCA) in an ip dose of 150 mg/kg in aqueous ethanol, followed 1 wk later by a dose of 100 mg/kg given sc in dimethylformamide. Four rats which survived for more than 17 months after this treatment developed tumours. These consisted of two nasal squamous carcinomas (found in two rats killed after 20 and 24 months, respectively), a sarcoma in the peritoneal cavity with metastatic nodules on the omentum, and a mesothelioma of the tunica albuginea of both testes. In view of the rarity of nasal tumours in rats, these findings were regarded as significant. It is not known whether TMCA as such is present in wood lignins, but it could be formed metabolically *in vivo* from its phenolic congeners by *O*-methyl-transferase, and/or be *p*-*O*-demethylated to sinapaldehyde and other products. Epoxides, formed by metabolic oxidation of the side-chain double bond, could well provide the proximate carcinogen, as in the case of several other known carcinogens.

2693. Contact dermatitis from cedar wood dust

Bleumink, E., Mitchell, J. C. & Nater, J. P. (1973). Allergic contact dermatitis from cedar wood (*Thuja plicata*). *Br. J. Derm.* **88,** 499.

Cases of contact dermatitis have been reported from time to time among people whose work entails the handling of certain types of wood. One such incident involved men engaged in the processing of African mahogany (*Cited in F.C.T.* 1966, **4,** 553) and the more recent report cited above relates the case of a workman who reacted to the heart-wood of western red cedar (*Thuja plicata*).

After exposure to various types of wood in a saw mill for about 2 yr, this workman developed an itching, vesicular dermatitis on his face and forearms and the back of his hands. The dermatitis cleared when he avoided contact with wood but appeared again within a few days of his return to the factory. Patch tests with extracts of 19 different species of wood revealed a strong delayed cutaneous reaction to the extract of western red cedar and a weaker allergy to deal (*Picea abies*) extract. The man's work brought him into regular contact with the fine sawdust of both of these woods. A further series of patch tests was conducted on a range of compounds known to be present in the woods of various species of Thuja, 0·10–0·15 mg of these compounds being applied on each patch in a 1% solution in ethanol. Positive reactions occurred with 7-hydroxy-4-isopropyltropolone and γ-thujaplicin, both of which are present in western red cedar, and also with thymoquinone, which occurs in various other woods. The first two compounds, both tropolones, are closely related chemically. Thymoquinone is a strong skin irritant, but additional testing in two other patients indicated that it is a potent allergen. Carvacrol, which is present in western red cedar as well as various other species of wood, has also been shown to be an active allergen, although the saw-mill worker discussed in this paper did not react positively to it.

2694. Worcester sauce for the rat

Kovacs, K. & Lazar, G. (1973). Observations on the nontoxicity of Worcestershire sauce in the rat kidney. *Int. Z. VitamForsch.* **43,** 81.

A link between kidney damage and the excessive consumption of Worcester sauce was first suggested in 1956, when a businessman's chronic nephritis and severe albuminuria rapidly disappeared when he gave up his curious but long-standing habit of drinking anything between a half and a full bottle of the sauce each day (Douthwaite. *Br. med. J.* 1956, **ii,** 958). Two further cases of bilateral renal calculi with aminoaciduria were attributed to the same cause in 1967 (*Cited in F.C.T.* 1967, **5,** 836), and three others have since been reported (Murphy, *Med. J. Aust.* 1971, **1,** 1119). One of the last three, who had drunk Worcester sauce "by the glassful" for many years, progressed to terminal renal failure. Kovacs & Lazar (cited above) have now made an unsuccessful attempt to reproduce these effects in rats.

Female rats given Worcester sauce twice daily by stomach tube in volumes of 5 ml for 4 days, or 1 ml for 31 days, showed no gross or histological kidney changes, apart from a scattered incidence of mild focal mononuclear cell infiltration which was considered to be unrelated to the treatment. Nor was there any evidence of the renal deposition of calcium salts. Moreover, two oral doses of 1 ml Worcester sauce failed to increase the renal calcinosis and mortality induced by the administration 4 days later of a potent nephrotoxin (mercuric chloride, 2 mg/kg given iv, or dihydrotachysterol, 30 ml/kg orally). The authors suggest that differences in kidney sensitivity or in metabolic pathways may be responsible for the differences in response between rat and man, or that Worcester sauce may have been erroneously implicated in the case histories mentioned above.

[Since some of the human cases had been drinking Worcester sauce by the glass or bottleful for many years, it would seem more likely that in the above study the treatment periods were too short for the dose levels given to affect the kidneys, since the total intake was relatively low. The results are reassuring, however, for those who consume Worcester sauce in more normal quantities—if any such reassurance were needed!]

BIOCHEMICAL PHARMACOLOGY

2695. Species variations in drug metabolism

Flynn, E. J., Lynch, M. & Zannoni, V. G. (1972). Species differences and drug metabolism. *Biochem. Pharmac.* **21,** 2577.

Use of the so-called "hundred-fold safety factor" in assessments of human hazard on the basis of results of animal studies has been much criticized in recent years, on the grounds that no practical or theoretical basis exists for this concept. A more 'scientific' approach to the problem stems from a study of the mechanisms underlying foreign-compound detoxication, in an effort to find those factors that determine species variations in rates of metabolism. The study cited above, for example, is concerned with finding which liver microsomal electron-transport components may be responsible for observed species differences in the oxidation of three types of drug.

Three archetypical drug-oxidation reactions—aminopyrine *N*-demethylation, *p*-nitroanisole *O*-demethylation and aniline hydroxylation—were examined in rats, mice and guinea-pigs. *In vitro* studies showed that although there were wide interspecies variations

in enzyme activities, there was no apparent correlation between microsomal oxidation activity and animal species. Investigation of the amount and activity of liver microsomal electron-transport components showed that cytochrome *P*-450 and NADPH-cytochrome *P*-450 reductase were far more important determinants of overall drug-oxidation activity than, for example, cytochrome b_5 and NADPH-cytochrome *c* reductase.

Binding studies of the reaction between the drug substrates and cytochrome *P*-450 showed qualitative interspecies differences in this cytochrome, suggesting that this initial step in drug oxidation may be especially important in rate determination. Such qualitative differences have not been apparent in previous studies, since comparisons were normally made between the reduced cytochrome *P*-450/CO complex or the usual type I (aminopyrine) and type II (aniline) substrate/cytochrome *P*-450 binding spectra, which, as verified in this study, show no clear species variation. Nevertheless, prolonged treatment of the liver-microsome preparation with aminopyrine or aniline for at least 20 hr resulted in an atypical type Ia or IIa spectrum with a species-specific absorption maximum. It was possible to convert these atypical spectra to the usual cytochrome *P*-450 spectrum.

Studies using phenobarbitone or 3-methylcholanthrene to induce individual electron-transport components indicated that *N*- and *O*-demethylase activities were dependent on the level of cytochrome *P*-450, and that aniline hydroxylase activity depended upon the activity of cytochrome *P*-450 reductase.

CANCER RESEARCH

2696. Nitrosamine susceptibility of regenerating liver tissue

Pound, A. W., Lawson, T. A. & Horn, Lorraine (1973). Increased carcinogenic action of dimethylnitrosamine after prior administration of carbon tetrachloride. *Br. J. Cancer* **27**, 451.

Carcinogenicity studies on dimethylnitrosamine (DMNA) have shown that the yield of tumours and the target organ can be modified in a number of ways. Apart from the obvious means of achieving this, such as a change in the route or frequency of administration or in the dose level, it has been shown that the nutritional state of the animal can have a profound influence on the final outcome. Thus rats fed a protein-deficient diet have been shown to tolerate larger doses of DMNA than adequately fed controls, so that a larger yield of tumours was eventually obtained (*Cited in F.C.T.* 1971, **9**, 584).

Craddock (*J. natn. Cancer Inst.* 1971, **47**, 889) showed that the production of liver tumours could be modified by hepatectomy prior to treatment with a single dose of DMNA. Administration of a single ip dose of 9 mg DMNA/kg to otherwise untreated rats failed to elicit any liver tumours, but when this dose was given 24 hr after partial hepatectomy, 40% of the treated rats developed liver-cell carcinoma. When the time between hepatectomy and dosing was increased to 31 hr, the incidence of these tumours was 17%. This author concluded that the induction of hepatocellular carcinoma in this experimental model underlined the susceptibility of the dividing liver cell to chemical carcinogens.

Further support for this hypothesis is provided in the paper cited above, whose authors induced hepatocellular necrosis with a dose of carbon tetrachloride (CCl_4) and studied the effect of cell division during the subsequent repair process on the hepatocarcinogenicity of DMNA. Male Sprague–Dawley rats were treated with a single ip dose of 20 mg DMNA/kg 42 or 60 hr after a non-lethal, hepatotoxic dose of 2·5 ml CCl_4/kg had been

administered by stomach tube. Controls were given either the DMNA injection or the dose of CCl_4. The animals were killed 12 months later. CCl_4 given alone produced no tumours, whereas DMNA alone produced a hepatocellular carcinoma in two of the 27 survivors. Three carcinomas were found among 27 rats treated with DMNA 42 hr after CCl_4 administration while a total of ten carcinomas was found in seven of 34 rats given DMNA 60 hr after the CCl_4 treatment.

No kidney tumours were found in rats treated with DMNA alone. In the animals treated with both compounds, an intervening period of 42 hr between the two treatments resulted in a total of ten renal neoplasms among the 27 surviving rats, whereas with a 60-hr gap only four such tumours occurred in 34 survivors. A total of 26 kidney tumours developed in a group of 17 rats given a double dose of DMNA (40 mg/kg) 42 hr after treatment with CCl_4.

Aims and Scope

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

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