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[The articles and abstracts listed above have been contributed by the staff of the British Industrial Biological Research Association. Comments on these items are welcomed and should be sent to the Assistant Editor at BIBRA.]

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Research Section

METABOLISM OF DI-(2-ETHYLHEXYL) PHTHALATE BY THE CONTENTS OF THE ALIMENTARY TRACT OF THE RAT

I. R. ROWLAND

British Industrial Biological Research Association, Woodmansterne Road, Carshalton, Surrey, SM5 4DS, England

(*Received 17 December 1973*)

Abstract—Di-(2-ethylhexyl) phthalate (DEHP) was degraded by the contents of the rat caecum or small intestine to a single metabolite, which was tentatively identified as mono-(2-ethylhexyl) phthalate. Cells derived from the intestinal mucosa seem to be an important source of DEHP-degrading enzymes, although there is evidence that bacterial and extracellular enzymes are also involved. The caecal and intestinal contents of Sprague–Dawley but not Wistar rats fed a diet containing DEHP metabolized the diester at greatly increased rates compared with rats fed on a standard diet. Bacterial numbers in the stomach and small intestine were reduced in the DEHP-fed rats.

INTRODUCTION

For over 20 years, di-(2-ethylhexyl) phthalate (DEHP) has been widely used as a plasticizer, notably in medical devices made from polyvinyl chloride, in which it contributes 20–40% of the final weight of the plastics material (Jaeger & Rubin, 1973). Research into the toxic effects of DEHP has intensified since the discovery that this ester was extracted from PVC blood-storage bags by blood at a rate of 0·25 mg/100 ml blood/day (Jaeger & Rubin, 1970). Of the phthalate esters used in plastics, DEHP appears to be one of the least toxic, although Rubin & Jaeger (1973) and Stein, Caasi & Nair (1973) have reported that it has subtle pharmacological and toxicological effects in mice and rats.

Until recently, information on the metabolism of DEHP was sparse, but Schulz & Rubin (1973) have demonstrated its rapid metabolism and excretion after iv administration, and subsequently Albro, Thomas & Fishbein (1973) isolated and identified several urinary metabolites of DEHP after oral administration of the ester to rats. The site of production of these metabolites has not been identified and because at least three major sites of xenobiotic transformation are known, namely the liver, intestinal wall and intestinal contents (Conney, 1967; Gillette, 1966; Scheline, 1968; Wattenberg, Leong & Strand, 1962; Williams, 1972), it was thought essential to explore which of these sites was primarily responsible for the metabolism of DEHP. In the present paper, the degradation of [^{14}C]DEHP by the intestinal contents of rats is described and the effects of orally administered DEHP on the microbial flora of the rat alimentary tract are reported.

EXPERIMENTAL

Materials. DEHP and mono-(2-ethylhexyl) phthalate were obtained from BP Chemicals International Ltd., Epsom, Surrey, and di-(2-ethylhexyl) phthalate labelled with ^{14}C in the carboxyl group ([^{14}C]DEHP) was purchased from the Radiochemical Centre, Amersham, Bucks. The Boehringer Corporation (London) Ltd., Ealing, supplied tetracycline hydrochloride, streptomycin sulphate and chloramphenicol. Neomycin sulphate was bought from the Sigma Chemical Co., Kingston-upon-Thames, Surrey, and silica gel HF_{254} from E. Merck AG, Darmstadt. Nuclear Enterprises Ltd., Edinburgh, supplied 2,5-diphenyloxazole (PPO) and *p*-bis-(5-phenyloxazol-2-yl)benzene (POPOP). Bacteriological media were bought from Oxoid Ltd.

Animals and diets. Male Wistar and Sprague–Dawley rats, weighing approximately 300 g, were maintained for up to 16 wk on water and Spillers' Laboratory Small Animal Diet given *ad lib.*, the latter being supplemented in some experiments with 2% (w/w) DEHP. Rats fed on the DEHP diet were transferred to the standard diet for 5 days before being killed, to reduce the concentration of DEHP in the alimentary tract.

Incubation with suspensions of gut contents. The animals were killed, at the same hour each day, by a blow on the head, and the alimentary tract was ligatured to separate the stomach, small intestine and caecum. The contents of the three regions were each expressed into 1-oz McCartney bottles, weighed and dispersed in 4 vols medium, which contained 0·2 M-potassium phosphate buffer (pH 7·0), 25% (v/v) Ringers solution and 1% (w/v) D-glucose. In some experiments the pH 7·0 buffer was replaced by 0·2 M-sodium acetate/acetic acid buffer (pH 4·0 or 5·0).

The resulting suspensions of material from stomach, caecum or small intestine were centrifuged at 16 **g** for 1 min to remove large debris. An aliquot (5 ml) of each supernatant was transferred to a 15 ml screwcap bottle containing 0·05 ml [^{14}C]DEHP (1·15 μCi/μmol; 100 mg/ml, prepared in absolute alcohol). The bottles were incubated at 37°C under a stream of oxygen-free nitrogen (British Oxygen Co. Ltd., London), which in addition to providing an anaerobic atmosphere ensured that the DEHP (final concentration 1 mg/ml) was adequately dispersed.

At the end of the incubation (16 hr), the mixture was acidified to pH 2 with 5 N-hydrochloric acid, saturated with sodium chloride and extracted three times with 5 ml ethyl acetate (Ogata, Tomokuni & Takatsuka, 1969).

In some experiments, the caecal or intestinal suspensions were heated (at 100°C for 25 min) or were centrifuged (at 10,000 **g** for 15 min at 4°C), the supernatants being passed through Sartorius membrane filters (25 mm in diameter, 0·2 μm pore size) before the addition of [^{14}C]DEHP. The effect of antibiotics on DEHP breakdown was studied by adding tetracycline hydrochloride, streptomycin sulphate, chloramphenicol and neomycin sulphate to the incubation mixture each at a final concentration of 2 mg/ml.

Chromatography

The ethyl acetate extracts were applied to plates of silica gel HF_{254} (0·25 mm in thickness) and the chromatograms were developed using the solvent system *n*-hexane–ethylacetate, 90:10, v/v (Nematollahi, Guess & Autian, 1967). The zones on the developed chromatograms were detected by ultraviolet light, scraped off into vials containing 10 ml of the scintillant PPO (5 g/litre) and POPOP (0·3 g/litre) in toluene with 4% (w/v) Cab-o-Sil, and counted using a Nuclear Chicago Mark 1 liquid scintillation counter. On some of the chromatograms, areas outside the zones that fluoresced under UV light were scraped off and

counted for radioactivity to determine whether any further radioactive metabolites, invisible under UV light, had been formed.

In some experiments, the radioactive material remaining at the origin of chromatograms developed in *n*-hexane–ethyl acetate was collected and eluted with diethyl ether and applied to plates of silica gel HF_{254}, which were developed in 95% (v/v) ethanol–water–NH_4OH, 100:12:16 by vol. (Braun & Geenen, 1962) or chloroform–methanol–acetic acid, 143:7:2 by vol. (Albro *et al.* 1973).

Gastro-intestinal flora

Viable counts of bacteria in the alimentary tract of the rat were performed as described by Drasar (1967), except that numbers of *Streptococcus faecalis* were estimated using the methylene-blue medium of Schaedler, Dubos & Costello (1965) and bifidobacteria and bacteroides were counted using the china-blue/horse-blood medium described by Van der Wiel-Korstanje & Winkler (1970). Strictly anaerobic bacteria were counted in roll tubes (Astell Laboratory Service Co., London) using a modification of the technique of Hungate (1950).

RESULTS

Spontaneous breakdown of DEHP under anaerobic conditions at acid, neutral or slightly alkaline pH was negligible (Table 1). However, when incubated with the contents of the small intestine or caecum of Wistar rats, [^{14}C]DEHP was degraded at the rate of approximately 1300 or 700 μg/g intestinal or caecal contents respectively/16 hr (Table 1). The maximum rate of degradation occurred at pH 7·0, which is close to the pH values observed for the caecal and small intestinal contents, i.e. pH 6·7 and 6·8 respectively (I. R. Rowland, unpublished observation 1973). At pH 4·0, the value recorded for the stomach contents, DEHP was not metabolized, and even when the pH was raised by buffer to 7·0 the amount of ester degraded by the stomach contents was negligible compared with that degraded by the caecal or intestinal contents (Table 1).

Table 1. *[^{14}C]DEHP metabolism by gut contents of Wistar rats*

Region of gut	pH of incubation*	Metabolite formed* (μg/g gut contents/16 hr) in experiment no. 1	2
Control (buffer only)	4·0	1	2
	5·0	4	7
	7·0	5	7
	7·4	17	27
Stomach	4·0	4	6
	5·0	17	20
	7·0	34	80
Small intestine	7·0	1170	1395
Caecum	7·0	565	775

*The contents of stomach, small intestine or caecum (1 g wet weight/5 ml incubation mixture) were incubated for 16 hr at 37°C with [^{14}C]DEHP (1 mg/ml; 1·15 μCi/μmol) in 0·2 M-potassium phosphate buffer, pH 7·0 (or 0·2 M-sodium acetate/acetic acid buffer, pH 4·0 or 5·0) containing 25%-strength Ringers solution and 1% (w/v) D-glucose. After 16 hr, the incubation mixture was extracted with ethyl acetate and the amount of metabolite formed was calculated from the radioactivity remaining at the origin of chromatograms of the ethyl acetate extract developed in *n*-hexane–ethyl acetate (90:10).

Table 2. [^{14}C]*DEHP metabolism by gut contents of Wistar rats*

Region of gut	Treatment	Metabolite formed* (μg/g gut contents/16 hr) in experiment no.		
		1	2	3
Small intestine	None	1170	1395	—
	Heat (100°/25 min)	5	3	—
	Antibiotics†	505	530	—
	Filtration‡	605	675	900
Caecum	None	565	775	—
	Heat (100°/25 min)	7	3	—
	Antibiotics†	265	330	—
	Filtration‡	20	27	—

*Calculated from the radioactivity remaining at the origin of chromatograms of the ethyl acetate extract developed in *n*-hexane–ethyl acetate (90:10).

†Tetracycline hydrochloride, neomycin sulphate, chloramphenicol and streptomycin sulphate, each at 2 mg/ml final concentration, were added to the incubation mixture.

‡The caecal or small intestinal contents, suspended in 4 vols buffer, pH 7·0, was centrifuged (10,000 g for 15 min at 4°C) and the supernatant was passed through a Sartorius membrane filter (0·2 μm pore size).

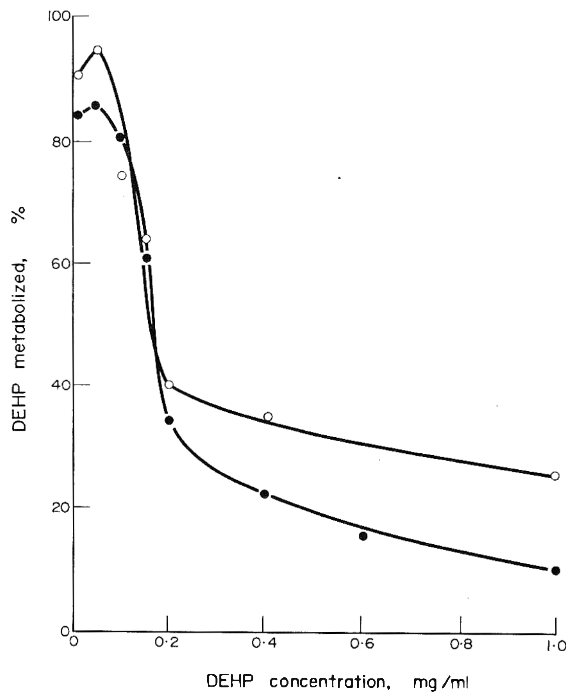

Fig. 1. Extent of DEHP metabolism when the contents of the caecum (●) or small intestine (○) suspended at a density of 0·2 g/ml in 0·2 M-potassium phosphate buffer (pH 7·0), 25% (v/v) Ringers solution and 1% D-glucose were incubated for 16 hr at 37°C with [^{14}C]DEHP (1·15 μCi/μmol) in the concentrations shown.

The ability of the caecal contents to metabolize DEHP was abolished by boiling the caecal suspension before use or by passing the suspension through a Sartorius membrane filter. When antibiotics were present in the incubation mixture, the rate of metabolism of DEHP appeared to be reduced from about 700 to 300 μg/g caecal contents/16 hr (Table 2). It may be noted that neomycin or tetracycline (2 mg/ml) alone lowered the rate of breakdown of DEHP by the caecal contents, but the effect was not as marked as with the mixture of four antibiotics. The presence of antibiotics had a similar effect on the degradation of DEHP by the contents of the small intestine. On the other hand, filtration of a suspension of intestinal contents reduced, but did not entirely abolish, the ability of the suspension to degrade DEHP (Table 2).

Table 3. [^{14}C]*DEHP metabolism by Sprague–Dawley rats fed a standard diet or diet containing 2% (w/w) DEHP for 16 wk*

Region of gut	Diet	Treatment of contents	Metabolite formed* (μg/g gut contents/16 hr) in experiment no. 1	2
Small intestine	Standard	None	930	1030
	DEHP	None	3075	3250
		Antibiotics†	3135	3520
		Filtration‡	715	830
Caecum	Standard	None	175	315
	DEHP	None	535	645
		Antibiotics†	723	803

*Calculated from the radioactivity remaining at the origin of chromatograms of the ethyl acetate extract developed in *n*-hexane–ethyl acetate (90:10).

†Tetracycline hydrochloride, neomycin sulphate, chloramphenicol and streptomycin sulphate, each at 2 mg/ml final concentration, were added to the incubation mixture.

‡The gut contents, suspended in 4 vols buffer, pH 7·0, were centrifuged (10,000 **g** for 15 min at 4°C) and the supernatant was passed through a Sartorius membrane filter (0·2 μm pore size).

At a DEHP concentration of 1 mg/ml, only 10–25% of the diester was metabolized in 16 hr by 1 g of caecal or small intestinal contents. However, at lower concentrations (below 0·1 mg/ml) virtually all added DEHP was degraded (Fig. 1).

When Sprague–Dawley rats were fed a diet containing 2% (w/w) DEHP for 16 wk, the rate of breakdown of DEHP by caecal and intestinal contents was increased approximately threefold (Table 3). The increase in rate was most marked in the contents of the small intestine, over 60% of added DEHP being metabolized by such material from DEHP-fed rats compared with only 18% by the intestinal contents of rats fed on the standard diet.

Filtration of the intestinal contents from DEHP-fed rats reduced the rate of degradation of DEHP to approximately the value recorded for rats fed on the standard diet, whereas treatment of the incubation mixture with antibiotics had no effect on the rate of DEHP breakdown by either the caecal or intestinal contents (Table 3).

It was found that 4 wk on the DEHP diet was sufficient for maximum induction of the DEHP-degrading enzyme(s) in Sprague–Dawley rats. However, in the Wistar strain even after 16 wk on the DEHP diet, no increase in the rate of DEHP metabolism by gut contents was observed.

Attempts were made to determine whether pure strains of bacteria isolated from the rat alimentary tract could degrade DEHP. Representatives of each of the major groups of bacteria found in the gut, i.e. bacteroides, bifidobacteria, lactobacilli, streptococci, *Escherichia coli*, Proteus and staphylococci, were grown in nutrient broth (for aerobes), reinforced clostridial medium (for anaerobes) or MRS broth (for lactobacilli). The cultures were centrifuged at 10,000 **g** for 15 min at 4°C and the cells were resuspended in the 0·2 M-buffer–Ringers solution (pH 7·0) described earlier (p. 294) at a density equivalent to 2×10^9 viable cells/ml. Such bacterial suspensions metabolized DEHP (1 mg/ml) at a maximum rate of only 10 μg/ml of culture medium/16 hr. The diester was metabolized at a similar rate when added directly to the growing cultures of bacteria. Attempts to isolate bacteria able to grow in a medium containing DEHP as the sole carbon-energy source were also unsuccessful.

The products of metabolism of DEHP by the caecal and intestinal contents were not fully identified. After chromatography in the solvent system *n*-hexane–ethyl acetate (90:10), the radioactivity in the ethyl acetate extract was distributed entirely between two fluorescent zones on the chromatogram, one remaining at the origin, the other migrating at the same rate as a DEHP standard. The radioactive material at the origin, after elution with diethyl ether, migrated in the solvent systems 95% ethanol–water–NH_4OH (100:12:16) and chloroform–methanol–acetic acid (143:7:2) as a single zone with R_F values of 0·90 and 0·80 respectively. The radioactive material that migrated with the DEHP standard in the *n*-hexane–ethyl acetate solvent could not be resolved into more than one component by chromatography in either of the other two solvent systems. In addition, radioactivity associated with areas outside the two major fluorescent zones on plates developed in *n*-hexane–ethyl acetate was at or near the background level. When a sample of mono-(2-ethylhexyl) phthalate was co-chromatographed with the metabolite, both migrated at the same rate in all three solvent systems described above, indicating that the metabolite was probably the monoester.

Sprague–Dawley but not Wistar rats fed a diet containing 2% (w/w) DEHP for 16 wk showed distinct changes in the microbial flora of the alimentary tract compared with rats

Table 4. *Effect of DEHP on flora of stomach and small intestine of Sprague-Dawley rats*

	No. of viable organisms ($\log_{10}$/g sample)			
	Stomach		Proximal small intestine	
Organism	Control*	DEHP-fed†	Control*	DEHP-fed†
Enterobacteria	3·3–4·4	<2	3·0–5·0	<2–3·9
Staphylococci	3·5–4·6	<2	3·7–4·8	<2–3·3
Streptococcus faecalis	4·7–5·8	<2–4·9	4·5–5·5	<2–5·1
'Viridans' streptococci	4·5–5·3	<2	4·3–6·7	<2
Lactobacilli	8·0–8·6	6·0–8·1	8·3–8·6	5·0–8·1
Yeast	<2	<2	<2	<2
Clostridia	<2	<2	<2	<2
Bifidobacteria	7·9–8·9	6·7–8·3	7·5–8·5	7·2–8·8
Bacteroides	7·7–8·7	6·7–7·4	7·5–8·5	6·9–8·4
Total ...	8·1–9·1	7·1–8·3	8·3–8·6	7·2–8·8

*Values shown indicate the range for seven animals fed on standard diet.

†Values shown indicate the range for three animals fed for 16 wk on standard diet containing 2% (w/w) DEHP.

fed on the standard diet (Table 4). The changes were confined to the upper section of the alimentary tract, i.e. the stomach and the region of the small intestine proximal to the stomach. In both regions the total number of bacteria was lower by a factor of ten in the DEHP-treated rats. More striking were the qualitative differences in microbial flora: in the control rats the stomach and proximal small intestine harboured a wide variety of bacterial types (Table 4; see also Draser, Hill & Williams, 1970), whereas the flora of the stomach and proximal small intestine of the DEHP-fed rats was composed almost entirely of bifidobacteria, bacteroides and lactobacilli. Aerobic organisms such as enterobacteria, streptococci and staphylococci were present in greatly reduced numbers compared with their count in control animals. It must be noted that the method of enumeration of bacteria did not allow numbers fewer than 10^2/g intestinal contents to be detected. In the distal region of the small intestine and in the caecum, differences between the flora of the control and DEHP-fed rats were negligible.

DISCUSSION

The contents of the caecum or small intestine of the rat convert [^{14}C]DEHP to only one radioactive metabolite, which was tentatively identified by TLC in three different solvent systems as mono-(2-ethylhexyl) phthalate. The maximum rate of metabolism of DEHP was faster in the contents of the small intestine than in that of the caecum, although at DEHP concentrations below 100 μg/ml both the caecal and intestinal contents converted virtually all added diester to the monoester in 16 hr. In contrast, the products of metabolism of DEHP by the rat *in vivo* are quite different. Schulz & Rubin (1973) found that virtually all of an iv-administered dose of DEHP in the rat was excreted, in the form of four water-soluble metabolites, in the faeces and urine. Subsequently, Albro *et al.* (1973) isolated and identified five metabolites of DEHP in the urine of rats that had been fed the ester. In both of the above investigations, mono-(2-ethylhexyl) phthalate was not found as a metabolite. However, the DEHP metabolites identified by Albro *et al.* (1973) were all derivatives of mono-(2-ethylhexyl) phthalate, so it would seem that only the first step in DEHP breakdown occurs in the alimentary tract and that the monoester is metabolized further at other sites, probably in the liver.

It is possible that under aerobic conditions some or all of the DEHP metabolites found by Albro *et al.* (1973) may be formed by the intestinal contents. However the very low redox potential of the rat alimentary tract renders this possibility highly unlikely *in vivo* (Schröder & Johansson, 1973).

The degradation of DEHP cannot be ascribed solely to the metabolic activity of the bacterial flora of the alimentary tract, since in the presence of antibiotics the contents of the caecum and small intestine still metabolized DEHP, although at only about 50% of the control rate. In addition, the inability of pure cultures of bacteria to degrade DEHP suggests that the gut flora plays only a minor role in the metabolism of the diester in the alimentary tract. Possibly extracellular enzymes and enzymes associated with cells of the intestinal mucosa are involved, the former being indicated by the fact that some DEHP-degrading activity remains after the intestinal contents are passed through a membrane filter (Table 2).

Feeding Sprague–Dawley rats on a diet containing DEHP markedly increased the rate of breakdown of the diester by the caecal or intestinal contents. The increase appeared to be associated with a cellular component of the gut contents, since membrane filtration of the intestinal contents reduced the rate of degradation of DEHP to the value for control

rats fed on the standard diet (Table 3). On the other hand, treatment of the contents of the small intestine or caecum with antibiotics during the incubation did not affect the rate of DEHP metabolism, a finding which suggests that the increase in DEHP metabolism after the feeding of a diet containing the diester was not due to a change in the metabolic activity of the intestinal flora. In fact there was a marked decrease in the numbers and types of bacteria in the upper regions of the alimentary tract of Sprague–Dawley rats fed on the DEHP diet (Table 4).

It is interesting to note that both the increase in rate of DEHP degradation and the change in microbial flora in the alimentary tract after feeding DEHP did not occur in Wistar rats.

The microbial flora of the alimentary tract of the rat has been extensively studied by several groups, including Draser *et al.* (1970), Raibaud, Dickinson, Sacquet, Charlier & Mocquot (1966) and Smith (1965). The intestinal flora of the Sprague–Dawley rats used in the present investigation was essentially similar to that reported by Draser *et al.* (1970) in that the predominant organisms in all parts of the alimentary tract were the anaerobic bifidobacteria and bacteroides and the lactobacilli, with the aerobic organisms such as the enterobacteria, staphylococci and streptococci forming a much smaller proportion of the total population. Clostridia and yeasts were not found. In the stomach and proximal small intestine of the DEHP-fed rats, the total bacterial count was slightly lower than that of the control rats, but whereas in the latter the flora consisted of a wide variety of bacterial types, the stomach and intestinal flora of the DEHP-treated animals consisted almost exclusively of bacteroides, bifidobacteria and lactobacilli.

Thus the results of the enumeration of bacterial types in the intestinal tract support the conclusion that bacterial members of the gut flora are not responsible for the increased ability of DEHP-fed rats to degrade DEHP. It is possible that the increase is due to enzyme induction in the mucosal cells of the intestine. The fact that increased hydrolysis occurred in the gut contents is compatible with this hypothesis as the turnover of epithelial cells of the mucosa is rapid, their turnover rate in the rat ileum being 1·35 days (Leblond & Stevens, 1948).

Although not likely to be involved in enzyme induction, the basal level of DEHP degradation may also involve intestinal lipases, as indicated by Albro & Thomas (1973).

Recently, B. G. Lake (personal communication 1973) has demonstrated that mono-(2-ethylhexyl) phthalate (1426·5 mg/kg administered daily to rats for 7 days by gastric intubation) produces a type of liver damage virtually identical to that caused by equimolar amounts of the diester, notably liver enlargement accompanied by depression of the activity of several mitochondrial and microsomal enzymes, such as succinate dehydrogenase and glucose-6-phosphatase. Thus the intestinal hydrolysis of the diester to the monoester may be an important factor in the hepatotoxic effect associated with DEHP.

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Métabolisation du phtalate de di-(2-éthylhexyle) par le contenu du tractus intestinal du rat

Résumé—Le contenu du caecum ou de l'intestin grêle du rat dégrade le phtalate de di-(2-éthylhexyle) (DEHP) en un métabolite unique, identifié expérimentalement comme phtalate de mono-(2-éthylhexyle). Des cellules provenant de la muqueuse intestinale semblent être une source importante d'enzymes dégradant le DEHP, mais l'intervention d'enzymes bactériens et extracellulaires est également prouvée. Le contenu du caecum et de l'intestin grêle de rats Sprague-Dawley—mais non de rats Wistar—soumis à un régime alimentaire comportant du DEHP a métabolisé le diester dans des proportions beaucoup plus élevées que chez des rats soumis à un régime standard. Les nombres de bactéries présentes dans l'estomac et dans l'intestin grêle avaient diminué chez les rats qui avaient consommé le DEHP.

Stoffwechsel von Di-(2-äthylhexyl)phtalat durch den Inhalt der Magendarmtrakt der Ratte

Zusammenfassung—Di-(2-äthylhexyl)phtalat (DEHP) wurde durch den Inhalt des Rattenblind- darms oder -dünndarms abgebaut zu einem einzigen Abbauprodukt, welches versuchsweise als Mono-(2-äthylhexyl)phtalat identifiziert wurde. Zellen, die aus dem Darmschleim gewonnen wurden, scheinen eine wichtige Quelle für DEHP-abbauende Enzyme zu sein, trotzdem Beweise vorhanden sind, daß bakterielle und extrazelluläre Enzyme auch mitspielen. Der Blinddarm und Darminhalt von Sprague–Dawley aber nicht Wistar Ratten, die mit DEHP-haltiger Kost gefüttert wurden, baute den Diester viel schneller ab, verglichen mit standardkostgefütterten Ratten. An DEHP-gefütterten Ratten war die Bakterienzahl in Magen und Darm reduziert.

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ACTIVITE ENZYMATIQUE, AU NIVEAU DES MICROSOMES HEPATIQUES DU RAT, APRES ADMINISTRATION D'ACIDES PHENOLIQUES

D. GAILLARD, S. MITJAVILA et R. DERACHE

Groupe de Recherches sur la Toxicologie des Aliments et des Boissons, INSERM-U87, Institut de Physiologie, 84 Grande Rue Saint-Michel, 31400 Toulouse, France

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Résumé—L'activité enzymatique au niveau des microsomes hépatiques est évaluée après traitements de rats femelles à l'acide gallique, au gallate d'éthyle, à l'acide syringique, à l'acide vératrique, à l'acide vanillique et à l'acide 3,4,5-triméthoxybenzoïque. Ces substances, administrées par voie ip à la dose de 150 mg/kg/jour, à 24 hr d'intervalle, pendant 4 jours ne produisent aucun effet sur l'activité enzymatique microsomale. Par contre, l'acide tannique, à la dose de 25 mg/kg/jour pendant 4 jours inhibe significativement l'hydroxylation aromatique de l'aniline et la *N*-déméthylation du pyramidon mais il n'a pas d'effet significatif sur l'*O*-déméthylation du *p*-nitroanisole et sur la concentration en cytochrome *P*-450. Il y a simultanément prolongation *in vivo* du temps de sommeil des rats traités au pentobarbital. Ces résultats mettent en évidence une altération, dans ces conditions expérimentales, au niveau subcellulaire, corroborée par une diminution du taux des protéines microsomales. Mais après administration orale d'acide tannique à la dose de 500 mg/kg/jour pendant 4 jours il n'y a aucune modification de l'activité des enzymes microsomales.

INTRODUCTION

Beaucoup de substances contenues dans les aliments, composés ajoutés intentionnellement comme les additifs, ou polluants tels les pesticides ou encore produits naturels ne possédant pas de propriétés nutritives, sont absorbées journellement par l'homme et par l'animal. Ces substances, ne couvrant pas les besoins nutritionnels essentiels comme les besoins de croissance ou caloriques, sont métabolisées par une grande diversité d'enzymes localisées au niveau des microsomes hépatiques. Il est bien connu aujourd'hui que l'activité des enzymes microsomales de détoxication peut être stimulée ou inhibée par de nombreux médicaments, pesticides, additifs alimentaires et hydrocarbures cancérigènes de l'environnement entre autres (Conney, 1967; Gaillard et Derache, 1972). Ces substances peuvent perturber non seulement leur propre métabolisme mais aussi celui d'autres composés (Fouts, 1964), et il faut noter que très peu d'études portent sur les substances naturelles. Quelques rares exemples sont connus: la caféine (Mitoma, Lombrozo, Le Valley et Dehn, 1969; Mitoma, Sorich et Neubauer, 1968), quelques terpènes (Parke et Rahman, 1969) et le safrole et l'isosafrole (Parke et Rahman, 1971) sont des inducteurs de la détoxication par les microsomes hépatiques alors que certains acides gras à longue chaîne et leurs dérivés contenus dans les huiles végétales alimentaires sont des inhibiteurs (Gaillard, Pipy et Derache, 1974).

A la suite de ces données, nous nous sommes demandés si des acides phénoliques et composés polyphénoliques que l'on trouve abondamment dans les aliments (végétaux divers et fruits) ou dans les boissons (vin, thé et café) ne peuvent pas jouer un rôle dans les phénomènes de détoxication enzymatique hépatique.

Dans le présent travail, nous nous sommes donc proposés d'étudier les effets des acides gallique, syringique, vératrique, vanillique, 3,4,5-triméthoxybenzoïque et tannique ainsi

que du gallate d'éthyle sur le système de détoxication localisé au niveau des microsomes hépatiques. Nous nous sommes également demandés si des modifications éventuelles ne pouvaient pas être liées à des variations des constituants essentiels de la cellule hépatique: protéines et acide ribonucléique.

METHODES EXPERIMENTALES

Animaux et traitement

Nous avons utilisé des lots de 16 rats femelles OFA (Sprague–Dawley) âgés de 40–60 jours; un aliment complet (provenance UAR) et l'eau sont donnés *ad lib.* Les rats de chaque groupe reçoivent pendant 4 jours, à 24 hr d'intervalle, une injection ip (150 mg/kg) d'acide gallique (acide 3,4,5-trihydroxybenzoïque), de gallate d'éthyle, d'acide syringique (acide 3,5-diméthoxy-4-hydroxybenzoïque), d'acide vératrique (acide 3,4-diméthoxybenzoïque), d'acide vanillique (acide 4-hydroxy-3-méthoxybenzoïque) et d'acide 3,4,5-triméthoxybenzoïque. Ces substances sont solubilisées (1,5 g/100 ml), sous forme de sel de sodium, dans du chlorure de sodium à 0,9% à pH 7. Une solution d'acide tannique pur (E. Merck AG. Darmstadt), neutralisée à pH 7, est administrée soit par voie ip à la dose de 25 mg/kg/jour, à 24 hr d'intervalle, pendant 4 jours (solution à 0,250%) soit par voie orale à la dose de 500 mg/kg/jour (solution à 5%). Les lots témoins reçoivent parallèlement la solution de chlorure de sodium équivalente.

Vingt heures après la dernière administration, on mesure la durée d'action du pentobarbital de sodium, injecté à la dose de 30 mg/kg; celle-ci est évaluée par le temps de sommeil de l'animal, témoin de son métabolisme. Le réflexe de redresement sert de test de réveil.

Une heure après le réveil, les animaux sont sacrifiés par décapitation; le foie est prélevé immédiatement, pesé et broyé au moyen d'un Potter dans 4 vols de tampon phosphate de potassium (0.1 M, pH 7,4) glacé. Une première centrifugation à 9000 g, pendant 15 min à 4°C, permet d'éliminer les mitochondries, les noyaux et les débris cellulaires. Le surnageant est alors centrifugé 4°C à 105.000 g pendant 1 hr (ultra-centrifugeuse MSE). Le culot, contenant les microsomes, est mis en suspension dans le tampon phosphate glacé de façon à avoir une concentration en protéines de l'ordre de 8 mg/ml.

Déterminations biochimiques

On détermine les taux de protéines microsomales par une méthode à la bromosulfophtaléine (Paul, 1961) et de l'acide ribonucléique (ARN) microsomal selon la technique de Wannemacher, Banks et Wunner (1965). Les standards de protéines sont effectués avec de la sérum albumine humaine, fraction V (Nutritional Biochemicals Corp., Cleveland, Ohio); l'ARN est évalué par rapport à de l'ARN de foie de mouton (Choay, Paris). La teneur en cytochrome *P*-450 est également déterminée (Omura et Sato, 1964).

L'activité des enzymes microsomales est estimée après incubation dans un milieu contenant 0,75 μM de triphosphopyridine nucléotide, 50 μM de glucose-6-phosphate, 0,5 UI de glucose-6-phosphate déshydrogénase, 25 μM de chlorure de magnésium, 100 μM de nicotinamide, 5 μM de diphosphopyridine nucléotide réduit et 290 μM de tampon phosphate de potassium, pH 7,4. Les concentrations en substrats sont de 5 μM pour l'aniline et le pyramidon et de 3 μM pour le *p*-nitroanisole. La quantité de protéines microsomales présente dans les 6 ml de milieu d'incubation est approx. de 20 mg. L'incubation est menée, pendant 30 min sous agitation (150 oscillations/min), dans un appareil Gallenkamp thermostaté à 37°C, l'air servant de phase gazeuse.

Tableau 1. *Effet de divers acides phénoliques, administrés par voie ip pendant 4 jours sur les poids du foie et sur les taux des protéines et de l'ARN des microsomes hépatiques du rat*

Traitements	Dose (mg/kg/jour)	Poids corporel (g)	Poids du foie (g/100 g poids corporel)	Protéines microsomales (mg/g foie)	ARN microsomal (mg/g foie)
Témoins	—	152,9 ± 2,2 (16)	4,31 ± 0,08 (16)	19,58 ± 0,57 (15)	3,34 ± 0,12 (16)
Acide gallique	150	148,1 ± 2,5 (16)	4,24 ± 0,06 (16)	19,05 ± 0,56 (16)	3,39 ± 0,08 (16)
Gallate d'éthyle	150	153,9 ± 2,3 (16)	4,16 ± 0,08 (16)	20,43 ± 0,37 (16)	3,38 ± 0,06 (16)
Acide syringique	150	150,6 ± 3,5 (16)	4,20 ± 0,03 (16)	19,61 ± 0,27 (16)	3,45 ± 0,08 (16)
Acide vératrique	150	155,5 ± 2,8 (16)	4,12 ± 0,05 (16)	20,00 ± 0,34 (16)	3,31 ± 0,05 (15)
Acide vanillique	150	151,8 ± 2,1 (16)	4,39 ± 0,10 (16)	10,68 ± 0,47 (16)	3,18 ± 0,07 (16)
Acide 3,4,5-triméthoxybenzoïque	150	157,0 ± 2,1 (16)	4,16 ± 0,07 (16)	20,52 ± 0,47 (16)	3,49 ± 0,07 (14)
Acide tannique	25	143,8 ± 2,3 (16)**	3,94 ± 0,10 (16)*	18,24 ± 0,24 (15)*	3,55 ± 0,10 (16)

Les valeurs représentent la moyenne ± l'erreur standard. Celles marquées avec des astériques diffèrent significativement (test de *t* de Student) par rapport au lot témoin: $*P < 0{,}05$; $**P < 0{,}01$. Les chiffres entre parenthèses indiquent le nombre d'animaux ayant servi à l'étude correspondante.

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Les quantités de métabolites formés à partir des trois substrats utilisés sont ensuite déterminées. L'hydroxylation aromatique de l'aniline est évaluée par l'apparition de *p*-aminophénol (Kato et Gillette, 1965), l'*O*-déméthylation du *p*-nitroanisole est estimée par la formation de *p*-nitrophénol (Kato et Gillette, 1965) et la *N*-déméthylation du pyramidon est déterminée par la formation de 4-aminoantipyrine (La Du, Gaudette, Trousof et Brodie, 1955). Les résultats sont exprimés en nanomoles de métabolite formé, en 30 min d'incubation, par mg de protéines microsomales. Les comparaisons sont faites entre les animaux témoins et les animaux traités (test de *t* de Student).

RESULTATS

Effets de divers acides phénoliques

Les résultats donnés dans les tableaux 1, 2 et 3 montrent que l'acide gallique, le gallate d'éthyle, l'acide syringique, l'acide vératrique, l'acide vanillique et l'acide 3,4,5-triméthoxybenzoïque administrés à la dose de 150 mg/kg/jour pendant 4 jours, à 24 hr d'intervalle, ne produisent pas de modifications au niveau microsomal dans nos conditions expérimentales; les taux des protéines et de l'ARN des microsomes hépatiques ne varient pas comparativement au lot témoin. Les activités enzymatiques *in vivo* du métabolisme du pentobarbital et *in vitro* de l'hydroxylation aromatique de l'aniline, de la *N*-déméthylation du pyramidon et de l'*O*-déméthylation du *p*-nitroanisole ne sont pas modifiées par rapport à celles des témoins. Les divers traitements n'ont également pas d'influence sur le taux du cytochrome *P*-450 des microsomes.

Effet de l'acide tannique

L'acide tannique, administré par voie ip à une dose six fois moins forte, provoque une diminution de 6% du poids des animaux traités par rapport aux témoins ainsi que du poids du foie pour 100 g de poids corporel de 8,5%. Il y a également chute du taux des protéines microsomales de 7% alors que celui de l'ARN n'est pas modifié (Tableau 1).

Le tableau 2 permet de constater que l'acide tannique provoque une augmentation très nette du temps de sommeil induit par le pentobarbital de sodium (50%) témoignant ainsi une plus lente métabolisation *in vivo* de ce barbiturique chez les rats traités comparativement aux témoins.

Les résultats rapportés dans le tableau 3 montrent que l'hydroxylation aromatique de l'aniline est fortement diminuée chez les rats soumis à l'acide tannique (de 30%); il en est

Tableau 2. *Effet de divers acides phénoliques, administrés par voie ip, pendant 4 jours, sur le temps de sommeil provoqué par le pentobarbital de sodium (30 mg/kg) chez le rat*

Traitements	Dose (mg/kg/jour)	Durée du sommeil (min)
Témoins	—	67 ± 3
Acide gallique	150	72 ± 2
Gallate d'éthyle	150	64 ± 3
Acide syringique	150	74 ± 4
Acide vératrique	150	75 ± 4
Acide vanillique	150	64 ± 2
Acide 3,4,5-triméthoxybenzoïque	150	65 ± 1
Acide tannique	25	140 ± 8**

Les valeurs représentent la moyene ± l'erreur standard. Celle marquée avec des astérisques diffère significativement (test de *t* de Student) par rapport au lot témoin: $^{**}P < 0{,}01$. Chaque lot expérimenté comprend 16 rats.

Tableau 3. *Effets de divers acides phénoliques administrés par voie ip pendant 4 jours, sur les métabolismes de l'aniline, du pyramidon et du p-nitroanisole et sur le taux du cytochrome* P-*450, au niveau des microsomes hépatiques du rat*

Traitements	Dosage (mg/kg/jour)	Activité enzymatique (nmoles de métabolite formé/mg protéines microsomales/30 min) — Aniline (hydroxylation aromatique)	Pyramidon (*N*-déméthylation)	*P*-Nitroanisole (*O*-déméthylation)	Cytochrome *P*-450 (ΔDO 450–490 nm/mg protéines microsomales)
Témoins	—	11,41 ± 0,73 (8)	4,73 ± 0,10 (8)	11,79 ± 0,62 (8)	0,045 ± 0,004 (16)
Acide gallique	150	13,02 ± 0,92 (8)	5,10 ± 0,19 (8)	12,01 ± 0,27 (8)	0,047 ± 0,002 (16)
Gallate d'éthyle	150	9,83 ± 0,78 (8)	5,22 ± 0,41 (8)	10,29 ± 0,54 (8)	0,049 ± 0,004 (16)
Acide syringique	150	11,75 ± 0,19 (8)	4,25 ± 0,42 (8)	12,99 ± 0,62 (8)	0,054 ± 0,004 (15)
Acide vératrique	150	12,90 ± 1,06 (8)	4,76 ± 0,32 (8)	12,46 ± 0,46 (8)	0,051 ± 0,004 (16)
Acide vanillique	150	11,07 ± 0,72 (8)	4,35 ± 0,13 (8)	11,40 ± 0,37 (8)	0,046 ± 0,002 (16)
Acide 3,4,5-triméthoxybenzoïque	150	9,79 ± 0,78 (8)	4,91 ± 0,26 (8)	11,91 ± 1,30 (8)	0,043 ± 0,003 (16)
Acide tannique	25	7,87 ± 0,24 (8)**	3,91 ± 0,10 (8)**	9,90 ± 0,92 (8)	0,040 ± 0,004 (16)

Les valeurs représentent la moyenne ± l'erreur standard. Celles marquées avec des astérisques diffèrent significativement (test de *t* de Student) par rapport au lot témoin: $**P < 0{,}01$. Les chiffres entre parenthèses indiquent le nombre d'animaux ayant servi à l'étude enzymatique correspondante.

de même de la *N*-déméthylation du pyramidon qui est, elle aussi, significativement inhibée de 17%. Bien que l'on observe une diminution de l'*O*-déméthylation du *p*-nitroanisole, l'inhibition n'est pas statistiquement significative en la comparant aux valeurs trouvées pour les témoins. L'acide tannique n'a pas d'influence sur le taux du cytochrome *P*-450 des microsomes.

Par contre, l'administration orale d'une dose 20 fois plus forte d'acide tannique (500 mg/kg/jour) à 24 hr d'intervalle et pendant 4 jours ne provoque aucune modification de l'activité des enzymes hépatiques dans nos conditions expérimentales, comme le montrent les résultats rapportés dans le tableau 4. Seul le poids du foie pour 100 g de poids corporel est diminué significativement de 6% par rapport aux témoins.

Tableau 4. *Activités enzymatiques au niveau des microsomes hépatiques, chez le rat, après administration orale d'acide tannique à la dose de 500 mg/kg/jour, pendant 4 jours*

Détermination	Témoins	Après traitement à l'acide tannique
Poids corporel (g)	148,9 ± 3,5 (16)	148.5 ± 3.1 (16)
Poids du foie (g/100 g poids corporel)	4,33 ± 0,05 (16)	4,08 ± 0,03 (16)**
Protéines microsomales (mg/g foie)	19,09 ± 0,43 (16)	19,13 ± 0,50 (16)
ARN microsomal (mg/g foie)	3,33 ± 0,10 (16)	3,49 ± 0,09 (16)
Durée du sommeil (min)	68 ± 2 (16)	72 ± 2 (16)
Aniline (hydroxylation aromatique)†	11,65 ± 0,46 (8)	11,11 ± 0,70 (8)
Pyramidon (*N*-déméthylation)†	5,00 ± 0,20 (8)	5,09 ± 0,26 (8)
p-Nitroanisole (*O*-déméthylation)†	11.63 ± 0,65 (8)	11,75 ± 0,63 (8)
Cytochrome *P*-450‡	0,047 ± 0,005 (16)	0,049 ± 0,004 (16)

†Exprimé en nmoles de métabolite formé/mg de protéines microsomales/30 min.
‡ΔDO 450–490 nm/mg de protéines microsomales.
Les valeurs représentent la moyenne ± l'erreur standard. Celle marquée avec des astérisques diffère significativement (test de *t* de Student) par rapport au lot témoin: $**P < 0{,}01$. Les chiffres entre parenthèses correspondent au nombre d'animaux ayant servi à l'étude correspondante.

DISCUSSION

Nos résultats montrent que seul l'acide tannique, parmi la série des acides phénoliques administrés par voie ip, a un effet inhibiteur, dans nos conditions expérimentales, sur l'activité de quelques enzymes microsomales de détoxication. Nous avons observé une prolongation du temps de sommeil induit par le pentobarbital témoignant une inhibition de son métabolisme *in vivo* et aussi une diminution de l'activité enzymatique microsomale *in vitro*. Il faut noter cependant qu'il y a une certaine spécificité dans cette action puisqu'il y a inhibition de l'hydroxylation aromatique de l'aniline et de la *N*-déméthylation du pyramidon alors que l'*O*-déméthylation du *p*-nitroanisole n'est pas diminuée significativement ainsi que le taux du cytochrome *P*-450. Ce phénomène confirme le fait que l'acide tannique pénètre dans la cellule hépatique; en effet Badawy, White et Lathe (1969) ont montré que 24 hr après injection ip d'acide tannique la majorité de cette substance se retrouve dans le noyau cellulaire, mais aussi une forte proportion est présente dans la fraction microsomale. Aussi, ne faut-il pas exclure *a priori* la possibilité d'une action directe de l'acide tannique sur les microsomes provoquant ainsi l'inhibition de l'activité enzymatique observée; mais, par contre, la chute du taux des protéines dans la fraction microsomale ne peut pas s'expliquer par ce mécanisme.

Les lésions hépatiques observées lors d'injections de solutions d'acide tannique débuteraient par la pénétration de ce polyphénol dans les hépatocytes (Cameron, Milton et Allen, 1943). A ce sujet, il est à noter que d'une part Horváth, Sólyom et Korpássy (1960) ont constaté un parallèlisme entre les lésions cellulaires et la chute du taux d'ARN qui atteint un minimum 48 hr après l'injection et d'autre part Badawy *et al.* (1969) ont localisé l'acide tannique dans le noyau cellulaire. Etant donné la nature polyanionique de ce polyphénol et le caractère cationique des acides nucléiques avec lesquels il forme des complexes insolubles (Baranowski, Kochman et Szewczuk (1963), il est probable que l'acide tannique se trouve dans le noyau intimement lié à l'ADN, interférant ainsi dans la transcription ADN–ARN (Racela, Grady et Svoboda, 1967). Le blocage de la transcription d'ARN-messager expliquerait la diminution de la synthèse protéique observée dans la cellule entière, diminution que nous avons retrouvée au niveau des protéines microsomales-conjointement à l'inhibition de l'activité de quelques enzymes présentes dans cette fraction. Ces phénomènes seraient donc le reflet d'une agression cellulaire car il est connu que lors d'intoxication hépatique le réticulum endoplasmique est altéré (Rouiller et Jézéquel, 1963).

Ces résultats seraient à rapprocher de l'inhibition de l'induction enzymatique observée avec d'autres hépatotoxiques comme l'actinomycine D (Gelboin, 1964) et l'aflatoxine (Kato, Takanaka, Onoda et Omori, 1970) qui empêcheraient toutes deux les synthèses d'ARN-messager (Clifford et Rees, 1966; Gelboin, Wortham, Wilson, Friedman et Wogan, 1966).

Cependant, à une dose 20 fois plus forte, l'acide tannique administré par voie orale ne produit pas le même phénomène au niveau des microsomes hépatiques. Ceci confirme le fait que l'acide tannique ne traverse pas la barrière intestinale en quantité suffisante pour produire des agressions au niveau du foie; il faudrait atteindre des doses de 2 g/kg de poids corporel, en une seule ingestion, pour voir apparaître une altération fonctionnelle de cet organe comme l'infiltration lipidique ou l'augmentation de l'activité de la fonction granulopéxique du système réticulo-endothélial (SRE) (Mitjavila, Gaillard et Derache, 1971).

A cet effet nous avons montré, en outre, qu'il existe une intéraction de l'activité physiologique du SRE et de la détoxication enzymatique par les microsomes hépatiques, mettant en évidence le rôle du SRE dans les mécanismes de détoxication métabolique (Gaillard *et al.* 1974). Ces altérations, réponse du SRE et diminution de l'activité enzymatique, semblent également liées à l'agression induite par des substances cytotoxiques, agression non seulement au niveau cellulaire mais aussi à l'échelle subcellulaire. Par ailleurs, on sait que sous l'action d'agents modifiant l'activité du SRE il y a rupture des membranes des lysosomes (Weissmann et Thomas, 1962) et il n'est pas exclu que les enzymes de détoxication puissent être aussi dégradées par les enzymes protéolytiques.

Avec les autres acides phénoliques étudiés il ne semble pas y avoir agression cellulaire; les activités microsomales ne sont pas modifiées, il en est de même des taux des protéines microsomales et du cytochrome *P*-450. Ces acides, de formule relativement simple, possédant un groupement carboxylique libre et des fonctions phénoliques, doivent être rapidement conjugués puis excrétés. Selon Gilbert, Martin, Gangolli, Abraham et Golberg (1969), des modifications de l'activité microsomale sont observées lorsque différentes chaînes aliphatiques ou aromatiques sont substituées au groupement phénolique de ces composés.

En conclusion, l'acide tannique administré oralement ne traverse pas la barrière intestinale en quantité suffisante pour produire des altérations au niveau microsomal. Lors d'injections intrapéritonéales, il doit vraisemblablement se comporter comme une sub-

stance cytotoxique provoquant une inhibition de la synthèse protéique ayant pour conséquence une diminution de l'activité des enzymes de détoxication. Notre expérimentation ne permet pas de définir le mécanisme par lequel se produisent les phénomènes d'inhibition et des études ultérieures sont nécessaires pour éclaircir ce problème.

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Hepatic microsomal-enzyme activity in the rat after administration of phenolic acids

Abstract—Enzyme activity at the level of the hepatic microsomes has been evaluated after treatment of female rats with gallic acid, ethyl gallate, syringic acid, veratric acid, vanillic acid or 3,4,5-trimethoxybenzoic acid. These substances, administered ip in a dose of 150 mg/kg/day at 24-hr intervals for 4 days had no effect on microsomal-enzyme activity. In contrast, tannic acid given in a dose of 25 mg/kg/day for 4 days caused a significant inhibition of aniline hydroxylation and amidopyrine *N*-demethylation but had no significant effect on *p*-nitroanisole *O*-demethylation or the concentration of cytochrome *P*-450. At the same time, pentobarbitone sleeping was prolonged *in vivo* in the treated rats. These results indicate a change at the sub-cellular level, under the conditions of the experiment, and this was supported by a reduction in the level of microsomal protein. However, after oral administration of tannic acid in a dose of 500 mg/kg/day for 4 days, there was no change in microsomal-enzyme activity.

Mikrosomale Enzymaktivität der Leber bei Ratten nach der Behandlung mit Phenolcarbonsäuren

Zusammenfassung—Nach Behandlung weiblicher Ratten mit Gallussäure, Äthylgallat, Syringinsäure, Veratrinsäure, Vanillinsäure oder 3.4.5-Trimethoxybenzoesäure wurde die Enzymaktivität im Bereich der hepatischen Mikrosomen beobachtet. Diese Substanzen, täglich ip in Dosen von 150 mg/kg über vier Tage im Abstand von 24 Stunden verabreicht, zeigten keinerlei Auswirkung auf die mikrosomale Enzymtätigkeit. Im Gegensatz dazu bewirkte die Verabreichung von Tanninsäure über einen Zeitraum von vier Tagen in Dosen von 25 mg/kg täglich eine signifikante Hemmung der Anilin-Hydroxylierung und Amidopyrin-*N*-Demethylierung, hatte jedoch keine signifikante Wirkung auf die *p*-Nitroanisol-*O*-Demethylierung oder die Konzentration von Cytochrom *P*-450. Gleichzeitig dauerte der Pentobarbitalschlaf der behandelten Ratten *in vivo* länger an. Diese Ergebnisse lassen unter den Bedingungen des vorliegenden Versuches auf eine Veränderung im subzellulären Bereich schließen, und dieses wird gestützt durch eine Reduktion im Bereich des mikrosomalen Proteins. Die orale Verabreichung von Tanninsäure in Dosen von 500 mg/kg täglich für die Dauer von vier Tagen bewirkte hingegen keinerlei Veränderung der mikrosomalen Enzymtätigkeit.

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BIDRIN: PERINATAL TOXICITY AND EFFECT ON THE DEVELOPMENT OF BRAIN ACETYLCHOLINESTERASE AND CHOLINE ACETYLTRANSFERASE IN MICE

J. S. Bus and J. E. Gibson

Department of Pharmacology, Michigan State University, East Lansing, Michigan 48824, USA

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Abstract—The purpose of this study was to investigate the perinatal toxicity of Bidrin and the effect of the insecticide on the developmental patterns of brain acetylcholinesterase (AChE; EC 3.1.1.7) and choline acetyltransferase (ChAc; EC 2.3.1.6) in mice. Bidrin (1, 2, 5 and 7·5 mg/kg) was given by ip injection to pregnant mice during organogenesis in teratology studies, while 5 mg Bidrin/kg was given ip on days 8–16 of gestation in AChE and ChAc developmental studies. AChE and ChAc were determined radiometrically. Bidrin caused no morphological anomalies at any doses and times of administration. Brain AChE levels of the embryo or foetus were depressed when measured 32 min after Bidrin administration to the mother in a dose of 5 mg/kg on day 11 or day 19 of gestation, but enzyme activity was fully recovered by day 19 of gestation after Bidrin had been given daily on days 8–16 of gestation. ChAc development patterns in embryos and foetuses were not changed after single doses or repeated Bidrin administration during gestation. Bidrin was concluded to have a low potential for induction of terata in mice, even though it transiently reduced embryonic or foetal AChE activity to 1·8% of control levels following acute administration on day 11 of gestation. Furthermore, prenatal exposure to Bidrin does not alter the development of AChE or ChAc.

INTRODUCTION

The use of organophosphate insecticides in agriculture has become increasingly widespread over the past two decades. Several of these compounds have been found to be teratogenic, particularly in chicks (Marliac, 1964; Marliac & Mutchler, 1963; Walker, 1971). Bidrin (3-hydroxy-*N*-*N*-dimethyl-*cis*-crotonamide dimethylphosphate) is one of these agents which has potent anticholinesterase activity and has been shown to be teratogenic in chicks in doses as low as 30 μg/egg (Roger, Chambers & Casida, 1964; Roger, Upshall & Casida, 1969). The teratogenicity of Bidrin in chicks has been attributed to acylation of an unknown protein of the yolk-sac membrane and not to a depression of cholinesterase activity as the nature of the compound would initially suggest (Upshall, Roger & Casida, 1968; Flockhart & Casida, 1972).

The purpose of this investigation was to evaluate the teratogenicity of Bidrin in mice and to examine the effects of repeated prenatal administration of Bidrin on the development of acetylcholinesterase (AChE; EC 3.1.1.7) and choline acetyltransferase (ChAc; EC 2.3.1.6) in the brain. The developmental patterns of these two enzymes and their response to Bidrin may aid in confirming or denying the possibility that Bidrin's teratogenicity in chicks is by a non-cholinergic mechanism. Further evalutation may also be possible regarding the potential for Bidrin as a harmful environmental contaminant.

EXPERIMENTAL

Animals. Virgin Swiss Webster mice (from Spartan Research Animals, Inc.) were used in all experiments and were housed in groups of five in stainless-steel cages with wire-mesh

bottoms and allowed food and water *ad lib*. Mating was accomplished by placing one male in a cage of five females for 1 hr starting at 08.00 hr. The finding of a vaginal plug designated the female as at day 1 of gestation. Pregnant mice were either killed on day 19 of gestation for teratogenicity studies or housed individually in clear plastics shoe-box cages for developmental studies. Control and treated litters were weaned on postnatal day 28.

Teratogenicity study. Bidrin (86% commercial formulation with 14% inert material; Shell Development Corp., Modesto, Calif.) was administered ip in a dose of 0, 1, 2, 5 or 7·5 mg/kg (corrected for concentration in the commercial formulation) in a single injection to pregnant mice on day 11 or day 13 only, or daily on days 10–12 of gestation. On day 19 of gestation, the females were killed by ether anaesthesia and the uterine horns were externalized. The numbers and positions of live, dead and resorbed foetuses were recorded. Foetuses were removed by cautery of the umbilical cord, dried, weighed and examined for gross defects. Half of the litter was fixed in Bouin's solution and half in 95% ethanol. Foetuses fixed in Bouin's were hand-sectioned and examined under a dissecting microscope for soft-tissue anomalies (Wilson, 1965) and those fixed in ethanol were cleared, stained with Alizarin Red S and examined for skeletal anomalies.

Developmental study of AChE and ChAc. Unless otherwise noted, Bidrin (97·2% analytical standard; Shell Chemical Co., Princeton, N. J.) was administered in single ip injections of 5 mg/kg daily on days 8–16 of gestation in the developmental studies of AChE and ChAc. Enzyme determinations were carried out on brain homogenates of varying concentrations at times from day 11 of gestation to 42 days postnatally. Brain homogenates from mice less than 21 days old were prepared by pooling brains of animals within a litter. AChE activity was also determined in placental homogenates taken on day 19 of pregnancy 32 min after ip administration of 5 mg Bidrin/kg.

AChE assay. AChE was determined by a modification of the method of Potter (1967). Brain tissue was homogenized in buffer containing 0·32 M-sucrose and 1 mM-Na-phosphate buffer adjusted to pH 7·4. The incubation mixture contained 0·1 ml whole homogenate, 0·1 ml 1% Triton-X 100 containing 0·3 M-NaCl and 1 mM-Na-phosphate buffer at pH 7·4, 0·05 ml [1-^{14}C]acetyl-β-methylcholine (MeCh, 2·3 × 10^{-3} μCi; New England Nuclear Corp., Boston, Mass.) and 0·05 ml distilled water to a final volume of 0·3 ml. Final concentration of MeCh in the incubate ranged from 10^{-3} to 10^{-4} M. The incubation was initiated by addition of the [^{14}C]MeCh and the placing of the tube in a 37 C oscillating Dubnoff incubator for 20–40 min. The incubation was stopped with 0·3 ml 0·2 M-HCl followed by addition of 6 ml 5:1 (v/v) toluene–isoamyl alcohol and a 15-sec mix on a vortex stirrer. The incubation tubes were centrifuged and 2 ml of the supernatant was removed to 5 ml PCS (Amersham-Searle, Arlington Heights, Ill.) and counted in a Packard 3380 liquid scintillation counter.

ChAc assay. A modification of the methods of Schrier & Schuster (1967) and Glover & Green (1972) was used for the assay of ChAc. The incubation mixture contained 0·02 ml of brain homogenized in 150 mM-NaCl containing 1% butanol, 0·02 ml of a solution containing 0·05 μmoles acetylcoenzyme A (AcCoA; lithium salt; P-L Biochemicals Milwaukee, Wisc.) and 0·05 ml of a stock solution containing 21 μmoles NaCl, 7·0 μmoles Na-phosphate buffer (pH 7·5), 0·05 μmoles eserine sulphate (Sigma Chemical Corp., St. Louis, Mo.), 0·1 mg bovine serum albumin (Sigma Chemical Corp. St. Louis, Mo.) and 0·006–0·6 μmoles choline (Matheson, Coleman and Bell, Norwood, Ohio). The tubes were preincubated at 37 C for 2 min and the incubation started by the addition of 0·01 ml [^{14}C]AcCoA, 0·091 μCi (New England Nuclear, Boston, Mass.). After 10–20 min, the incu-

Table 1. *Effect of Bidrin (tech.) given by ip injection, on resorption rate and foetal body weight*

Time of treatment (day(s) of gestation)	Bidrin dose (mg/kg/day)	No. of pregnant mice		Mean response/litter ± SEM			
		Treated	Surviving	No. of implantations	No. of foetuses	Resorption (%)	Foetal body weight (g)
11	0	8	8	12 ± 1	10 ± 1	13·0 ± 3·4	1·318 ± 0·069
	1	8	8	12 ± 1	11 ± 1	6·8 ± 4·0	1·301 ± 0·037
	2	9	9	14 ± 1	13 ± 1	6·2 ± 2·0	1·243 ± 0·025
	5	8	8	12 ± 2	12 ± 2	6·1 ± 3·2	1·287 ± 0·025
13	2	4	4	13 ± 1	12 ± 1	9·8 ± 4·3	1·321 ± 0·044
	5	4	4	16 ± 0	15 ± 1	1·8 ± 1·8	1·358 ± 0·032
	7·5	2	1	13	13	0	1·125
10–12	5	7	7	12 ± 2	11 ± 2	12·3 ± 3·8	1·234 ± 0·027

bation was stopped by addition of 0·5 ml cold water and removal to an ice-bath. Incubation-tube contents were removed to a 4 × 0·5 cm column of Dowex Ag 1-X8 200–400 mesh anion-exchange resin (Bio-Rad Corp., Richmond, Calif.) followed by three 0·5 ml distilled-water washes. Effluent was collected in scintillation vials. 15 ml PCS was added and the samples were counted.

Protein assays. Samples of each homogenate were assayed for protein by the method of Lowry, Rosebrough, Farr & Randall (1951).

Statistical evaluation. Statistical analyses were by analysis of variance (completely randomized design) and Student's t test. The level of significance was chosen as $P < 0·05$.

RESULTS

Bidrin (tech.), administered ip at 1, 2, 5 or 7·5 mg/kg at varying times during organogenesis had no significant effects on foetal resorption rate or foetal weight as determined on day 19 of gestation (Table 1). At a dose of 7·5 mg/kg, Bidrin (tech.) increased maternal mortality and appeared to decrease foetal body weight, although neither change was significant. This dose was approximately 75% of the ip LD_{50} in mice, which has been determined (unpublished observations in this laboratory) as 10·4 mg/kg (95% confidence limits: 9·1–11·6 mg/kg).

Tables 2, 3 and 4 show, respectively, the effects of Bidrin (tech.) on the development of gross, soft-tissue and skeletal anomalies. No significant effects were observed with any of the Bidrin (tech.) dose levels given during the various times of gestation. Bidrin (tech.) may, however, show a tendency to increase the incidence of internal hydrocephaly at 7·5 mg/kg on day 13 of gestation.

Table 2. *Gross anomalies in offspring of pregnant mice given Bidrin (tech.) ip during organogenesis*

Time of treatment (day(s) of gestation)	Bidrin dose (mg/kg/day)	No. of litters examined	Incidence of gross anomalies (mean % response/litter ± SEM)	
			Kinky tail	Open eyes
11	0	8	0	0
	1	8	0	0
	2	9	2·4 ± 1·2	0
	5	8	0	0
13	2	4	0	0
	5	4	0	0
	7·5	1	0	0
10–12	5	7	1·1 ± 1·1	1·1 ± 1·1

The effects of Bidrin (anal.), 5 mg/kg, on the various biochemical parameters of foetal brain AChE and ChAc as measured on day 19 of gestation are shown in Table 5. Acute injection of Bidrin (anal.) on day 19 of gestation followed by enzyme determinations 32 min later resulted in large decreases in K_m, V and velocity at 10^{-3} M-MeCh for AChE and no changes in the same parameters (velocity determined at 6×10^{-3} M-choline) for ChAc. In contrast, the kinetic parameters of foetal brain AChE after repeated treatment with Bidrin (anal.) on days 8–16 of gestation had almost returned to control values when measured on day 19 of gestation. Again, the biochemical parameters of foetal brain ChAc were not changed. The K_m and V for AChE were obtained by using MeCh as the variable

Table 3. *Soft-tissue anomalies in offspring of pregnant mice given Bidrin (tech.) ip during organogenesis*

Time of treatment (day(s) of gestation)	Bidrin dose (mg/kg/day)	No. of litters examined	Incidence of soft-tissue anomalies (mean % response/litter ± SEM)			
			Bladder hypertrophy	Hydronephrosis	Internal hydrocephaly	Brain oedema
11	0	8	6·2 ± 6·2	4·1 ± 4·1	6·2 ± 6·2	0
	1	8	5·9 ± 4·2	0	8·9 ± 5·6	0
	2	9	15·3 ± 5·3	0	13·3 ± 6·1	13·8 ± 8·0
	5	7	9·4 ± 6·1	0	13·9 ± 6·0	17·4 ± 9·6
13	2	4	4·2 ± 4·2	0	18·0 ± 8·4	0
	5	4	9·2 ± 6·0	0	12·5 ± 9·0	0
	7·5	1	0	14·0	100	0
10–12	5	7	6·4 ± 4·7	20·3 ± 13·8	16·7 ± 14·1	0

Table 4. *Skeletal anomalies in offspring of pregnant mice given Bidrin (tech.) ip during organogenesis*

Time of treatment (day(s) of gestation)	Bidrin dose (mg/kg/day)	No. of litters examined	Incidence of skeletal abnormalities (mean % response/litter ± SEM)							
			Ribs		Sternebrae		Vertebrae		Long bones	
			Supernumerary	Fused	Supernumerary	Absent or not ossified	Supernumerary	Fused	Absent	Not ossified
11	0	8	12·5 ± 6·8	0	0	4·1 ± 4·1	0	0	0	0
	1	8	12·0 ± 5·3	0	3·1 ± 3·1	9·4 ± 6·6	0	0	1·5 ± 1·5	0
	2	9	10·6 ± 7·4	0	0	13·6 ± 7·7	1·9 ± 1·9	0	0	0
	5	8	11·9 ± 8·2	0	0	5·2 ± 3·7	0	0	0	0
13	2	4	12·5 ± 12·5	0	0	4·2 ± 4·2	0	0	0	0
	5	4	27·2 ± 17·1	0	0	10·8 ± 10·8	2·8 ± 2·8	0	0	0
	7·5	1	50·0	0	0	0	0	0	0	0
10–12	5	7	14·4 ± 6·8	2·0 ± 2·0	2·8 ± 2·8	3·7 ± 2·4	0	4·7 ± 4·7	0	0

Table 5. *Effect of ip administration of Bidrin (anal.) on day 19 or daily on days 8–16 of gestation on foetal brain acetylcholinesterase and choline acetyltransferase measured on day 19 of gestation*

Time of treatment (day(s) of gestation)	Bidrin dose (mg/kg/day)	Acetylcholinesterase			Choline Acetyltransferase		
		K_m(M)	V*	v*†	K_m(M)	V‡	v‡§
—	0	$5·0 \times 10^{-3}$	10·0	1·800	$1·8 \times 10^{-4}$	0·111	0·112
19‖	5	$7·2 \times 10^{-4}$	0·52	0·274	$1·7 \times 10^{-4}$	0·132	0·131
8–16	5	$3·3 \times 10^{-3}$	7·84	1·742	$1·8 \times 10^{-4}$	0·134	0·133

*Activity expressed as nmoles [^{14}C]MeCh hydrolysed/mg protein/min.
†Velocity measured at 10^{-3} M-MeCh.
‡Activity expressed as nmoles [^{14}C]acetylcholine synthesized/mg protein/min.
§Velocity measured at 6×10^{-3} M-choline.
‖Enzyme activity determined 32 min after Bidrin treatment.

substrate and in the case of ChAc, choline was the variable substrate. The K_m and V values were obtained graphically from Lineweaver–Burke and Eadie–Hofstee plots.

Figure 1 illustrates the development of brain AChE from day 19 of gestation to 42 days postnatally as influenced by repeated Bidrin (anal.) treatment at 5 mg/kg on days 8–16 of gestation. It appears that Bidrin (anal.) treatment did not alter the steady rise in the activity of AChE from 1·8 nmoles MeCh hydrolysed/mg protein/min at day 19 of gestation to 13·9 nmoles MeCh hydrolysed/mg protein/min at 42 days after birth.

The effect of repeated Bidrin (anal.) administration on the development of brain ChAc is seen in Fig. 2. As with AChE, Bidrin (anal.) did not alter the developmental pattern of ChAc from its lowest value of 0·112 nmols acetylcholine synthesized/mg protein/min on day 19 of gestation to its value on postnatal day 42 of 1·7 nmoles acetylcholine synthesized/mg protein/min.

In other experiments, Bidrin (anal.) administered in a dose of 5 mg/kg on day 11 of gestation depressed AChE levels in whole-head sections of embryos to 1·8% of the control level, 32 min after injection. Bidrin (anal.), given ip to mice on day 19 of pregnancy in a

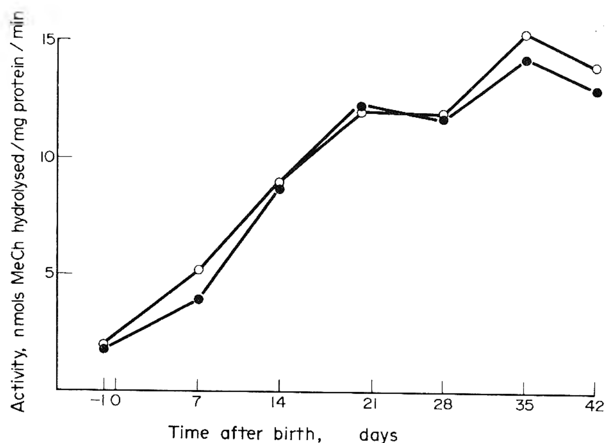

Fig. 1. Foetal and neonatal development of brain AChE (activity measured at 10^{-3} M-MeCh) in controls (○) and in the offspring of mice given Bidrin (anal.) in an ip dose of 5 mg/kg daily on days 8–16 of gestation (●). Each point is the mean of three determinations and there were no significant differences between the treated and control groups.

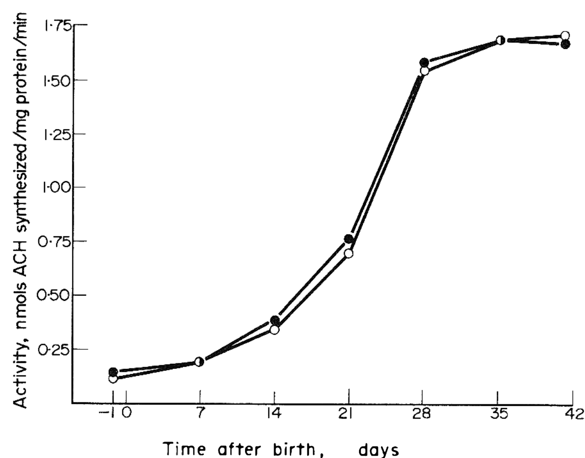

Fig. 2. Foetal and neonatal development of brain ChAc (activity measured at 6×10^{-3} M-choline) in controls (○) and in the offspring of mice given Bidrin (anal.) in an ip dose of 5 mg/kg daily on days 8–16 of gestation (●). Each point is the mean of three determinations and there were no significant differences between the treated and control groups.

dose of 5 mg/kg, reduced placental AChE activity measured 32 min later to 11·0% of control values. When Bidrin (anal.) was administered ip to adult females and AChE was determined 32 min later, brain-enzyme activity was reduced to 16·1% by a dose of 5 mg/kg, 15·2% by 4 mg/kg, 22·3% by 3 mg/kg, 53·5% by 2 mg/kg and 67·3% by 1 mg/kg. Finally, brain AChE activity in adult mice given 5 mg Bidrin (anal.)/kg ip was observed to return to control levels 24 hr after Bidrin administration.

DISCUSSION

The teratogenicity of Bidrin (tech.) in mice was found to be non-significant when considered at the doses and times of administration used in this study. The time of administration was selected to maximize the potential for disruption of organogenesis, the dose being limited by acute maternal toxicity. This lack of teratogenicity is in striking contrast to the results reported in chicks (Roger *et al.* 1964 & 1969) in which doses as low as 30 μg/egg induced anomalies. Upshall *et al.* (1968) observed that nicotinamide alleviated Bidrin-induced teratogenesis in chicks, but did not protect against the chronic inhibition of AChE in eggs treated with Bidrin. Consequently, they postulated that the teratogenicity may have resulted from a nutritional deficiency to the embryo due to an inability of certain ester-hydrolysis products to cross the yolk-sac membrane. This hypothesis was strengthened by the observation that Bidrin and another teratogenic cholinesterase inhibitor, eserine (Agarwal, 1956), were able to inhibit phenyl phenylacetate-hydrolysing esterases found in the yolk-sac membrane (Flockhart & Casida, 1972). Furthermore, Budreau & Singh (1973) found two other organophosphates, demeton and fenthion, to be embryotoxic rather than teratogenic in mice and this effect may have been associated with decreased maternal nutrition.

In our investigations it appeared that Bidrin (tech.) was not teratogenic in mice despite large decreases in AChE activity in the embryonic brain during organogenesis (1·8% of controls 32 min after 5 mg Bidrin (anal.)/kg on day 11). This was indirect evidence that

Bidrin could cross the placenta and act upon the embryo. Our results may illustrate possible differences between the placenta in mice and the yolk-sac membrane in chicks and their function in allowing vital nutrients to reach the embryo. The inhibition of placental AChE by Bidrin (anal.) did not appear to have any developmental consequences for the mouse embryo, in contrast to the results of Flockhart & Casida (1972) suggesting that inhibition of specific yolk-sac membrane esterases may have induced chick teratogenicity. It was noted from Table 1 that Bidrin (tech.) given in a dose of 7·5 mg/kg on day 13, may have reduced foetal body weight. This weight reduction may be attributed to the stress to the mother from acute or chronic reduction of AChE, i.e. decreased food and water consumption and possible dehydration from severe diarrhoea. Other organophosphates such as methyl parathion (Tanimura, Katsuya & Nishimura, 1967) and diisopropyl fluorophosphate (Glow & Richardson, 1966) have been shown to reduce maternal food and water consumption in rats and mice when administered during gestation. Doses of Bidrin (tech.) (1 and 2 mg/kg), which did not produce any teratogenicity or overt signs of maternal cholinesterase inhibition, were associated with 32·7 and 46·5% AChE inhibition, respectively, in the whole maternal brain.

The absence of any teratogenic effects of Bidrin (tech.) in mice led to the examination of foetuses repeatedly treated with Bidrin (anal.) for possible subtle alterations in the biochemical maturation of AChE and ChAc. Bidrin (anal.) administered daily on days 8–16 gestation, however, did not alter the K_m, V or velocity measured at a single substrate concentration for foetal brain AChE determined on day 19 of gestation. The failure of repeated Bidrin (anal.) treatment (on days 8–16 of gestation) to alter either the K_m or V of AChE is indirect evidence that the compound did not alter selectively the development of any individual or group of isozymes of AChE. The isozyme pattern of AChE has been reported by Karczmar, Srinivasan & Bernsohn (1973) to change during development, although the possible functional consequences of these changes are not known. Only acutely administered Bidrin (anal.) was able significantly to alter the K_m and V. The apparent explanation for these observations is that AChE inhibition by Bidrin is of short duration, complete enzyme recovery within 24 hr following a single 5 mg/kg dose of Bidrin (anal.). The effect of Bidrin (anal.) on foetal brain ChAc was much the same as with AChE, except that not even acute doses of the Bidrin altered the enzyme parameters. Thus acute inhibition of foetal brain AChE by Bidrin (anal.) with probable subsequent increases in free acetylcholine levels *in vivo* does not appear to be compensated by a decrease in the activity of ChAc, measured 32 min after the Bidrin injection, when AChE activity was 16·1% of the control value. This appears to be contrary to reports of a negative feedback system existing for ChAc in adult mice and controlled by levels of acetylcholine (Datta & Wajda, 1972; Sharkawi & Schulman, 1969). It is possible, however, that the negative feedback system is not as responsive in prenatal or newborn animals, as physiological and enzymatic systems are often immature in young animals.

The postnatal development of brain AChE and ChAc was not altered by repeated treatment with Bidrin (anal.) during gestation. Both AChE and ChAc showed a slow continuous rise in brain tissue during development despite the Bidrin treatment, a plateau being attained at approximately 35 days after birth. AChE activity on postnatal day 1 was approximately 17% of the value at 42 days, while day 1 activity of ChAc was about 12% of 42-day levels. McGeer, Fibiger & Wickson (1971), examining the development of AChE and ChAc in the caudates of rats, found 4-day levels of AChE and ChAc to be 70·8 and 3·8% of the 21–34-days average levels, respectively. Although Bidrin (anal.) did not alter

AChE and ChAc development, these enzyme systems are capable of alteration by conditions such as hypothyroidism, which has been shown to diminish the development of ChAc (Ladinsky, Consolo, Peri & Garattini, 1972).

The potential for Bidrin as an environmental contaminant harmful to mammalian systems appears to be minimal. Exposure of pregnant mice to Bidrin does not produce any terata or any biochemical lesions in the AChE and ChAc levels of the offspring.

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Toxicité périnatale de la Bidrine et son effet sur le développement de l'acétylcholinestérase cérébrale et de la choline-acétylase chez la souris

Résumé—On s'est proposé d'étudier la toxicité périnatale de la Bidrine et l'effet de cet insecticide sur les profils de développement de l'acétylcholinestérase cérébrale (AChE; EC 3.1.1.7) et de la choline-acétylase (ChAc; EC 2.3.1.6) chez la souris. La Bidrine était injectée par voie intrapéritonéale: à raison de 1, 2, 5 et 7,5 mg/kg aux souris gestantes et pendant l'organogenèse pour les recherches tératologiques et à raison de 5 mg/kg du 8ème au 16ème jour de la gestation pour les recherches sur le développement de l'AChE et de la ChAc. Ces deux enzymes ont été déterminés par la méthode radiométrique. A aucune dose et à aucune époque d'administration la Bidrine n'a provoqué d'anomalies morphologiques. Les taux d'AChE cérébrale de l'embryon ou du foetus avaient dimunué quand on les mesurait 32 min après l'administration de 5 mg/kg à la mère au 11éme ou 19ème jour de la gestation, mais l'activité enzymatique avait pleinement repris le 19ème jour de gestation quand on avait administré la Bidrine quotidiennement du 8ème au 16ème jour. Les profils de développement de la ChAc chez les embryons et foetus n'ont pas changé après l'administration de Bidrine en doses uniques ou répétées pendant la gestation. On conclut de ceci que la Bidrine a un faible potentiel d'induction tératogénique chez la souris, même si, après l'administration à dose aiguë au 11ème jour de gestation il réduit passagèrement l'activité de l'AChE chez l'embryon ou chez le foetus à 1.8% du taux d'activité témoin. De plus, l'exposition prénatale à la Bidrine n'altère pas la production d'AChE ou de ChAc.

Bidrin: perinatale Toxizität und Effekt auf die Entwicklung von Gehirnacetylcholinesterase und Cholinacetyltransferase in Mäusen

Zusammenfassung Der Zweck dieser Studie war, die perinatale Toxizität von Bidrin und den Effekt des Insektizides auf das Entwicklungsbild von Gehirnacetylcholinesterase (AChE; EC 3.1.1.7) und Cholinacetyltransferase (ChAc; EC 2.3.1.6) in Mäusen zu untersuchen. Bidrin (1, 2, 5 und 7,5 mg/kg) wurde durch ip Injektion an trächtige Mäuse verabreicht während der Organogenese für teratologische Studien, während 5 mg Bidrin/kg ip an den Tagen 8–16 der Gestation gegeben wurde für AChE- und ChAc-Entwicklungsstudien. AChE und ChAc wurden radiometrisch bestimmt. Bidrin verursachte keine morphologischen Anomalitäten in allen Dosen und Zeiten der Verabreichung. Der Gehirnspiegel der AChE in Embryo oder Foetus war erniedrigt, wenn er gemessen wurde 32 Minuten nach der Bidringabe an die Mutter in einer Dosis von 5 mg/kg am Tag 11 oder 19 Gestation; aber die Enzymaktivität war wieder völlig erreicht bis zum Tag 19 der Gestation, nachdem Bidrin täglich gegeben wurde an den Tagen 8–16 der Gestation. ChAc-Entwicklungsmuster in Embryos und Foeten war nicht verändert nach Einzelgaben oder wiederholter Bidringabe während der Gestation. Es wurde geschlossen, daß Bidrin ein niedriges Potential hat, Terata in Mäusen zu induzieren, obwohl es vorübergehend die Embryo- oder Foetal-AChE-Aktivität bis zu 1.8% der Kontrollwerte folgend der akuten Gabe am Tag 11 der Gestation reduziert. Außerdem ändert prenatale Bidrinaussetzung nicht die Entwicklung von AChE oder ChAc.

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EFFECT OF PROLONGED INGESTION OF POLYCHLORINATED BIPHENYLS ON THE RAT

J. V. BRUCKNER, K. L. KHANNA and H. H. CORNISH

The University of Michigan, Department of Environmental and Industrial Health, School of Public Health, Ann Arbor, Michigan 48104, USA

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Abstract—Aroclor 1242, a commercial polychlorinated biphenyl mixture, was fed to rats at dietary levels of 0, 5 and 25 ppm for 2, 4 and 6 months. Significant elevations of urinary coproporphyrin excretion, hepatic lipid and hepatic microsomal-hydroxylase activity were observed in rats ingesting 25 ppm for 2 months. In rats given the 5 or 25 ppm regimens for 4 or 6 months, urinary coproporphyrin excretion and liver weight, lipid content and microsomal-hydroxylase activity were higher than in controls. Histopathological examination revealed lipid vacuolation and proliferation of smooth endoplasmic reticulum in the hepatocytes of treated animals. These findings indicate that long-term, low-level ingestion of Aroclor 1242 may result in changes in a variety of biological parameters.

INTRODUCTION

Polychlorinated biphenyls (PCBs), a series of chlorinated polycyclic hydrocarbons, have become the subject of widespread concern in recent years. Commercial PCB products consist of mixtures of isomers, differing from one another in degree of chlorination. As PCBs are water-insoluble and inert, they have been used extensively as surface coatings, flame retardants, stabilizers, capacitors, heat-transfer agents and components of plasticizers, adhesives and printing inks. Such industrial applications have apparently resulted in world-wide environmental contamination (Nisbet & Sarofim, 1972) and accumulation in the tissues of many biological species (Risebrough & de Lappe, 1972).

Investigations have demonstrated the presence of PCBs in human foodstuffs and adipose tissue. Price & Welch (1972) found that 41–45% of human adipose samples, obtained from the general population of the United States, contained >1·0 ppm PCBs. Potential dietary sources of PCBs include fish, poultry, milk, eggs and cereals (Kolbye, 1972). The FDA interim guideline for PCB contamination of human foodstuffs has been set at 5 ppm, with lower levels recommended for long-term ingestion.

Though current environmental and dietary levels of PCBs are not felt to constitute an immediate health hazard, limited information is available concerning biological manifestations of long-term, low-level exposure in mammals. Kimbrough, Linder & Gaines (1972) reported significant increases in liver to body-weight ratios in rats fed 5 ppm Aroclor 1254 or Aroclor 1260 for 8 months. These same commercial PCB products at dietary levels as low as 0·5 and 5 ppm may induce certain hepatic microsomal-enzyme activities in rats within 4 wk (Litterst, Farber, Baker & Van Loon, 1972). A 1-yr 20 ppm dietary regimen of Aroclors 1242, 1254 or 1260 has been reported to reduce pentobarbitone sleeping time in rats (Villeneuve, Grant & Phillips, 1972).

The present study was initiated to elucidate biological responses in the rat upon prolonged ingestion of a common PCB product, Aroclor 1242. Dosage levels corresponding to the contemporary FDA guideline and to a level encountered in fish were selected. Emphasis was placed upon biological parameters shown to be altered by acute and subacute dosage regimens in previous investigations (Bruckner, Khanna & Cornish, 1973 & 1974).

EXPERIMENTAL

Animals. Male Sprague–Dawley rats (mean body weight 125 g) were obtained from Spartan Research Farm, Lansing, Mich. The rats were randomly divided into groups of six animals and housed in pairs in stainless-steel cages in air-conditioned quarters, with tap-water and ground Rockland rat and mouse chow available *ad lib.* Only during collection of urine specimens were the rats confined individually in metabolism cages.

Materials and dosage. Aroclor 1242 (lot no. KA 419) was supplied by Monsanto Chemical Company, St. Louis, Mo. Appropriate quantities of Aroclor 1242 were dissolved in acetone and mixed thoroughly with a small amount of ground chow. After the acetone had been allowed to evaporate, the Aroclor-chow mixture was mixed by geometric dilution with increasing amounts of chow until desired Aroclor 1242 concentrations of 5 and 25 ppm were attained. The groups of six rats were fed the diets containing 0, 5 or 25 ppm Aroclor 1242 for 2, 4 or 6 months. Body weights and food consumption were measured at weekly intervals.

Urine analysis. Urinary excretion of coproporphyrin was determined by the method of Schwartz, Zieve & Watson (1951) 1 wk before the rats were killed.

Haematology. At the end of the treatment period, blood samples were taken from tail veins for determinations of haematocrit, erythrocyte counts and haemoglobin levels. Erythrocyte counts, utilizing a Coulter counter, were performed for the 6-month groups only. Haemoglobin levels were measured by a technique involving cyanmethaemoglobin formation (Davidsohn & Henry, 1969). Following anaesthetization with ether, blood was withdrawn by open-chest cardiac puncture. Serum glutamic–pyruvic transaminase (SGPT) and serum glutamic–oxalacetic transaminase (SGOT) activities were determined by standard procedures. The method of Solem & Brinck-Johnsen (1965) was used for analysis of plasma corticosteroids.

Microsomal-enzyme assays. Hydroxylase activity was estimated in the 13,000 **g** supernatant of 5 g liver samples by measurement of *N*-acetyl-*p*-aminophenol formation from acetanilide, and *N*-demethylase activity by measurement of 4-aminoantipyrine formation from aminopyrine. Methods utilized were outlined in a previous investigation (Bruckner *et al.* 1973).

Hepatic studies. Liver weights were recorded and total liver lipid was assayed by the procedure of Bligh & Dyer (1959).

Histopathology. Liver and kidney specimens were processed and stained with haematoxylin and eosin for light microscopic examination. Frozen sections of liver and kidney were stained for fat with Sudan IV. Cubes of liver were minced and fixed in cold 3% phosphate-buffered glutaraldehyde for 2 hr, stained in 2% osmium tetroxide for 2 hr, dehydrated in an ethanol and propylene oxide series and embedded in Epon plastic. Sections were post-stained with uranyl acetate and lead citrate and examined with an AEI Cornith 275 electron microscope.

Statistical analysis. The significance of all results was assessed by Student's *t* test.

RESULTS

Body weight and food consumption

No significant differences in body-weight gain were seen at any dietary level after 2, 4 or 6 months. Food consumption was comparable at each dietary level throughout the course of the study. With the average food intake of 25 g/rat/day, the 5 ppm groups ingested approximately 0·3 mg Aroclor 1242/kg body weight/day, the 25 ppm groups approximately 1·5 mg Aroclor 1242/kg/day. Total Aroclor 1242 consumption during the course of the studies is expressed in Table 1.

Table 1. *Approximate total consumption of Aroclor 1242 by test groups*

Dose (ppm)	Total Aroclor 1242 consumption* (mg/rat) during test period of (months) 2	4	6
5	7	14	21
25	35	70	105

*Values are based on an approximate average consumption of 25 g food/400 g rat/day.

Haematology

Results of blood assays other than SGOT, SGPT and plasma corticosteroid determinations are given in Table 2. No significant alterations in SGOT, SGPT or plasma corticosteroid levels were seen at any dietary level after 2, 4 or 6 months. Only in the group fed 25 ppm Aroclor 1242 for 2 months were significant reductions in haematocrit recorded. A significant reduction in haemoglobin levels occurred in the groups fed 5 or 25 ppm for 2 months. Slight reductions from control values in haematocrit and erythrocyte count were noted in the group given 25 ppm for 6 months, although mean values were not statistically different from those of the controls.

Table 2. *Effect of prolonged, low-level ingestion of Aroclor 1242 on haematocrit, erythrocyte count and haemoglobin level*

Duration of test (months)	Dose (ppm)	Haematocrit† (%)	Erythrocyte count† ($10^6/mm^3$)	Haemoglobin (g/100 ml)
2	0	51·0 ± 1·0		16·1 ± 0·1
	5	50·6 ± 0·6		15·1 ± 0·1*
	25	48·0 ± 0·6*		14·8 ± 0·2**
4	0	52·6 ± 1·3		17·1 ± 0·3
	5	51·7 ± 0·9		16·2 ± 0·4
	25	53·2 ± 0·9		16·8 ± 0·3
6	0	55·2 ± 1·3	10·3 ± 0·4	16·8 ± 0·4
	5	56·0 ± 0·2	10·1 ± 0·3	16·2 ± 0·4
	25	54·0 ± 1·1	9·7 ± 0·1	15·9 ± 0·5

†Mean values represent an average of two determinations/rat.
Values are means ± SEM for groups of six rats and those marked with asterisks differ significantly (Student's t test) from the control values: $^*P < 0·01$; $^{**}P < 0·001$.

Microsomal-enzyme assays

Table 3 presents the effects of the dietary regimens on the activities of hepatic microsomal hydroxylase and *N*-demethylase. A significant induction of hydroxylase activity was manifest in rats that had consumed 25 ppm Aroclor 1242 for 2 months. Hydroxylase activity was induced in some members of the group given 5 ppm for 2 months but not in others. Dose-dependent increases in hydroxylase activity were measured at each sampling period. The 5 ppm diet did not alter *N*-demethylase activity significantly at any sampling period, though the 25 ppm diet induced significant increases in the 4- and 6-month groups.

Table 3. *Effect of prolonged, low-level ingestion of Aroclor 1242 on hepatic microsomal hydroxylase and* N-*demethylase activities*

		Hydroxylase activity		*N*-Demethylase activity	
Duration of test (months)	Dose (ppm)	*N*-Acetyl-*p*-aminophenol formed (μg/mg protein/20 min)	% of control value	4-Aminoantipyrine formed (μg/mg protein/20 min)	% of control value
2	0	0·14 ± 0·01	100	0·13 ± 0·01	100
	5	0·19 ± 0·02*	140	0·13 ± 0·02	100
	25	0·53 ± 0·04***	376	0·17 ± 0·02	133
4	0	0·31 ± 0·03	100	0·30 ± 0·05	100
	5	0·51 ± 0·03***	163	0·36 ± 0·07	123
	25	1·06 ± 0·03****	342	0·59 ± 0·04***	197
6	0	0·34 ± 0·08	100	0·49 ± 0·00	100
	5	0·51 ± 0·03**	147	0·45 ± 0·03	93
	25	1·38 ± 0·06****	401	0·88 ± 0·03****	181

Results are expressed as the mean ± SEM for groups of six rats and as a percentage of the control value. Values marked with asterisks differ significantly (Student's *t* test) from those for controls: *$P < 0·1$; **$P < 0·05$; ***$P < 0·01$; ****$P < 0·001$.

Urine analysis

The effect of repeated low-level exposure to Aroclor 1242 on urinary coproporphyrin excretion is summarized in Table 4. A significant rise in coproporphyrin excretion was

Table 4. *Effect of prolonged low-level ingestion of Aroclor 1242 on urinary coproporphyrin, liver weight and liver lipids*

Duration of test (months)	Dose (ppm)	Urinary coproporphyrin (μg/24 hr)	Liver weight (g/100 g body weight)	Total lipids (mg/g liver wet wt)
2	0	4·7 ± 0·3	3·7 ± 0·2	31·2 ± 0·7
	5	5·8 ± 0·4*	3·6 ± 0·2	35·3 ± 1·1***
	25	9·9 ± 0·6****	3·8 ± 0·1	38·2 ± 1·0****
4	0	5·9 ± 0·9	2·9 ± 0·1	33·4 ± 2·0
	5	8·5 ± 1·4*	3·1 ± 0·1*	40·0 ± 2·1**
	25	16·6 ± 4·4**	3·4 ± 0·1***	41·5 ± 2·0***
6	0	3·9 ± 0·7	3·3 ± 0·2	31·9 ± 0·7
	5	7·9 ± 1·4**	3·9 ± 0·2*	34·1 ± 1·3*
	25	6·3 ± 0·9*	3·9 ± 0·2*	36·0 ± 1·1***

Values are the means ± SEM for groups of six rats and those marked with asterisks differ significantly (Student's *t* test) from the control value: *$P < 0·1$; **$P < 0·05$; ***$P < 0·02$; ****$P < 0·001$.

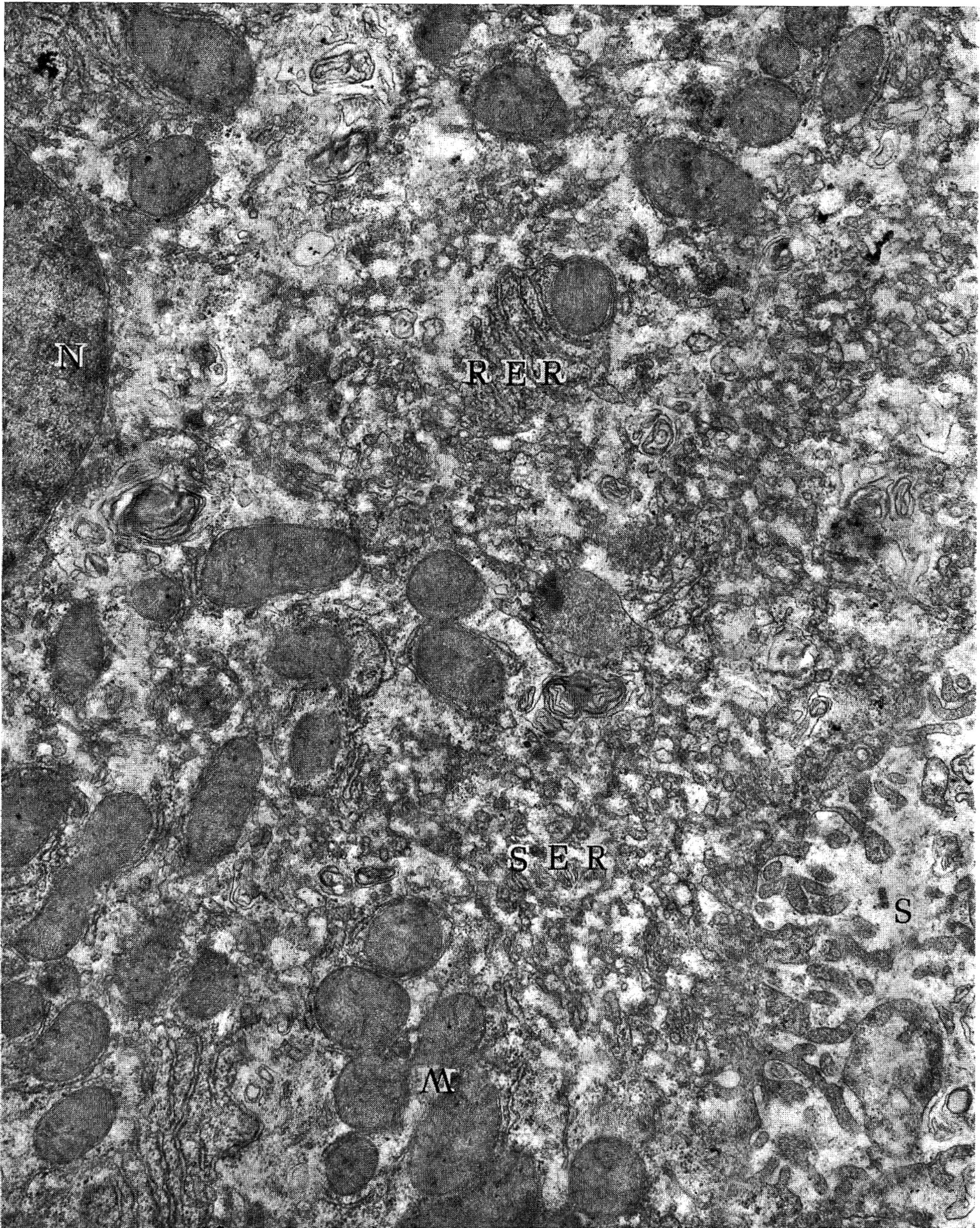

Fig. 1. Part of hepatocyte from a rat fed 5 ppm Aroclor 1242 for 4 months, showing a preponderance of smooth endoplasmic reticulum (SER) and paucity of rough endoplasmic reticulum (RER), together with the nucleus (N) mitochondria (M) and space of Disse (S). × 24,000.

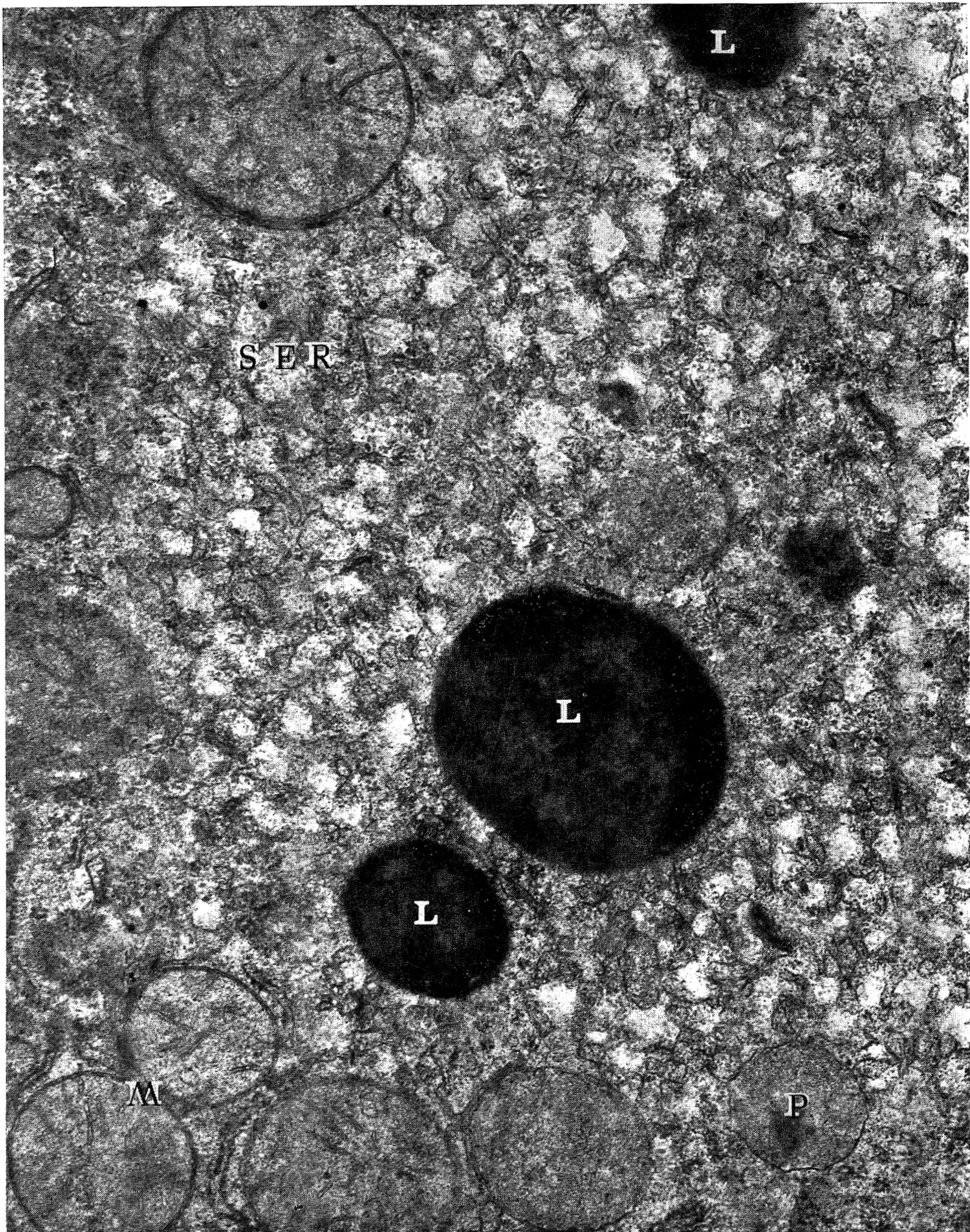

Fig. 2. Part of hepatocyte from a rat fed 25 ppm Aroclor 1242 for 4 months, showing lipid vacuoles (L) similar in size to the mitochondria (M) and cytoplasmic in distribution, smooth endoplasmic reticulum (SER) and a peroxysome (P). × 45,000.

observed in the group that consumed 25 ppm for 2 months. Although a definitely significant increase was not seen until 6 months of feeding the 5 ppm diet, some animals in both the 2- and 4-month groups fed 5 ppm exhibited high urinary coproporphyrin levels.

Hepatic studies

Although increases in liver weight were not apparent in response to either the 5 or 25 ppm regimens after 2 months, liver lipid was significantly higher than the control level in each group (Table 4). Both liver weight and lipid content were raised in the 5 and 25 ppm groups at 4 and 6 months.

Histopathology

No discernible histopathological changes were present in haematoxylin/eosin-stained liver and kidney specimens of any rat after 2, 4 or 6 months of dosing. The Sudan IV stain, however, revealed increased lipid deposition in the livers of rats fed 5 or 25 ppm Aroclor 1242 for 2, 4 or 6 months. The distribution of vacuolated hepatocytes in the liver lobules was primarily peripheral, with encroachment into the midzonal regions in more severe cases. The sudanophilic vacuoles were quite small and were distributed uniformly within the cytoplasm of afflicted hepatocytes. Limited vacuolation of the renal convoluted tubules was observed only after the feeding of 25 ppm Aroclor 1242 for 4 or 6 months. Minute sudanophilic vacuoles were observed in the peripheral portion of affected tubular epithelial cells.

A proliferation of smooth endoplasmic reticulum and an increase in lipid vacuolation were seen with the electron microscope in hepatocytes of the rats fed 25 ppm Aroclor 1242 for 2 months. Both the 5 and 25 ppm diets produced these effects in the 4- and 6-month groups (Figs 1 & 2). No increases in myelin figures, microbodies or lysosomes were observed.

DISCUSSION

The small reductions in haematocrit and haemoglobin seen in the present study after 2 months are analogous to responses produced in previous studies (Bruckner *et al.* 1973 & 1974) by much higher doses of Aroclor 1242. Reduction in the erythrocyte count and haemoglobin content by PCB exposure has also been reported in severely poisoned humans (Okumura & Katsuki, 1969) and in rhesus monkeys (Abrahamson & Allen, 1973). Abrahamson & Allen (1973) observed hypoplasia of the bone marrow in these anaemic monkeys. Reasons for the lack of anaemic response in the 4- and 6-month groups in the present study are unclear. An adaptive mechanism capable of negating the haematological effects of low levels of Aroclor 1242 may have become operative.

Urinary coproporphyrin excretion proved to be a sensitive indicator of Aroclor 1242 ingestion. Though relatively high doses of PCBs are known to cause porphyria, we are not aware of any previous demonstration that ingestion of low dietary levels may elicit this condition in mammals. The dosage threshold for porphyria induction was 5 ppm Aroclor 1242. Variation of individual responses within groups was pronounced, as evidenced by the rather large standard errors.

Hepatic microsomal-hydroxylase induction also proved to be a sensitive index of Aroclor 1242 ingestion. A threshold effect was observed in response to 2 months' consumption of the 5 ppm diet. As in a previous study (Bruckner *et al.* 1974), hydroxylase activity was

dose-dependent. A similar phenomenon was reported by Litterst *et al.* (1972) in 4-wk rat-feeding studies, in which Aroclors 1254 and 1260 showed clear dose-response relationships with *N*-demethylase, nitroreductase and hydroxylase activities. *N*-Demethylase activity, however, proved a much less sensitive index of Aroclor 1242 ingestion than hydroxylase activity in the present investigation.

The level of hydroxylase activity in rats fed 25 ppm Aroclor 1242 varied directly with the length of dietary exposure. Such a phenomenon is evidence of a cumulative effect, as would be predicted from the lipid solubility and the persistence of PCBs in biological systems. Other investigations have demonstrated that a single dose of PCBs will induce microsomal enzymes for weeks (Bruckner *et al.* 1974; Fujita, Tsuji, Kato, Saeki & Tsukamoto, 1971). Upon administration of a single oral dose of 25 mg [^{3}H]Kanechlor-400/rat, Yoshimura, Yamamoto, Nagai, Yae, Uzawa, Ito, Notomi, Minakami, Ito, Kato & Tsuji (1971) found that significant amounts of radioactivity remained in some tissues after 8 wk. Dietary levels of 100 ppm Aroclor 1254 have been shown to be cumulative in rats, with a steady build-up in all tissues over an 8-month feeding period (Curley, Burse, Grim, Jennings & Linder, 1971).

The liver hypertrophy with lipid vacuolation and proliferation of smooth endoplasmic reticulum observed in hepatocytes in the present study are typical manifestations of exposure to chlorinated aromatic hydrocarbons. Similar changes have been reported in feeding studies on PCB in mice and monkeys (Nishizumi, 1970) and in rats (Kimbrough *et al.* 1972), though not in response to such low dietary levels. Norback & Allen (1972) proposed that proliferated membranes provided sites for storage, isolation and contact of membrane enzymes with lipophilic aromatic hydrocarbons. Microsomal membranes have been shown to contain the highest amount of radioactivity of any subcellular fraction following oral administration of [^{3}H]Kanechlor-400 to rats (Fujita *et al.* 1971).

Hepatomegaly resulting from exposure to PCBs may be attributed to increases in several hepatic components. Norback & Allen (1972) measured significant increases in hepatic microsomal protein and phospholipid in rat feeding studies. Triglyceride content in the rat liver may also be elevated by ingestion of Aroclors (Litterst *et al.* 1972) as indicated by findings in the present investigation. Fat stains and electron-microscopic studies revealed many small lipid vacuoles in affected hepatocytes, though corresponding haematoxylin/eosin-stained specimens appeared unaltered. The lipid vacuoles were apparently small enough to escape detection by the latter histopathological procedure. Alteration of hepatocytes by Aroclor 1242 was not so extensive that SGOT and SGPT levels were elevated.

Evaluation of the potential hazards posed to man by long-term exposure to PCBs must use, as criteria, incidents of short-term human exposure and animal studies. On the basis of findings in the Yusho incident (Kuratsune, Yoshimura, Matsuzaka & Yamaguchi, 1972), the minimal toxic dose of Kanechlor-400 (chlorine content 48%) in man was estimated to be about 0·5 g/patient, or 8 mg/kg body weight for a 60-kg adult. The minimal effective dietary level of Aroclor 1242 in the present study was 5 ppm, at which dose level a 400-g rat consumed a total dose of approximately 18 mg/kg body weight in 2 months. Although such a comparison suggests a similar bioeffect level for man and rat, one must appreciate the limitations on extrapolation of rodent data to man, the variation in chlorine content and potential contaminants between Kanechlor-400 and Aroclor 1242, the dissimilarity of dosage regimens, the imprecise approximation of total doses and the differences in biological parameters evaluated in the two studies.

Our findings indicate that prolonged low-level exposure to 5 or 25 ppm Aroclor 1242 may result in changes in a variety of biological parameters. Although these alterations do not constitute an immediate threat to life, they may, in addition to their direct effects, modify biological responses to numerous chemical agents metabolized by the microsomal-enzyme system.

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Effet de l'ingestion prolongée de biphényles polychlorés chez le rat

Résumé—On a fair consommer à des rats, pendant 2, 4 et 6 mois et à raison de 0,5 et 25 ppm de leur régime alimentaire, de l'Aroclor 1242, mélange commercial de biphényles polychlorés. Des augmentations significatives de l'excrétion urinaire de coproporphyrine, des lipides hépatiques et de l'activité de l'hydroxylase microsomique hépatique ont été observées chez les animaux qui avaient consommé 25 ppm pendant 2 mois. L'excrétion urinaire de coproporphyrine, le poids du foie, sa teneur en lipides et l'activité de son hydroxylase microsomique étaient plus élevés chez les rats soumis pendant 4 ou 6 mois aux régimes à 5 ou à 25 ppm que chez les animaux témoins. A l'examen histopathologique on a constaté une vacuolation des lipides et une prolifération de réticulum endoplasmique dans les hépatocytes des animaux traités. Ces constatations signifient que l'ingestion prolongée de faibles doses d'Aroclor 1242 peut modifier un certain nombre de paramètres biologiques.

Effekt der Langzeiteinnahme der polychlorierten Biphenylen bei der Ratte

Zusammenfassung– Aroclor 1242, eine handelsübliche polychlorierte Biphenylmischung, wurde an Ratten verfüttert in Konzentrationen von 5 und 25 ppm in der Kost während 2, 4 und 6 Monaten. Bermerkenswerte Erhöhung der Coproporphyrinausscheidung im Harn, Leberlipid- und Lebermicrosomalhydroxylase-Aktivität wurden festgestellt in Ratten, die 25 ppm während 2 Monaten einnahmen. An Ratten, die 5 oder 25 ppm Kost während 4 oder 6 Monaten erhielten, war die Coproporphyrinausscheidung im Harn, das Lebergewicht, der Lipidgehalt und die Microsomalhydroxylaseaktivität höher als in Kontrolltieren. Die histopathologische Untersuchung zeigte eine Lipidvacuolation und Proliferation des glatten endoplastischen Reticulums in den Hepatocyten der behandelten Tiere. Diese Befunde weisen darauf hin, daß Langzeiteinnahme von Aroclor 1242 in niedrigen Dosen in Veränderungen einer Vielfalt von biologischen Parametern resultieren könnte.

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PENICILLIUM VIRIDICATUM MYCOTOXICOSIS IN THE RAT. IV. ATTEMPTS TO MODIFY THE TISSUE RESPONSES

M. D. McCracken and W. W. Carlton

Department of Veterinary Microbiology and Pathology, School of Veterinary Science and Medicine;

and

J. Tuite

Department of Botany and Plant Pathology, School of Agriculture, Purdue University, West Lafayette, Indiana 47907, USA

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Abstract—Male rats were fed a rice culture of *Penicillium viridicatum* at a dietary concentration of 50% and were administered tripelennamine hydrochloride, syrosingopine or prednisolone. Other groups fed the fungal diet at 25% dietary concentration were administered prednisolone or tetracycline. One group fed this diet was castrated prior to feeding. Tripelennamine, syrosingopine and tetracycline were not effective in reducing the incidence or severity of the ocular and scrotal lesions that developed in the test rats. Prednisolone was somewhat effective in reducing the incidence and severity of scrotal lesions and decreased the severity of the ocular lesions. Tetracycline, but not tripelennamine or syrosingopine, reduced the severity of the hepatic lesions. Castration effectively prevented the scrotal lesions, but did not alter those of the eye and liver.

INTRODUCTION

Male rats fed grain cultures or fungal mats of *Penicillium viridicatum* developed lesions of the eye (McCracken, Carlton & Tuite, 1974a), and of the scrotum and liver (Carlton & Tuite, 1970; McCracken, Carlton & Tuite, 1974b, c). Because the ocular, and especially the scrotal, lesions were markedly inflammatory in character, studies were completed to ascertain how the development of the lesions was affected by an antihistamine, tripelennamine, the serotonin antagonist, syrosingopine, the corticosteroid, prednisolone, and the antibiotic, tetracycline. In addition, one group of rats was castrated and fed the fungal diet. The results of these studies are presented in this report.

EXPERIMENTAL

Experimental animals. Male rats were purchased at a weight of about 250–300 g and were housed and fed as previously described (McCracken *et al.* 1974a). Rats were examined daily and the incidence and character of ocular and scrotal alterations were recorded. Orchidectomy was performed under ether anaesthesia on one group of rats in Trial II and the rats were allowed 2 wk for recovery.

Fungal diets. Rice cultures of *P. viridicatum* were prepared as previously described and mixed with a purified diet (McCracken *et al.* 1974a).

Experimental design and conduct. In Trial I, male rats were fed the fungal grain cultures at a dietary concentration of 50% and groups of these rats were given either tripelennamine

(Pyribenzamine, supplied by Ciba Pharmaceutical Co., Summit, N.J.) in a daily sc dose of 5 mg/kg body weight, syrosingopine (Singoserp, supplied by Ciba Pharmaceutical Co.) in a daily sc dose of 25 mg/kg or prednisolone in a daily sc dose of 5 mg/kg. Rats in Trial II were fed the fungal grain culture at a dietary concentration of 25% either alone or together with a daily sc dose of 5 mg prednisolone/kg or a daily intramuscular dose of 5 mg tetracycline/kg. The treatments were continued for up to 6 wk.

Autopsy. The eyes, scrotum, liver, stomach and kidneys were removed at autopsy. Eyes were processed as previously described (McCracken *et al.* 1974a). The other tissues were fixed in 10% buffered formalin, processed for paraffin sectioning and stained with haematoxylin and eosin for histopathological examination.

RESULTS

Trial I

Clinical signs. Signs of toxicity in the groups of rats fed the fungal diet were similar to those previously described and included loss of activity, roughened hair, anorexia, diarrhoea and progressive loss of body weight (Table 1). Administration of tripelennamine hydrochloride, syrosingopine or prednisolone had no effect on the incidence or severity of the clinical signs. Mortality was high in the test groups given tripelennamine or syrosingopine.

Table 1. *Mean body weights and incidence of ocular lesions in rats fed a culture of* P. viridicatum *and treated with tripelennamine, syrosingopine or prednisolone (Trial I)*

Experimental group	Mean body weight (g) at wk 0	1	4	Total gain (g)	Mortality*	Gross ocular lesions*
Purified diet†	363	387	455	92	0/10	0/18
50% PV‡	367	310	208	−159	2/15	30/30
Tripelennamine†§	331	344	407	76	0/5	0/10
50% PV‡ + tripelennamine§	334	283	236	−98	7/15	30/30
50% PV‡ + syrosingopine§	339	280	200	−139	10/15	28/30
Prednisolone†§	364	370	405	41	1/10	0/24
50% PV‡ + prednisolone§	357	306	190	−167	6/15	30/30

*Mortality is expressed as no. dead/no. in group and gross ocular lesions as no. of eyes affected/no. examined.
†Control groups.
‡Rice culture of *P. viridicatum* fed at a level of 50% in the diet.
§Tripelennamine or prednisolone given in daily sc doses of 5 mg/kg body weight and syrosingopine in daily sc doses of 25 mg/kg.

Ocular effects. Clinically detectable ocular lesions occurred in all rats fed only the fungal diet. Lesions, first noted after 7–10 days of feeding, were similar to those previously described (McCracken *et al.* 1974a), beginning as a slight haziness of the cornea and increasing in severity so that the cornea became opaque. Hypopyon and corneal vascularization occurred in severely affected eyes. Tripelennamine and syrosingopine had no apparent effect on the development or severity of the clinically detectable ocular changes (Table 1), but ocular disease appeared to be less severe in test rats given prednisolone.

The initial microscopic ocular lesions in the test rats consisted of corneal oedema and hydropic degeneration of the corneal epithelium. In mildly affected eyes, accumulations of a mixture of inflammatory cells were present around the limbal vessels and within the

substantia propria. In more severely affected eyes, diffuse interstitial keratitis was accompanied by iridocyclitis, hypopyon and corneal vascularization (Table 2). No difference in the incidence of corneal oedema was observed in test rats given prednisolone. However, the incidence of hypopyon and corneal vascularization was lower in this group than in that fed the fungal diet alone (Table 2). The incidence and severity of ocular lesions in the rats treated with tripelennamine or syrosingopine were comparable to those in the fungal control group (Table 2).

Effects on scrotum. Gross scrotal lesions in test rats were similar to those previously described (McCracken *et al.* 1974b). Oedematous swelling of the scrotum began after 2–3 wk of feeding and was followed by necrosis and ulceration of the scrotal epidermis. The administration of tripelennamine or syrosingopine had little observable effect on the incidence or severity of scrotal swelling and necrosis (Table 3). Scrotal necrosis followed sooner upon the initial swelling in test rats given tripelennamine. Scrotal oedema was observed in 7 of 15 rats treated with prednisolone but was severe in only one rat, and epidermal necrosis was observed in only two (Table 3).

Microscopic scrotal lesions occurred in almost all test rats and consisted of a severe necrotizing cellulitis characterized by foci and bands of necrotic leucocytes in the epididymal connective tissue and scrotal fascia. Necrosis and ulceration were present in the overlying epidermis. Differences in microscopic lesions between rats given tripelennamine or syrosingopine and those fed the fungal diet alone were a lower incidence of testicular lesions and of rats with lesions in the epididymis (Table 3).

Prednisolone decreased the incidence and severity of the scrotal lesions (Table 3); two of the 15 test rats given prednisolone had no lesions. Lesions of the epididymal fat and the scrotal fascia surrounding the tunicae were extremely variable, ranging from no lesions to very severe changes. Changes in the epididymal adipose tissue consisted of focal mononuclear cell accumulations and areas of necrosis small-to-moderate in size. Five rats had lesions of the epididymal fat, but there were no lesions outside the tunicae. Seven of these 15 rats had lesions of severe necrotizing cellulitis similar to that present in the rats given only the fungal diet. Necrosis and ulceration of the epidermis occurred in only three of the 15 prednisolone-treated test rats.

Hepatic changes. Gross hepatic lesions in test rats consisted of a variable number of yellow-to-white foci and often the smaller hepatic lobes were the more severely affected. There was no difference in the incidence or appearance of gross hepatic lesions among the various treatment groups (Table 4).

Microscopic hepatic changes in the test rats were characterized by a necrotizing cholangitis with pericholangitis, proliferation of the biliary epithelium and focal areas of hepatic cell necrosis, often adjacent to the bile ducts. Periductal fibrosis resulted in stenosis and obliteration of the bile ducts in the more severely affected livers. Neither the incidence nor severity of hepatic changes in rats given tripelennamine or syrosingopine differed significantly from those in the group fed the fungal diet alone (Table 4). Focal necrosis and periductal fibrosis of the liver were particularly severe in test rats given prednisolone. Necrotic foci were more numerous and larger in these rats, but bile-duct proliferation tended to be less severe.

Effects on other organs. Lesions in other organs included gastric erosions, extra-scrotal skin necrosis and biliary pigment in the renal tubular epithelium. There were no apparent differences among the various treatment groups in the development of these lesions.

Table 2. *Ocular lesions in rats fed a culture of* P. viridicatum *and treated with tripelennamine, syrosingopine or prednisolone (Trial I)*

Experimental group	No. of eyes examined microscopically	No. of eyes with microscopic lesions					
		Corneal oedema	Keratitis	Corneal vessels	Iritis	Synechia	Hypopyon
Purified diet*	14	0	0	0	0	0	0
50% PV†	26	25	26	12	18	6	17
Tripelennamine*‡	9	0	0	0	0	0	0
50% PV† + tripelennamine‡	27	27	27	7	13	2	8
50% PV† + syrosingopine‡	26	26	22	9	20	4	9
Prednisolone*‡	24	0	0	0	0	0	0
50% PV† + prednisolone‡	28	28	26	6	12	6	9

*Control groups.
†Rice culture of *P. viridicatum* fed at a level of 50% in the diet.
‡Tripelennamine or prednisolone given in daily sc doses of 5 mg/kg body weight and syrosingopine in daily sc doses of 25 mg/kg.
§Only 24 eyes examined.

Table 3. *Gross and microscopic scrotal lesions in rats fed a culture of* P. viridicatum *and treated with tripelennamine, syrosingopine or prednisolone (Trial I)*

Experimental group	No. of rats examined	No. of animals affected by						
		Gross scrotal lesions		Microscopic findings				
		Swelling	Necrosis	Cellulitis of scrotum	Epidermal necrosis	Testicular lesions	Epididymal lesions	Penile lesions
Purified diet*	10	0	0	0	0	0	0	0
50% PV†	15	14	12	13	10	13	15	11
Tripelennamine*†	5	0	0	0	0	0	0	0
50% PV† + tripelennamine‡	15	10	9	15	10	2	7	6
50% PV† + syrosingopine‡	15	12	10	14	10	1	7	9
Prednisolone*‡	10	0	0	0	0	0	0	0
50% PV† + prednisolone‡	15	7	2	7	3	0	5	7

*Control groups.
†Rice culture of *P. viridicatum* fed at a level of 50% in the diet.
‡Tripelennamine or prednisolone given in daily sc doses of 5 mg/kg body weight and syrosingopine in daily sc doses of 25 mg/kg.

Table 4. *Gross and microscopic hepatic lesions in rats fed a culture of* P. viridicatum *and treated with tripelennamine, syrosingopine or prednisolone (Trial I)*

Experimental group	No. of rats examined	No. of animals affected by					
		Gross lesions	Pericholangitis	Bile-duct proliferation	Periductal fibrosis	Necrotizing cholangitis	Scrotal necrosis
Purified diet*	5	0	0	0	0	0	0
50% PV†	15	9	14	15	14	14	13
Tripelennamine*‡	5	0	0	0	0	0	0
50% PV† + tripelennamine‡	15	10	14	14	14	14	13
50% PV† + syrosingopine‡	15	11	12	15	15	15	13
Prednisolone‡	10	0	0	0	0	0	0
50% PV† + prednisolone‡	15	7	3	9	12	12	12

*Control groups.
†Rice culture of *P. viridicatum* fed at a level of 50% in the diet.
‡Tripelennamine or prednisolone given in daily sc doses of 5 mg/kg body weight and syrosingopine in daily sc doses of 25 mg/kg.

Trial II

Signs of toxicity in rats fed the 25% fungal diet were minimal during the first 3 wk. During 4–6 wk of feeding, signs of toxicity appeared and were similar to those observed in Trial I. Test rats progressively lost weight and those given prednisolone showed the greatest reduction. Castrated rats fed the fungal diet maintained their body weights (Table 5).

Table 5. *Response of intact and castrated rats to a culture of* P. viridicatum *and to treatment with prednisolone or tetracycline (Trial II)*

Experimental group	Mean body weight at wk 0	1	5	Total gain (g)	Mortality*
Purified diet†	362	403	422	60	0/10
25% PV‡	367	374	323	−44	0/15
Prednisolone†	357	367	355	−2	0/5
25% PV‡ + prednisolone§	383	366	286	−97	1/15
Tetracycline†	363	401	461	98	1/5
25% PV‡ + tetracycline§	362	370	321	−41	0/15
Castrated rats†	355	379	417	62	0/5
25% PV‡ + castration	341	364	337	−4	0/15

*No. dead/no. in group.
†Control groups.
‡Rice culture of *P. viridicatum* fed at a level of 25% in the diet.
§Given in daily doses of 5 mg/kg body weight, prednisolone sc and tetracycline intramuscularly.

Ocular effects. Clinical ocular lesions were similar to those described in Trial I. Castration and treatment with tetracycline had no apparent effect on the incidence or severity of these lesions, but the lesions appeared less severe in test rats given prednisolone.

Microscopic ocular lesions were present in most eyes of test rats and were similar to those of Trial I (Table 6). Castration or treatment with tetracycline had no apparent effect on the incidence of ocular lesions. The incidence of iridocyclitis, synechia and hypopyon was less in the test rats given prednisolone (Table 6).

Effects on scrotum. Scrotal swelling took a longer time to develop in rats fed the 25% fungal culture, usually occurring after 3–5 wk of feeding. Scrotal epidermal necrosis developed within 2–11 days of the initial swelling. The pattern of necrosis was similar to that seen in Trial I, starting approximately 1·0 cm distal to the prepuce and progressively spreading over most of the scrotum. The incidence of scrotal swelling and epidermal necrosis was less in test rats given prednisolone (Table 7), but treatment with tetracycline had no effect on the incidence or severity of the scrotal alterations. Swelling and necrosis of the scrotum was observed in only three of 15 castrated test rats (Table 7) and, in these rats, the lesions were associated with remnants of the epididymal fat incompletely removed at surgery.

Microscopic scrotal lesions in test rats were similar to those observed in Trial I and consisted of necrotizing cellulitis of the scrotal fascia with necrosis and ulceration of the epidermis. Diffuse orchitis was observed in about half of the rats fed the fungal diet alone or in association with tetracycline treatment. Orchitis was characterized by oedema, marked infiltration of a mixture of inflammatory cells and necrosis and calcification of the seminiferous tubules.

The incidence of microscopic scrotal lesions was greatly decreased in the castrated test rats (Table 7) as 11 of 15 had very mild or no lesions. Lesions were generally confined

Table 6. *Ocular lesions in intact and castrated rats fed a culture of* P. viridicatum *and treated with prednisolone or tetracycline* (*Trial II*)

Experimental group	Incidence of gross ocular lesions	No. of eyes examined microscopically	Microscopic lesions	Corneal oedema	Keratitis	Vascularization	Iritis	Synechia	Hypopyon
Control†	0/30	30	0	0	0	0	0	0	0
25% PV‡	29/30	30	29	24	29	18	18	13	1
25% PV‡ + prednisolone§	28/30	22	20	18	18	12	7	4	0
25% PV‡ + tetracycline§	30/30	24	23	19	22	16	13	9	7
25% PV‡ + castration	26/30	28	21	21	19	14	15	7	5

*No. of eyes affected/no. examined.
†Including tetracycline, prednisolone and castrated controls.
‡Rice culture of *P. viridicatum* fed at a level of 25% in the diet.
§Given in daily doses of 5 mg/kg body weight, prednisolone sc and tetracycline intramuscularly.

Table 7. *Microscopic lesions of the scrotum and male reproductive organs in intact and castrated rats fed a culture of* P. viridicatum *and in rats fed the fungal diet and treated with prednisolone or tetracycline* (*Trial II*)

Experimental group	No. of rats examined	No. of animals affected by				
		Scrotal cellulitis	Epidermal necrosis	Testicular lesions	Epididymal lesions	Penile lesions
Control*	6	0	0	0	0	0
25% PV†	15	11	8	7	11	0
25% PV† + prednisolone‡	15	9	6	1	8	2
25% PV† + tetracycline‡	15	13	7	8	10	0
25% PV† + castration	15	5	3§	—	—	0

*Animals given purified diet.
†Rice culture of *P. viridicatum* fed at a level of 25% in the diet.
‡Given in daily doses of 5 mg/kg body weight, prednisolone sc and tetracycline intramuscularly.
§Necrosis usually associated with suture abscesses.

to the adipose connective tissue about the cremaster muscle and consisted of infiltration of lymphocytes and numerous pigment-laden macrophages. In three rats, the lesions were more severe, and exudate and necrosis was present around the suture material. No lesions were present in the scrotal epidermis in 12 of the 15 castrated test rats. Small foci of necrosis were confined to the distal portion of the scrotum in one rat. Necrosis and ulceration of the epidermis occurred in two rats, with more severe lesions in the subcutis.

Test rats given prednisolone showed a slight reduction in the incidence of microscopic scrotal lesions compared with those given the fungal diet alone and testicular lesions were present in only one rat (Table 7). Treatment with tetracycline had no apparent effect on the incidence or severity of microscopic scrotal lesions.

Hepatic changes. Gross hepatic changes in the test rats were usually less severe than in Trial I. Mild hepatic changes included mottling, varying from dark red or pale tan to yellow, with occasional small red foci. Yellow foci, 1–2 mm in diameter, were observed in some rats of all the groups given the fungal diet. The most severe gross changes were observed in the livers of test rats given prednisolone.

Microscopic hepatic changes in the test rats consisted of necrotizing cholangitis, pericholangitis, periductal fibrosis and bile-duct proliferation, lesions similar to those observed in Trial I. The incidence of focal necrosis of hepatocytes was low in all the fungal-diet groups except in the group given prednisolone (Table 8). Hepatic lesions appeared slightly less severe in test rats given tetracycline, as the incidence of necrotizing cholangitis was reduced and focal necrosis was not observed. Castration and treatment with prednisolone had no apparent effect on the severity of the hepatic lesions (Table 8).

DISCUSSION

The observation that treatment with an antihistamine or a catecholamine depressor had no apparent protective effect against the scrotal and ocular lesions described in this study suggests that histamine and serotonin may not play a significant or major role in the pathogenesis of these lesions. However, because only a single dose level was given, it is possible that effective blood and tissue levels of the drugs were not achieved. In the scrotum, the lesions begin in the perivascular areas in the adipose connective tissue and at an early stage

Table 8. *Microscopic hepatic lesions in intact and castrated rats fed a culture of* P. viridicatum *and treated with prednisolone or tetracycline*

Experimental group	No. of rats examined	No. of animals affected by				
		Pericholangitis	Bile-duct proliferation	Periductal fibrosis	Necrotizing cholangitis	Focal necrosis
Controls*	6	0	0	0	0	0
25% PV†	15	10	11	12	10	3
25% PV + prednisolone‡	15	6	9	8	7	9
25% PV + tetracycline‡	15	8	12	9	4	0
25% PV + castration	15	10	13	14	11	1

*Animals given purified diet.
†Rice culture of *P. viridicatum* fed at a level of 25% in the diet.
§Given in daily doses of 5 mg/kg body weight, prednisolone sc and tetracycline intramuscularly.

are largely confined to these areas, indicating that the toxin(s) may have a specific action especially on the vessels of loose connective tissue. Because of the established role of histamine and serotonin in increasing vascular permeability, the initial scrotal lesions could have originated through a mechanism involving these substances. The later lesion of epidermal necrosis appeared to be secondary to ischaemia.

Prednisolone decreased the incidence and reduced the severity of the scrotal lesions and decreased the incidence of some ocular lesions, but not the incidence of corneal oedema. Prednisolone can influence several phases of the inflammatory reaction and the particular phase was not ascertained by the methods of this study, but significant anti-inflammatory properties of glucocorticoids are the effects on vascular permeability and stabilization of lysosomal membranes (Weissmann & Thomas, 1964).

Hepatic lesions were generally more severe in prednisolone-treated test rats. The reason for this is unknown, but pathogenic mechanisms of lesion induction apparently differ from organ to organ. It is possible that prednisolone-induced 'stress' activated a latent bacterial infection, as the lesions in some of the livers resembled those produced by *Corynebacterium kutscheri* (LeMaistre & Tompsett, 1952). An extensive literature points to damage to Descemet's endothelium as most significant to the development of corneal oedema. Our observations are consistent with these data, as oedema preceded any other ocular change. The hepatic lesions appear to begin with damage to the biliary epithelium followed by secondary inflammatory and reparative changes. Because neither the ocular nor hepatic lesions appear to be initially vascular, it would not be unexpected for an antihistamine, a 5-hydroxytryptamine antagonist or a corticosteroid to be ineffective in preventing their development. Prednisolone did appear to reduce the more purely inflammatory ocular lesions, such as iridocyclitis and corneal vascularization.

Castration appeared to inhibit the development of scrotal lesions, as alterations were either absent or were very mild and confined to adipose connective-tissue remnants. Further experiments would be necessary to determine whether the protective effects were due only to the removal of androgen secretion. Testosterone is known to increase the blood supply to and stimulate the growth of male accessory organs, including the scrotum (Zarrow, Yockim and McCarthy, 1964). Because the lesions in the scrotum, stomach and skin begin around vessels and appear to be the result of a blood-borne toxin, castration

may have inhibited scrotal necrosis through atrophy of the scrotum and a resulting reduction in the amount of loose connective tissue and blood supply.

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Mycotoxicose par *Penicillium viridicatum* chez le rat. IV. Essais de modification de réactions des tissus

Résumé—On a administré du chlorhydrate de tripélennamine, de la syrosingopine ou de la prednisolone à des rats mâles dont le régime alimentaire comportait à raison de 50% une culture sur riz de *Penicillium viridicatum*. D'autres groupes, dont le régime ne comportait que 25% de cette culture, ont reçu de la prednisolone ou de la tétracycline. Les rats d'un des groupes soumis à ce régime alimentaire avaient été châtrés au préalable. La tripélennamine, la syrosingopine et la tétracycline n'ont pas été capables de diminuer la fréquence ou la gravité des lésions oculaires et scrotales contractées par les rats soumis à ces essais. La prednisolone a fait preuve d'une certaine efficacité en diminuant la fréquence et la gravité des lésions du scrotum et la gravité des lésions oculaires. La tétracycline—mais non la tripélennamine et la syrosingopine—a atténué la gravité des lésions hépatiques. La castration a efficacement prévenu les lésions du scrotum, mais n'a pas entraîné de modifications des lésions des yeux et du foie.

Penicillium viridicatum-Mycotoxicose bei der Ratte. IV. Versuchen die Gewerbereaktionen zu verändern

Zusammenfassung—Männlichen Ratten wurde eine Reiskultur von *Penicillium viridicatum* in einer Konzentration von 50% in der Kost verfüttert, und es wurde ihnen Tripelenaminhydrochlorid, Syrosingopin oder Prednisolon verabreicht. Anderen Gruppen wurde die Pilzdiät zu 25% in der Kost verfüttert, und es wurde ihnen Prednisolon oder Tetracyclin verabreicht. Eine mit dieser Kost gefütterte Gruppe wurde vor Beginn kastriert. Tripelenamin, Syrosingopin und Tetracyclin zeigten keinen Effekt, das Auftreten oder die Stärke der ocularen und scrotalen Läsionen zu reduzieren, die sich in den getesteten Ratten entwickelten. Prednisolon zeigte einigen Effekt in der Reduktion des Auftretens und in der Stärke der scrotalen Läsionen und verringerte die Stärke der ocularen Läsionen. Kastrierung verhinderte effektiv die scrotalen Läsionen, aber veränderte nicht die der Augen und Leber.

Fd Cosmet. Toxicol. Vol. 12, pp. 341–350. Pergamon Press 1974. Printed in Great Britain

LIVER RESPONSE TESTS. IX. CYTOPATHOLOGICAL CHANGES IN THE ENLARGED BUT HISTOLOGICALLY NORMAL RAT LIVER

P. GRASSO, M. G. WRIGHT, S. D. GANGOLLI and R. J. HENDY
British Industrial Biological Research Association, Woodmansterne Road, Carshalton, Surrey, SM5 4DS, England

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Abstract—At some dose levels, coumarin and 2,4-xylidine produce liver enlargement in rats without histological evidence of hepatocellular damage. When administered by gavage, coumarin produced this effect at a dose level of 135 mg/kg/day. Biochemically, cytochrome *P*-450 and amidopyrine demethylase were depressed by coumarin but remained at control values inrrespective of the dose of 2,4-xylidine administered. Histochemical and ultrastructural investigations on the enlarged livers produced by both coumarin and 2,4-xylidine revealed that the lysosomes in most of the centrilobular hepatocytes were translocated from their normal peribiliary distribution and many were grossly enlarged. Recognizable cell constituents were present in many of these lysosomes (autophagic vacuoles). In addition there was a centrilobular depression of glucose-6-phosphatase and aniline hydroxylase. Both the liver size and the severity of these cytological changes were related to the dose administered. They indicated the presence of liver damage despite a normal histological appearance. At a daily dose of 405 mg/kg, coumarin caused histologically demonstrable vacuolar degeneration and fatty change as well as liver enlargement.

Coumarin at dose levels of 15 and 45 mg/kg/day and 2,4-xylidine at 10 mg/kg/day did not produce liver enlargement. At these dose-levels, no differences from controls treated with arachis oil alone were detected biochemically, histochemically or ultrastructurally.

INTRODUCTION

The significance of the enlarged histologically normal liver has been a controversial issue in toxicology for several years (Gilbert & Golberg, 1965; Rowe, Wolf, Weil & Smyth, 1959; Weil & McCollister, 1963). Frequently, such enlargement is accompanied by an increase in drug-metabolizing enzymes, and there is considerable evidence that this increase in enzyme activity accelerates the metabolism of the chemical responsible for its induction. It is thought that this increased capacity of the liver is a physiological response to an increased demand for a particular metabolic process rather than a toxic manifestation—a so-called 'work hypertrophy' (Conney, 1967; Golberg, 1966). Examples of compounds that produce this type of liver enlargement are butylated hydroxytoluene, phenobarbitone, SKF 525A, DDT and dieldrin. These and other examples are discussed in reviews by Conney (1967), Gillette, Davis & Sasame (1972), Golberg (1966) and Remmer (1967).

However, there are some compounds, such as coumarin, which produce liver enlargement in rats without stimulating the activity of microsomal enzymes. This type of liver enlargement has not been investigated as fully as that associated with microsomal-enzyme induction, but the work of Feuer, Golberg & Le Pelley (1965) showed that some biochemical changes could be detected in the enlarged liver produced by coumarin. These involved a reduction of glucose-6-phosphatase (G6P) and glycogen and an increase in glucose-6-phosphate dehydrogenase (G6PD). The authors paid particular attention to the significance of G6P depression and came to the conclusion that depression of this enzyme might

indicate hepatocellular damage. This conclusion was reached because a number of known hepatotoxic compounds of diverse chemical structure also depressed the activity of G6P. However, there were some anomalous observations. Only a moderate depression of enzyme activity resulted from the administration of relatively large doses of the hepatotoxins safrole, allyl alcohol and aflatoxin, while 2-phenylindole, listed by the authors as non-hepatotoxic, produced a profound fall (Feuer *et al.* 1965). Moreover, the level of G6P activity may also be affected by a number of other factors, which may therefore interfere with the results obtained with a compound under test. Thus fasting (Ashmore, Hastings & Nesbett, 1954) and cortisone (Weger & Singhal, 1964) increase the biochemical level of enzyme activity, while the development of various tumours depresses the enzyme (Pitot, 1960).

In our study we have investigated the effects produced in rats by coumarin and 2,4-xylidine, each of which produces liver enlargement without inducing microsomal-enzyme activity. Because of the limitations of G6P as an index of hepatocellular damage, we have investigated changes in lysosomal acid phosphatase, since the lysosome is known to participate in the reaction of the cell to injury. We have adopted a histochemical approach which enables lysosomal changes to be identified in specific cells and allows complementary electron-microscopic investigations to be carried out on these organelles. We have also attempted to correlate these lysosomal changes with the degree of liver enlargement on the one hand and the depression of G6P activity on the other. A suggested classification of liver enlargement is shown in Fig. 1.

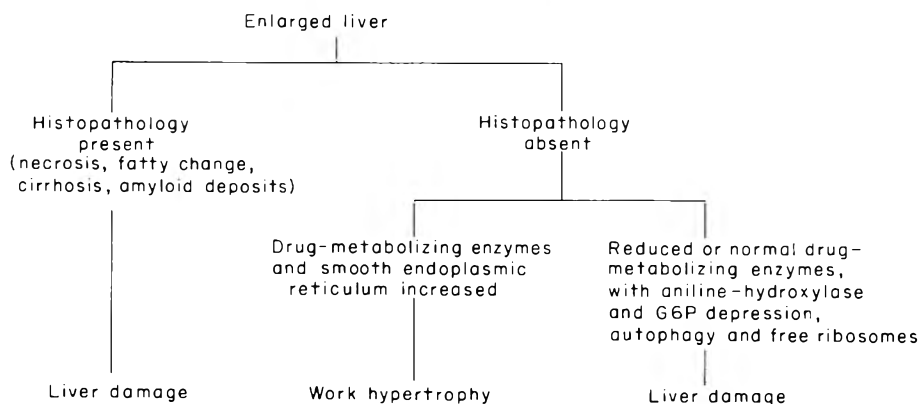


Fig. 1. Types of enlarged liver.

EXPERIMENTAL

Animals and diet. SPF male rats of the Carworth Farm E or Tuck albino strain were used. They weighed 150–250 g at the commencement of treatment. They were kept in cages of six and allowed free access to Spillers' Laboratory Small Animal Diet and water.

Treatment. Groups of six rats were used for each dose level in both the coumarin and 2,4-xylidine experiments. Coumarin was prepared as a 2% solution in arachis oil and was administered at dose levels of 15, 45, 135 and 405 mg/kg. 2,4-Xylidine was prepared as a 5 or 2·5% solution in arachis oil and was administered in doses of 10, 25, 50, 100 and 250 mg/kg. Each dose level of coumarin or 2,4-xylidine was given by oral intubation daily for 7 days. Controls were treated with 0·5 ml arachis oil daily. At the end of treatment,

rats were starved overnight and killed by decapitation. Each liver was removed and weighed and samples were taken for histological, histochemical, ultrastructural and biochemical examinations.

Methods of examination

Histology. Tissue was fixed in neutral buffered formalin, and paraffin sections, about 5 μm thick, were stained with haematoxylin and eosin. Frozen sections of formalin-fixed tissue were cut at 15 μm and stained with Oil Red O.

Histochemistry. Demonstration of G6P (Wachstein & Meisel, 1956) and aniline hydroxylase (Gangolli & Wright, 1971) was carried out on cryostat sections cut at 15 μm. Lysosomal acid phosphatase was demonstrated on liver tissue fixed in formol-calcium using a Gomori (1952) technique.

Electron microscopy. Cubes of liver (1 mm^3) were fixed in 1% osmium tetroxide buffered with sodium cacodylate (pH 7·4) for 1 hr at 4°C. The cubes were dehydrated in alcohol and embedded in Epon resin. Sections (1 μm) were cut and stained with toluidine blue for light microscopy. Selected areas were cut on an LKB ultramicrotome and ultra-thin sections were stained with uranyl acetate and lead citrate and examined in an AEI EM6B electron microscope.

Biochemical studies. Livers were homogenized in 3 vols ice-cold 1·15% KCl solution containing 1 mM EDTA at pH 7·4 in a teflon glass homogenizer. The microsomal fraction was obtained by the centrifugation method of Kato & Gillette (1965). The pellet was resuspended in ice-cold KCl–EDTA solution, 1·0 ml of this suspension being equivalent to 400 mg liver. The amidopyrine-demethylase activity of the microsomal suspension was determined by the method of Anders & Mannering (1966) and the cytochrome *P*-450 activity by the method of Omura & Sato (1963).

RESULTS

Liver enlargement

The liver weight expressed relative to body weight (RLW) was related to the dose of the compound administered. There was no increase in the RLW of rats treated with 10 mg 2,4-xylidine/kg or with 15 or 45 mg coumarin/kg. When higher doses of either coumarin or 2,4-xylidine were administered, however, the RLW was increased and showed a dose-related response (Figs 2 & 3). The increase in RLW was greater in rats treated with 2,4-xylidine than in those treated with coumarin despite the fact that, on a molar basis, the doses of xylidine were smaller than those of coumarin.

Histology

Histological changes were observed only at the highest dose level in coumarin-treated rats. These consisted of extensive fatty change and vacuolar degeneration in the centrilobular hepatocytes (Fig. 4). At other dose levels, no morphological changes were observed. No histological changes were observed in the livers of rats treated with 2,4-xylidine at any dose level.

Histochemical changes

G6P and aniline hydroxylase. A centrilobular loss of G6P and aniline hydroxylase was observed in the livers from all rats treated with the two higher dose levels of 2,4-xylidine

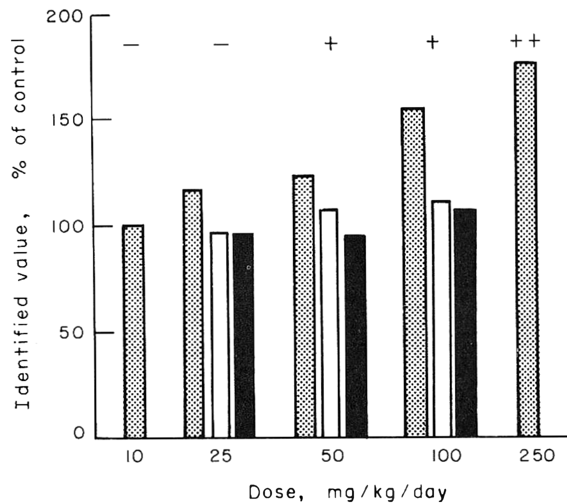


Fig. 2. Biochemical and morphological effects in the liver of rats intubated with 2,4-xylidine in doses of 10–250 mg/kg/day showing relative liver weight (▒), cytochrome P-450 level (□) and amidopyrine-demethylase activity (■) expressed as percentages of control values. The finding of centrilobular loss of glucose-6-phosphatase, aniline hydroxylase and acid phosphatase (Gomori) plus autophagy is indicated +/++ and its absence by —.

or coumarin. Two out of four rats given 50 mg 2,4-xylidine/kg showed a centrilobular loss of G6P and three of these four rats showed also a centrilobular loss of aniline hydroxylase. The loss of G6P activity was profound in the affected cells irrespective of the dose or compound given (Fig. 5). At the lowest dose at which this effect was observed, only a few cells around the central vein showed a loss of G6P activity, but with increasing doses the number of cells affected was progressively greater, so that at the highest dose almost half the cells forming the lobule had lost their activity (Fig. 5).

Loss of aniline hydroxylase activity was also seen in the centrilobular areas. While it was not nearly as profound as that of G6P, it showed a relationship to dose. The loss of

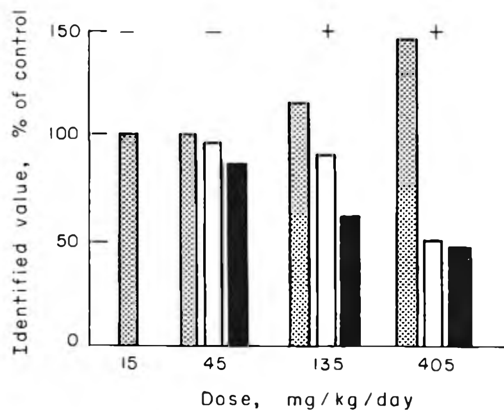


Fig. 3. Biochemical and morphological effects in the liver of rats intubated with coumarin in doses of 15–405 mg/kg/day, showing relative liver weight (▒), cytochrome P-450 level (□) and amidopyrine-demethylase activity (■) expressed as percentages of control values. The finding of centrilobular loss of glucose-6-phosphatase, aniline hydroxylase and acid phosphatase (Gomori) plus autophagy is indicated by +/++ and its absence by —.

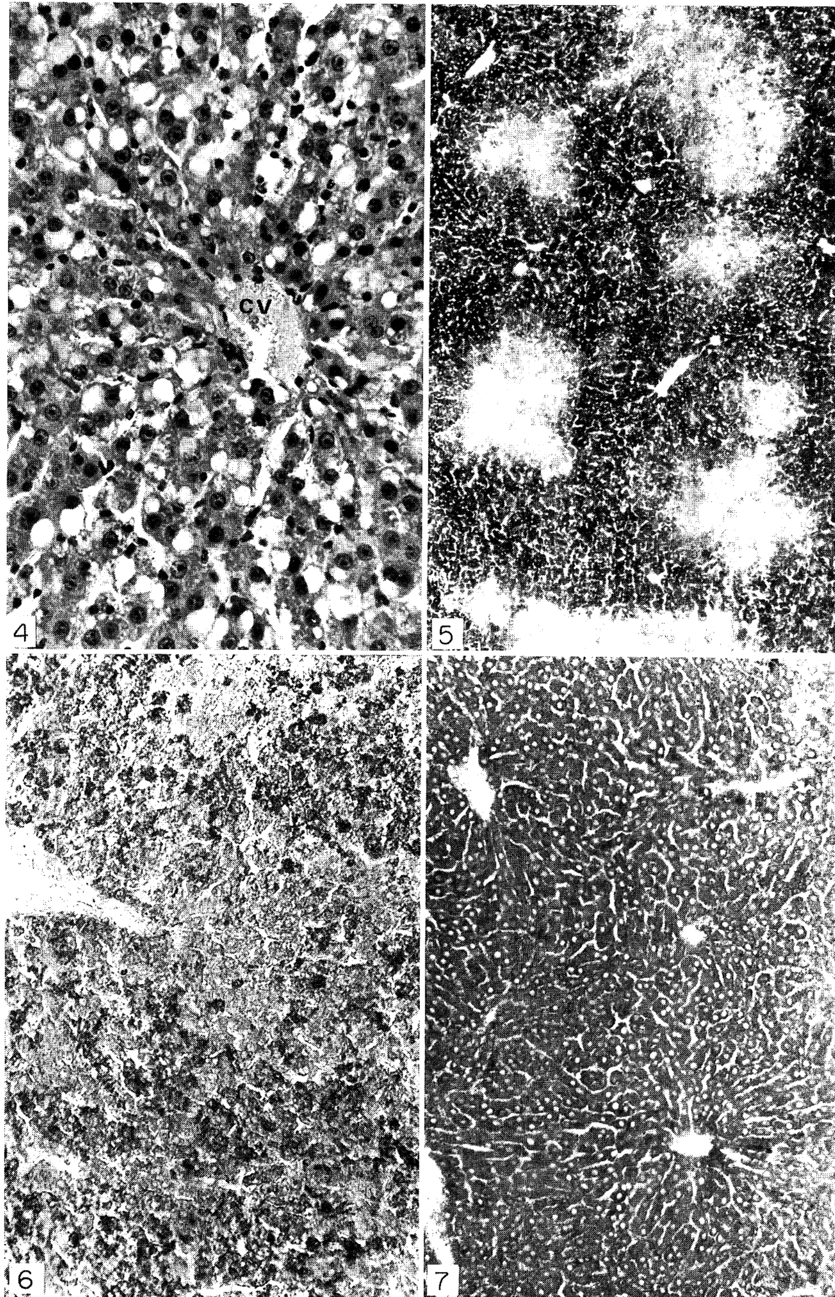
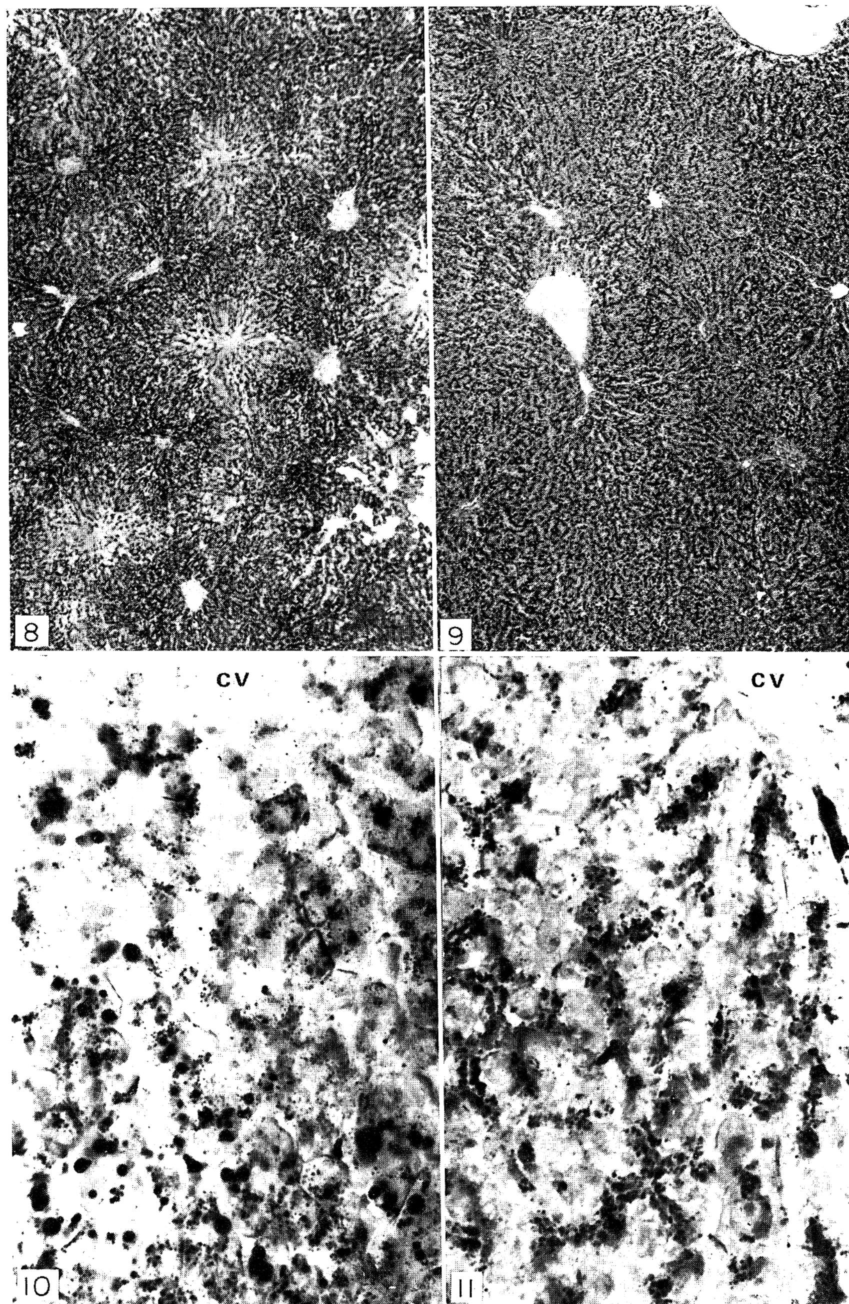


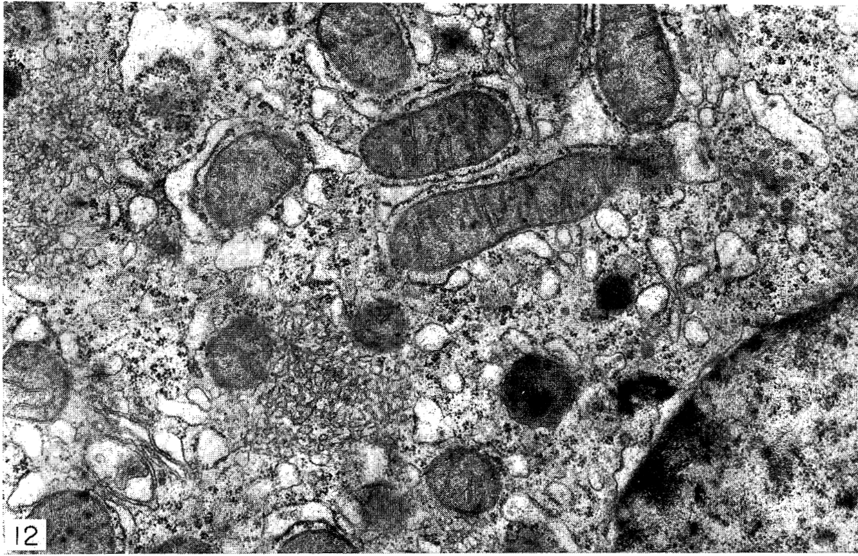
Fig. 4. Vacuolar degeneration and fatty change in centrilobular hepatocytes around the central vein (CV), from a rat intubated with 405 mg coumarin/kg/day. Haematoxylin and eosin × 400.

Fig. 5. Glucose-6-phosphatase activity in rat hepatocytes, showing centrilobular and mid-zonal loss after intubation with 250 mg 2,4-xylidine/kg/day. Wachstein & Meisel (1956) lead method × 40.

Figs 6 & 7. Aniline hydroxylase activity in rat hepatocytes. Extensive depression of enzyme activity in centrilobular cells following intubation with 405 mg coumarin/kg/day, but with some cells retaining their activity (Fig. 6), compared with the uniform lobular distribution in a control rat (Fig. 7). Gangolli & Wright (1971) method × 40.



Figs 8–11. Lysosomal acid-phosphatase activity (Gomori-type method) in rat liver. Centrilobular loss of activity after intubation with 405 mg coumarin/kg/day (Fig. 8) is compared with activity in a control liver (Fig. 9). Gomori-type method × 40. Figure 10 shows enlarged lysosomes and disturbance of the pericanalicular arrangement after dosage with 405 mg coumarin/kg/day and Fig. 11 the corresponding picture in a control liver. Gomori-type method × 800.



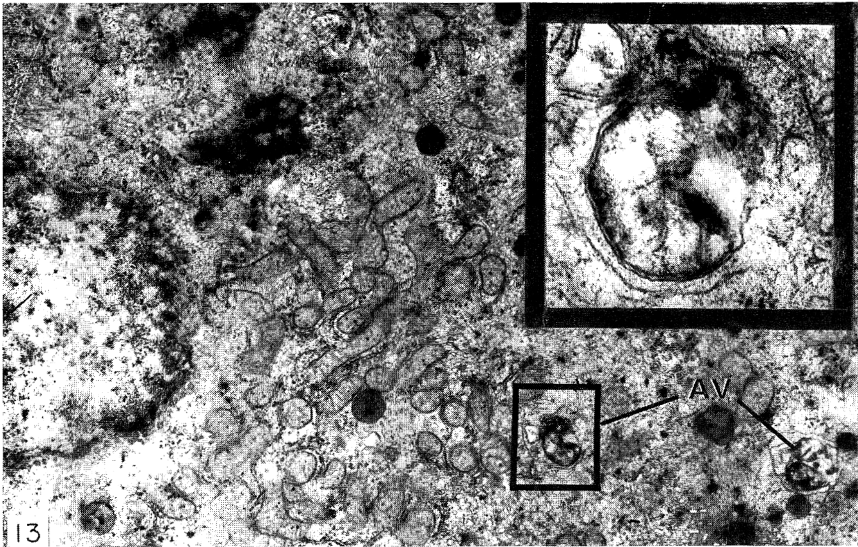


Fig. 12. Free ribosomes and collections of smooth endoplasmic reticulum in the liver of a rat intubated with 100 mg 2,4-xylidine/kg/day. × 20,000.

Fig. 13. Autophagy (AV) in the liver of a rat intubated with 250 mg 2,4-xylidine/kg/day. × 10,000. *Inset*: Autophagic vacuole. × 45,000.

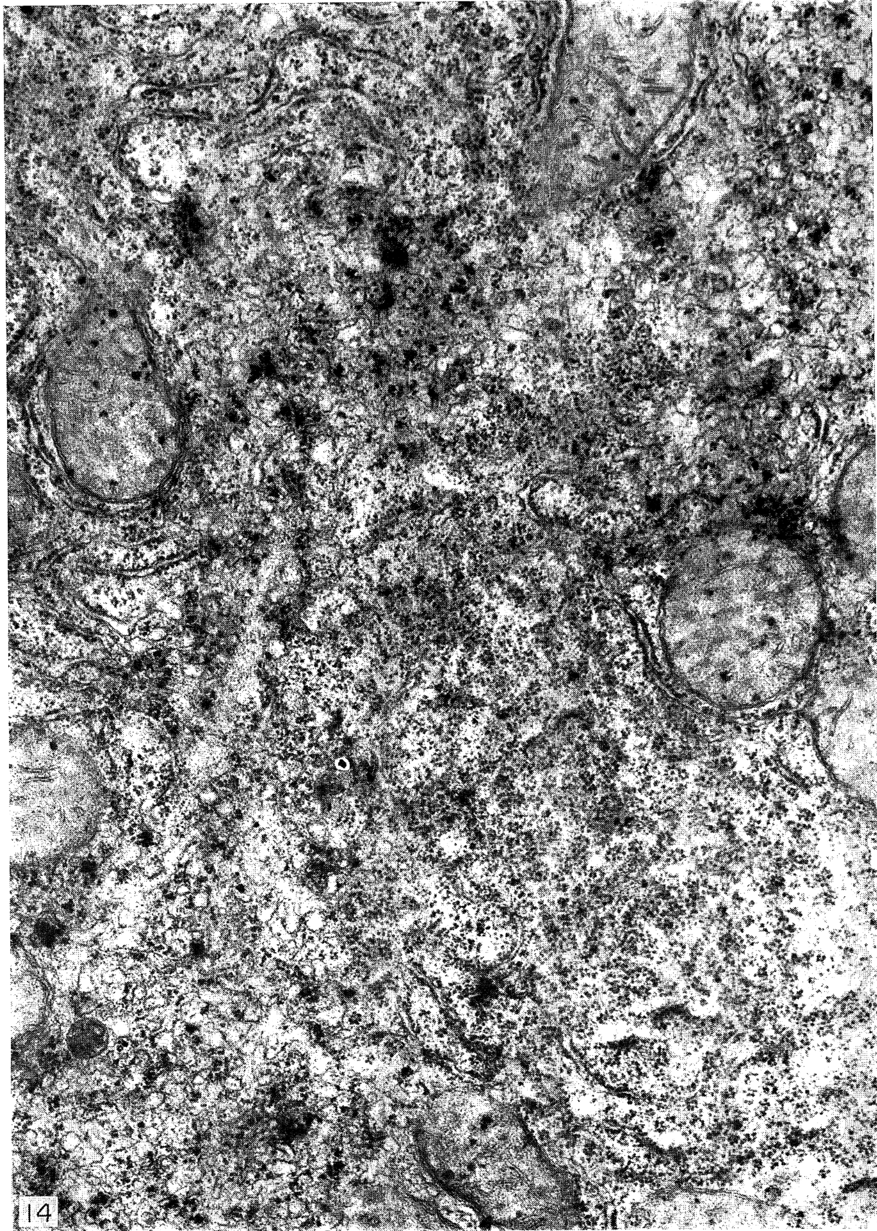


Fig. 14. Free ribosomes and hypertrophy of rough endoplasmic reticulum in the liver of a rat intubated with 405 mg coumarin/kg/day. × 40,000.

activity was apparent in the majority, but not all, of the cells of the affected regions (Figs 6 & 7). In the case of rats treated with 2,4-xylidine, some of the cells stained more intensely than controls, indicating a considerable increase in the activity of the enzyme.

The lower dose level of xylidine and the two lower dose levels of coumarin, which did not affect the RLW, caused no detectable changes in the activity or distribution of either enzyme.

Lysosomal acid phosphatase. Lysosomal changes, demonstrated by the Gomori technique, were observed in all the enlarged livers produced by coumarin or 2,4-xylidine. The greater the degree of liver enlargement, the more pronounced were the lysosomal changes. No such changes were seen in the absence of liver enlargement. The lysosomal changes were confined almost exclusively to the centrilobular areas and were similar in character with each test compound, despite the dissimilarity in the chemical structure of the two compounds used.

At low magnification, the centrilobular areas of livers showing lysosomal changes appeared pale (Fig. 8) compared with those of control livers (Fig. 9). At higher magnifications, the hepatocytes in the pale areas were seen to contain only a few lysosomes. These lysosomes were not confined to the pericanalicular area but were distributed haphazardly throughout the cell and were considerably larger than normal. At the dose levels producing minimal liver enlargement, both the centrilobular pallor and the lysosomal changes were limited to one or two layers of cells close to the central vein, but at the highest dose levels almost one-third of the lobule was involved, the majority of the cells in the centrilobular area exhibiting large lysosomes (Fig. 10). A corresponding area from a control liver is shown in comparable magnification in Fig. 11.

Ultrastructural studies

With both compounds, ultrastructural changes were seen at dose levels that induced liver enlargement. At doses that did not affect liver weight, no ultrastructural differences from controls were observed. The ultrastructural changes seen in the enlarged livers were confined to the hepatocytes from the centrilobular areas and consisted of gross hypertrophy and dilatation of the rough endoplasmic reticulum (Fig. 12) and increases in the number and size of the lysosomes and the incidence of autophagic vacuoles (Fig. 13). Hypertrophy of the rough endoplasmic reticulum was accompanied by a more frequent occurrence of 'pools' of free ribosomes (Fig. 14). In livers from rats treated with 2,4-xylidine an occasional cell exhibited hypertrophy and dilatation of the smooth endoplasmic reticulum (Fig. 12).

The Golgi apparatus was considerably hypertrophied and exhibited lipoprotein droplets, but no changes were observed in the mitochondria or nuclei. These ultrastructural changes were less marked with the lower doses.

Biochemical changes

The activities of cytochrome *P*-450 and amidopyrine demethylase did not increase above control levels in rats treated with 2,4-xylidine despite an increase in the liver weight. In the case of coumarin, dose-related depressions of cytochrome *P*-450 and of amidopyrine demethylase were observed. In rats given 135 mg coumarin/kg, these depressions (expressed as a percentage of the control values) were to 91 and 62%, and in those given 405 mg/kg they were to 51 and 48%, respectively.

DISCUSSION

Extensive pathological changes have been reported in the rat liver following the administration for several months of 2,4-xylidine or coumarin. 2,4-Xylidine caused cirrhosis in rats when added to the diet at a level of 10,000 ppm (600 mg/kg body weight) for 6 months (Lindstrom, Hansen, Nelson & Fitzhugh, 1963) while coumarin given at 5000 ppm in the diet produced extensive hepatocellular necrosis, cholangiofibrosis and bile-duct proliferation (Bär & Griepentrog, 1967). At a lower level (1000 ppm), however, there were no pathological effects (Bär & Griepentrog, 1967). The results of these feeding studies provide some indication of the hepatotoxic potential of the two compounds at the dose levels used in our experiments, so that despite the normal histological appearance of the liver after a short period of administration, the occurrence of adverse changes at the subcellular levels must be suspected. Our demonstration of the presence of autophagy and the depression of G6P and aniline hydroxylase provides evidence that, in fact, such changes were occurring.

Significance of autophagy

Autophagy is a process whereby the cell surrounds certain areas of its cytoplasm by a single or double membrane. These enclosed areas contain recognizable cytoplasmic organelles, which are either completely normal or display a certain degree of morphological disorganization (Ericsson, 1969; Ericsson & Trump, 1964). Lysosomal enzymes appear at an early stage in these vacuoles and can be demonstrated cytochemically by the appropriate procedures at light- and electron-microscopic level (Abraham, Dawson, Grasso & Golberg, 1968). The contents of the autophagic vacuoles are thought to be digested by the lysosomal hydrolases, so that eventually a small residual body of amorphous material remains (de Duve, 1963; Grasso, Muir, Golberg & Batstone, 1968).

Autophagy may occur in viable cells that have not been subjected to injury. It is thought to be a process associated with the removal of ageing or effete parts of the cell, and has been described not only in the rat liver but also in many tissues of vertebrate and invertebrate species, particularly in organs undergoing metamorphosis (Cole, Matter & Karnovsky, 1971; de Duve & Wattiaux, 1966; Weber, 1969; Woessner, 1969).

In the livers of untreated healthy rats, vacuoles containing recognizable cell organelles are found in only a very few cells (R. J. Hendy, unpublished observations 1972; Pfeifer, 1971 & 1972). If the liver is damaged by hypoxia, hyperoxia or a variety of chemical agents, the frequency with which autophagy is encountered is considerably increased, so that in severe cases up to three or four autophagic vacuoles/cell may be seen in a substantial proportion of hepatocytes (Abraham *et al.* 1968; Abraham, Golberg & Grasso, 1967; Arstila & Trump, 1968; Hendy & Grasso, 1972; Kerr, 1967; Kerr & Pound, 1966). These authors identified autophagy by electron microscopy, but in this study we obtained a good indication of an enhanced formation of autophagic vacuoles from light-microscope histochemical preparations of complementary pieces of liver tissue, in which we observed grossly enlarged lysosomes scattered randomly throughout the liver cells.

Light- and electron-microscopic changes similar to those described by the authors quoted were found in the enlarged livers produced by the administration of either coumarin or 2,4-xylidine, so that on this basis the liver enlargement produced by these two compounds must be regarded as a toxic response.

Significance of G6P depression

G6P is thought to form an integral part of the protein-phospholipid structure of the endoplasmic reticulum (Arion, Wallin, Carlson & Lange, 1972; Mahler & Cordes, 1969). It has been shown by a number of workers in the biochemical field that depression of G6P, whether produced *in vitro* by the treatment of microsomal fractions with phospholipases or *in vivo* by the treatment of rats with hepatotoxic agents, may be partially reversed by the addition of phospholipids derived from a variety of sources (e.g. plants or hepatic microsomes of beef) and is totally reversed by detergents (Arion *et al.* 1972; Feuer & Golberg, 1967; Kaschnitz & Mittermayer, 1969; Snoke & Nordlie, 1972).

Feuer & Golberg (1967) regarded a depression of G6P activity as a largely reversible alteration in the state of binding of the enzyme protein to the microsomal phospholipid or alternatively as the result of some equally reversible conformational change in the enzyme protein itself consequent upon alterations effected on the associated phospholipid. Kaschnitz & Mittermayer (1969) supported these interpretations but suggested that such changes were only likely to occur when the enzyme activity was severely depressed (to about 50% of control values). These interpretations do not appear to have been challenged despite the current controversy on the mechanism by which phospholipids and detergents restore the enzyme activity (Arion *et al.* 1972; Snoke & Nordlie, 1972).

If one assumes, on the basis of the work carried out by Chiquoine (1955), that the G6P demonstrated histochemically is the same as that assayed biochemically, the histochemical depression of G6P activity would have the same connotation as the depression of the enzyme observed biochemically, so that one would assume that both coumarin and 2,4-xylidine produced some change in the endoplasmic reticulum of the centrilobular cells. This change must have occurred at molecular level since we observed no dispersal of the endoplasmic reticulum. In this context, coumarin and 2,4-xylidine differ from carbon tetrachloride (Reynolds, Ree & Moslen, 1972) and carbon disulphide (Butler, Chandra & Magos, 1974). Both these compounds produce pronounced morphological changes in the endoplasmic reticulum as well as a profound histochemical and biochemical depression of G6P.

Despite the extensive work carried out on G6P, it is not yet possible to understand fully the significance of depression of this enzyme in terms of hepatocellular damage. The biochemical evidence is insufficient to provide an unequivocal indication that depression of G6P is indicative of membrane damage. In our studies, this change in activity was seen histochemically in the same zone of the liver lobule as autophagy. If one takes into account also the studies on carbon tetrachloride (Chopra, Roy, Ramalingaswami & Nayak, 1972), carbon disulphide (Butler *et al.* 1974) and diethyl- and dimethylnitrosamines (R. J. Hendy, unpublished observations 1973), studies which demonstrate that loss of G6P occurs only in that zone of the hepatic lobule in which necrosis subsequently appears, it seems that depression of the activity of this enzyme could be interpreted as one of the manifestations of a toxic effect on the liver cell.

Significance of depression of aniline hydroxylase

Aniline hydroxylase, too, is found in the endoplasmic reticulum (Gangolli & Wright, 1971). The depression of its activity observed histochemically in rats treated with coumarin is consistent with the biochemical depression of microsomal enzymes. The centrilobular depression of aniline hydroxylase found in the case of 2,4-xylidine does not appear to correlate with the apparently unaffected levels of P-450 and amidopyrene demethylase found

on biochemical assay. It would appear from our histochemical results, however, that the effect of 2,4-xylidine on the liver cells is variable, some cells showing a clear depression and others a marked elevation of activity. In this situation, it is possible that some cells may compensate for the depressed activity of other cells so that the overall level of enzyme activity is unaffected.

Ultrastructural changes

The hypertrophy of the rough endoplasmic reticulum resulting from treatment with either coumarin or 2,4-xylidine indicated that protein synthesis was enhanced (Campbell & Lawford, 1968). Our morphological observations agree with the biochemical results obtained by Nievel (1969), who demonstrated an increase in protein synthesis in the liver of rats given 50–450 mg coumarin/kg daily for 3–7 days. The increase involved both the microsomal and soluble fractions, suggesting that this activity was spread throughout the cytoplasm.

Hypertrophy of the smooth endoplasmic reticulum was found in some liver cells from rats treated with 2,4-xylidine. Such a change was consistent with microsomal-enzyme induction (Meldolesi, 1967) and its occurrence in only a few cells was in keeping with our histochemical findings of an increase in the activity of aniline hydroxylase in some of the hepatocytes.

Conclusion

Histochemical and electron-microscopic changes demonstrated in the enlarged liver produced by 2,4-xylidine or coumarin indicate that these compounds are producing toxic effects. At doses that do not produce liver enlargement, no such changes were observed.

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Tests de réactions du foie. IX. Modifications cytopathologiques du foie hypertrophié, mais histologiquement normal, chez le rat

Résumé—Chez le rat, la coumarine et la 2,4-xylidine provoquent, à certaines doses, une hypertrophie du foie sans aucun indice de lésions cellulaires de cet organe. La coumarine administrée par gavage a eu cet effet à la dose de 135 mg/kg/jour. Sur le plan biochimique, le cytochrome *P*-450 et l'amidopyrine-déméthylase ont diminué sous l'effet de la coumarine, mais n'ont pas changé sous celui de la 2,4-xylidine, quelle que fût la dose administrée. L'examen histochimique et ultrastructurel des foies hypertrophiés par les deux produits a montré que les lysosomes de la plupart des hépatocytes centrilobulaires ne se présentaient plus selon leur distribution péribiliaire normale et que beaucoup étaient fortement hypertrophiés. Des éléments cellulaires reconnaissables étaient présents dans beaucoup de ces lysosomes (vacuoles autophages). On a constaté en outre une diminution centrilobulaire de la glucose-6-phosphatase et de l'aniline-hydroxylase. La taille du foie et la gravité de ces modifications cytologiques étaient tous deux en corrélation avec la dose administrée et indiquaient la présence de lésions hépatiques nonobstant l'apparence histologique normale.

A la dose de 405 mg/kg/jour, la coumarine a provoqué, en plus de l'hypertrophie du foie, une dégénérescence vacuolaire et des altérations graisseuses histologiquement démontrables.

Des doses de 15 et de 45 mg/kg/jour de coumarine et de 10 mg/kg/jour de 2,4-xylidine n'ont pas fait augmenter la taille du foie. A ces dosages on n'a décelé à l'examen biochimique, histochimique ou ultrastructurel aucune différence avec les animaux témoins traités uniquement à l'huile d'arachide.

Untersuchungen der Leberreaktion. IX. Cytopathologische Veränderungen in der vergrößerten aber histologisch normalen Rattenleber

Zusammenfassung—Bei einigen Dosierungsspiegeln bewirkt Cumarin und 2,4-Xylidin Lebervergrößerung in Ratten ohne histologischen Beweis eines Hepatocellularschadens. Oral verabreicht, zeigt Cumarin diesen Effekt bei einer Dosis von 135 mg/kg pro Tag. Biochemisch gesehen wurden Cytochrom *P*-450 und Amidopyrin demethylase durch Cumarin herabgesetzt; aber sie verblieben bei den Kontrollwerten, unabhängig von der Gabe von 2,4-Xylidin. Histochemische und ultrastrukturelle Untersuchungen der durch sowohl Cumarin als auch 2,4-Xylidin vergrößerten Lebern ergaben, daß die Lysosomen in den meisten der centrilobularen Hepatocyten transloziert waren von ihrer normalen peribiliaren Verteilung und daß viele sehr vergrößert waren. Erkennbare Zellbaustoffe waren vorhanden in vielen dieser Lysosomen (autophagische Vacuolen). Außerdem war eine centrilobulare Depression von Glucose-6-phosphatase und Anilinhydroxylase vorhanden. Sowohl Lebergröße als auch Stärke der cytologischen Veränderungen standen im Zusammenhang mit der gegebenen Dosis. Sie weisen auf das Vorhandensein von Leberschäden hin, trotz eines normalen histologischen Bildes. Bei einer täglichen Gabe von 405 mg/kg bewirkte Cumarin histologisch darstellbare Vacuolardegeneration und Fettveränderungen als auch Leververgrößerung.

Bei einer Dosis von 15 und 45 mg/kg pro Tag produzierte Cumarin und 2,4-Xylidine bei 10 mg/kg pro Tag keine Lebervergrößerung. Bei diesen Dosierungen wurden keine biochemischen, histochemischen oder ultrastrukturellen Unterschiede gegenüber den Kontrolltieren gefunden, die mit Arachisöl allein behandelt wurden.

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GASTRO-INTESTINAL ABSORPTION AND TOXICOLOGY: INGESTA-EXCHANGE AND SIMULATED-MEAL TECHNIQUES

J. C. PEKAS

United States Department of Agriculture, Agricultural Research Service, Metabolism and Radiation Research Laboratory, Fargo, North Dakota 58102, USA*

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Abstract—The ingesta-exchange technique involves the homogeneous incorporation of a test substrate into ingesta recovered from donor animals and subsequent administration of the mixture to recipient animals. This and the simulated-meal technique, which involves incorporation of the test substrate into semi-liquid preparations of normal diets, overcome many of the limitations of conventional oral dosing of animals with microgram quantities of synthetic non-nutritive substances, especially if these substances are lipophilic and have high toxicity or biological activity. The techniques are compatible with time-course studies and are especially useful for studies involving non-absorbable markers. Equipment is described for the extrusion of semi-solid donor gastric ingesta or simulated meals. The rate and dynamics of absorption of a lipophilic carbamate were much slower and delayed after an ingesta-exchange dose than after a conventional dose given by oesophageal cannula, while the extent of metabolism was apparently greater after the ingesta-exchange dose.

INTRODUCTION

Methods for studying gastro-intestinal absorption using intact or surgically modified animals and isolated tissue preparations have been reviewed recently (Parsons, 1968; Wilson, 1962). A complete study of the absorption of non-nutritive synthetic substances includes the determination of the extent of absorption and the primary site(s) in the gut where absorption occurs. Preliminary experiments at this laboratory have shown that oral administration of microgram or smaller quantities of synthetic non-nutritive substances to fed animals is associated with a wide variation in measured absorption parameters. Lipophilic substrates generally permeate the gastro-intestinal and other cellular barriers more rapidly than hydrophilic substrates of similar molecular size (Brodie, 1964) and they are difficult to administer because they are insoluble in physiological solvents. Dietary ingredients, such as corn or cottonseed oil, dextrose, sucrose, casein and egg white, have been used as vehicles for dosing but have been found to be toxic themselves, when used in massive quantities and, consequently, to interfere with the study of the test chemical (Boyd & Boulanger, 1969; *Food and Cosmetics Toxicology*, 1970; Krijnen & Boyd, 1970). Organic solvents are poor vehicles because they are toxic and are liable to damage cell membranes, an effect which is particularly undesirable in studies of absorption across epithelial cells.

The techniques described in this communication were conceived to overcome these problems. They involve incorporation of microquantities of non-nutritive substrates (xenobiotics) into either ingesta or semi-liquefied meals, which are subsequently administered orally to animals in normal quantities.

*Reference to a company or product name does not imply approval or recommendation of the product by the US Department of Agriculture to the exclusion of others that may be suitable.

Gastro-intestinal absorption and/or metabolism of microquantities of xenobiotics after natural ingestion are likely to differ both qualitatively and quantitatively from those occurring after experimental administration of oral doses. Consequently, toxicity studies of xenobiotics may yield different results under the two conditions. Ingested plant and animal tissues are presumed to compete with the epithelial lining of the gastro-intestinal tissues for those substrates (i.e. lipophilic substances) which have an inherent affinity for tissues, and the ingesta bulk is assumed to act as a vehicle to carry the substrate down the gastro-intestinal tract and to dilute substrate concentration. This is in contrast to the role of organic solvents, which are absorbed and do not function as a bulk carrier or diluent.

The composition of ingesta from naturally consumed foodstuffs is relatively homogeneous, in sharp contrast to the situation existing in the gastric compartment of fed animals given an oral dose of liquid or crystalline substrates by conventional methods. High variability is certain after conventional oral doses. The sites at which injected liquid doses or swallowed capsules, tablets or pill doses are deposited are random and variable because they cannot be controlled. The dose deposits vary from sites directly on the gastric epithelium to sites in the centre of the ingesta mass of an engorged stomach. Absorption in the first instance can be instantaneous; in other instances, in the same animal under the same conditions, absorption will be severely delayed because the bolus of ingesta containing the dose must be mobilized to make contact with the gastric or intestinal epithelium, or the substrate must diffuse from the bolus to the epithelium. The average result of many tests must fall between these extreme limits.

These variations are likely to affect substrate concentrations within the ingesta bulk and tissues (and the degree of toxicity if the substrate is poisonous), the rates of passage and the resultant blood concentrations at various times. The variabilities associated with conventional oral dosing are acceptable in studies of absorption after therapeutic administration or after accidental ingestion. Unfortunately, the same variabilities apply when conventional oral dosing methods are employed in experiments intended instead to characterize absorption under natural circumstances (where the substrate would be ingested in contaminated foodstuffs or in premixed experimental diets). Consequently, results obtained after conventional oral dosing of fed or fasted animals can be grossly misleading as far as the absorption and toxicology of naturally ingested xenobiotics are concerned.

Obvious differences were observed in studies of the gastro-intestinal absorption and metabolism of the pesticide, naphthyl *N*-methylcarbamate (carbaryl), after conventional and ingesta-exchange dosage methods.

EXPERIMENTAL

Ingesta-exchange technique

In this procedure, ingesta recovered from rats *post mortem* was used as the dose-carrier vehicle. The donor ingesta, obtained from the engorged stomachs of rats that had just eaten after a fast, was solid and appeared as damp feed. Dilutions between 15 and 25%, using dilute HCl of the same pH as the undiluted ingesta, were required before the ingesta could be properly manipulated. However, gastric ingesta pooled from several non-fasted donor rats was satisfactory without dilution. Considerable variation in the degree of ingesta fluidity was observed, however, and was presumably related to the length of time since the rat had eaten. The donor ingesta was maintained over ice during preparation

for dosing and were brought to body temperature before administration to the recipient animal by extrusion of 5–7 g in semi-solid form under high pressure directly into the stomach through an oesophogeal cannula (Fig. 1a), thus eliminating the necessity for surgical intervention in the form of a gastric fistula. During administration of the ingesta, the cannula was protected by passing it through a perforated wooden rod which held the rat's jaws apart (Fig. 1b).

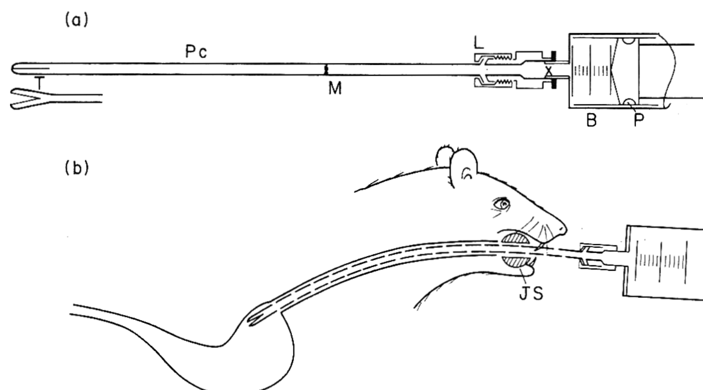

Fig. 1. (a) Equipment used to administer gastric donor-ingesta to a recipient rat, showing the polyethylene cannula, 1·67 mm ID × 2·42 mm OD (Pc), with an insertion mark (M) and an atraumatic tip (T) carrying a 1 cm slit for extrusion of ingesta. At the other end, the cannula is fitted with a glass syringe barrel (B), a rubber-tipped plastics syringe plunger (P) and a leur adapter (L), the ID at point × being 2·0 mm. (b) Illustration of the use of the wooden jaw spacer (JS) and the correct insertion of the oesophageal cannula to ensure that the slit-tip opens right in the stomach.

Cannulae were designed to minimize both discomfort to the animal during dosing and the ingesta dilution necessary for extrusion. Extrusion resistance was minimized by using the shortest possible cannula with the largest possible inside diameter. For rats of approximately 350 g body weight, the cannula described in Fig. 1a was prepared. The atraumatic tip allowed easy insertion without injury, and the slit ensured maximal opening for extrusion of the semi-solid ingesta. The cannula had to be inserted into the stomach to ensure that the slit tip did not injure the oesophagus when it spread open during extrusion of the ingesta (Fig. 1b). A glass syringe barrel and rubber-tipped plastics syringe plunger were used to exert safely the high pressure required to force the donor ingesta through the oesophageal cannula. Uptake of the lipophilic substrates by the rubber tip of the syringe plunger was prevented by a layer of ingesta which did not contain the test substrate. This overlay was adjusted to the dead volume of the syringe plus oesophogeal cannula. The actual administered dose was determined by subtracting the quantity of substrate retained by the ingesta in the syringe and cannula after the administration.

Studies on carbaryl absorption and metabolism

Comparison of ingesta-exchange and micro-dose techniques. A microquantity (7·5 μmol/kg body weight; 1×10^7 dpm) of the radiolabelled lipophilic substrate [^{14}C]naphthyl *N*-methylcarbamate ([^{14}C]carbaryl) was administered orally by the two methods to the same animal on successive days. A fasted rat was dosed with 7 g donor ingesta containing the [^{14}C]carbaryl and paired samples (50 μl) of portal blood were withdrawn via an indwelling cannula at timed intervals over a 20-hr period after dosing. The same rat was dosed again,

24 hr after receiving the ingesta-exchange dose, the same quantity of [^{14}C]carbaryl in 50 μl alcoholic solution ('micro-dose') being administered to the stomach via a polyethylene oesophageal cannula as described before (Casper, Pekas & Dinusson, 1973). In this case, paired blood samples were collected for 4 hr. The first of each pair of blood samples was immediately extracted with benzene for measurement of the content of non-metabolized [^{14}C]carbaryl, the second sample was used to determine total ^{14}C and the quantity of metabolized ^{14}C was estimated by difference.

Absorption of carbaryl given in ingesta-dose. The rate of absorption of [^{14}C]carbaryl (7·5 μmol/kg; 4 × 10^5 dpm) was estimated in five rats from the rate of disappearance of the gastro-intestinal contents after ingesta-dosing. One rat was killed with ether at 1, 4, 6, 15 and 24 hr after dosing and the gastro-intestinal tract was removed. The contents were carefully removed to minimize inclusion of tissue debris and ^{14}C taken up by it. The recovered ingesta and faeces were extracted and partitioned between water and benzene to separate the carbamate from its water-soluble metabolites. The quantities of benzene-extractable ^{14}C and of all other ^{14}C (including that in tissue, ingesta retained on tissue and glass-ware rinses) were measured by liquid scintillation and by the Packard Model 305 Oxidizer for dry samples. The total ^{14}C in the gastro-intestinal tract and its contents was calculated.

RESULTS

The levels of [^{14}C]carbaryl and its water-soluble ^{14}C-labelled metabolites in portal blood are shown in Fig. 2. The water-soluble ^{14}C-metabolities are presumed to be primar-

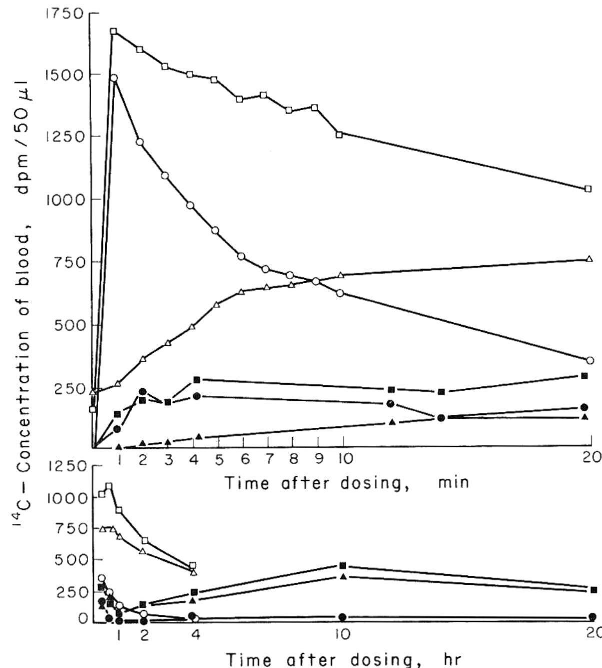

Fig. 2. Concentrations of total ^{14}C (■, □), benzene extractable ^{14}C (●, ○) and non-extractable ^{14}C (▲, △) in portal blood at varying times after a dose of 7·5 μmol [^{14}C]carbaryl/kg given to a rat in the form of an ingesta-exchange dose (■, ●, ▲) or a micro-dose in 50 μl alcoholic solution (□, ○, △). Blood levels immediately before micro-dosing were approximately the same as those 20 hr after the preceding ingesta dose (lower panel). Difficulty with the portal cannula prevented blood sampling between 5 and 10 min after the ingesta dose.

ily [^{14}C]naphthyl glucuronide, as determined in everted sacs of rat intestine (Pekas, 1971, 1972a & 1974). The obvious differences in the profiles in Fig. 2 demonstrate much slower absorption after the ingesta-exchange dose than after the micro-dose. The magnitude of the differences in the extent of metabolism and in the time of peak concentrations of ^{14}C in the blood suggests that the sites of absorption and/or metabolism in the gastro-intestinal tract may differ with the mode of dose administration.

In the second study, involving ingesta-dose administration to five rats, the disappearance rate of the ^{14}C-carbamate was much slower than that seen in experiments in which the micro-dose was used. The time required for half of the dose to disappear was between 2·5 and 4·5 hr (Fig. 3), compared with essentially instantaneous and complete absorption

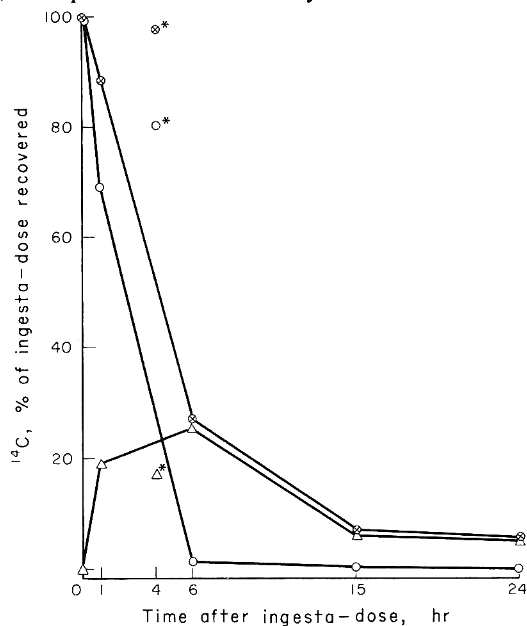

Fig. 3. Disappearance of [^{14}C]carbaryl from the gastro-intestinal tract of five rats at intervals of 1–24 hr after administration of an ingesta-exchange dose of 7·5 μmol/kg, as indicated by total ^{14}C recovered from the gastro-intestinal tract and its contents (⊕), benzene-extractable ^{14}C recovered from ingesta (○) and the remaining ^{14}C (△), which was primarily water-soluble and insoluble metabolites but possibly included some parent carbamate in tissue and retained ingesta not subjected to benzene partition. The rat studied at 4 hr (*) consumed a large quantity of food immediately after dosing. The ^{14}C in benzene (○), examined by thin-layer chromatography and radioscanning, was associated with intact [^{14}C]naphthyl *N*-methylcarbamate at 1 and 4 hr after the ingesta dose, but the quantities recovered at 6 hr and after were insufficient for further investigation.

after the micro-dose. Substantial quantities of ^{14}C in the ingesta were not benzene-extractable, demonstrating either that the gastro-intestinal tissue actively metabolized the carbamate under these conditions and/or that metabolites of the carbamate accumulated in the gastro-intestinal tract. Only the rat killed at 4 hr was allowed food. This rat was observed to consume a large but unmeasured quantity immediately after the dosing, and the additional volume and lipid content of this ingested food apparently suppressed absorption of the lipophilic carbamate. The slow disappearance of the carbamate and eventual appearance of ^{14}C-metabolites in the gastro-intestinal tract are in good agreement with the quantity and partition properties of the ^{14}C found in the portal blood at the same intervals after the ingesta-dose.

DISCUSSION

The possible shift in sites of absorption, resulting from the relatively slow absorption observed in both of the studies described here, could modify the availability, toxicity and/or biological activity of carbaryl and other lipophilic substrates. These factors would be important in experimental investigations. The reduced and delayed absorption of the same carbamate after an ingesta-exchange dose compared with that following a micro-dose has been further verified by measuring $^{14}CO_2$ expiration (J. C. Pekas, to be published). The same changes may be effected to some degree with microgram and sub-microgram quantities of water-soluble xenobiotics.

In other studies, the type of equipment used for administration of the ingesta-dose has been used to administer doses in semi-liquefied meals. For this simulated-meal technique, the conventional dry meal was rendered semi-liquid by dilution with drinking-water, and where appropriate the meal was ground more finely than normal to facilitate extrusion. This technique is recommended for studies of gastro-intestinal absorption and metabolism during the normal complete passage of microquantities of substrates down the gastro-intestinal tract. Incorporation of the test substrate into the dietary bulk and administration of this dietary bulk to the gastric compartment approach natural voluntary ingestion more closely than could be accomplished by the gastric ingesta-exchange technique.

Nevertheless, the ideal dose carrier for most absorption studies, whether for natural or synthetic non-nutritive substrates, is considered to be ingesta. The ingesta composition of lipids, water, inorganic ions, hydrogen ions, buffer ions and bile salts (and, consequently, micelles), as well as of specific enzymes derived from gastro-intestinal secretions or from the bacterial flora and of inert or reactive non-digestible materials, constantly changes as the ingesta passes down the tract. These changes undoubtedly affect the dispersion of substrates in the ingesta and their availability and rate of absorption, and therefore affect their metabolism and biological activity (toxicity). Consequently, in experimental investigations into the absorption of ingested substances, the substrate is ideally presented to the animal under conditions where these physico-chemical properties of the ingesta exert natural influences on the substrate at the site under study. Thus in studies on gastric absorption, the substrate should be incorporated uniformly into the gastric ingesta; similarly, studies in the small intestine should involve small intestinal ingesta, those in the caecum, caecal ingesta and so on. Obviously, in living animals, incorporation of substrates into ingesta *in situ* is impossible, but the ingesta-exchange technique allows experimentation under conditions which approach this ideal situation.

Donor ingesta harvested from several animals can be pooled to overcome some of the natural variation in composition. The quantity of ingesta bulk administered is likely to be important and the amount recovered from a specific site should determine the approximate quantity to be administered to the same site of recipient animals. Fortunately, the quantity can easily be standardized. Administration of typical quantities of gastric ingesta require less than 1 min, so the dose can be considered as a point source for time-course studies. Ingesta from the stomach, caecum, and colon of normal rats is substantial in bulk and semi-solid and is consequently difficult to mix and administer. Amounts of ingesta recovered from the small intestine, particularly the upper part, are very small and the material is liquefied. Problems are associated with handling both types of ingesta. When lipophilic substrates are used, as in this project, the materials used to construct administration equipment must be selected with care, as described before (Pekas, 1972b), to eliminate retention of the substrate on the materials.

Influences from those regions of the gastro-intestinal tract cranial to the selected site of study can be experimentally excluded by administration of the ingesta dose via strategically located fistulas. Fistulas may also be employed to obtain donor ingesta.

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Absorption gastro-intestinale et toxicologie: Technique de l'échange d'ingesta et du repas simulé

Résumé—La technique de l'échange d'ingesta implique l'incorporation homogène d'un substrat d'essai dans les ingesta qui sont récupérés à partir des animaux donateurs, puis l'administration du mélange à l'animal receveur. Cette technique, ainsi que celle du repas simulé qui nécessite l'incorporation du substrat d'essai dans des préparations semi-liquides de régimes normaux, permet de s'affranchir de nombreuses limitations que comporte l'administration des doses orales conventionnelles, en quantité de l'ordre du microgramme, de substances synthétiques non nutritives, spécialement si ces substances sont lipophiles et ont une haute activité toxique ou biologique. Ces techniques sont compatibles avec des études d'évolution dans le temps et sont spécialement utiles pour des études qui impliquent des marqueurs non absorbables. On décrit l'équipement nécessaire pour le prélèvement de l'ingesta gastrique semi-solide du donateur, ou pour les repas simulés. La vitesse et la dynamique de l'absorption d'un carbamate lipophile sont beaucoup plus lentes et retardées après une dose administrée par échange d'ingesta qu'après une dose conventionnelle donnée par cannule oesophagienne, tandis que l'importance du métabolisme est apparemment plus grande après une dose administrée par échange d'ingesta.

Gastrointestinale Absorption und Toxikologie: Ingesta-Austausch und simulierte Mahlzeittechniken

Zussammenfassung—Die Ingestaaustauschtechnik schließt die homogene Inkorporierung des Testsubstrates in die von den Spendertieren gewonnene Nahrung ein und die folgende Gabe der Mischung an die Empfängertiere. Dies und die simulierte Mahlzeittechnik, die die Inkorporierung des Testsubstrates in halbflüssige Präparationen normaler Nahrung umfaßt, überwältigen viele der Grenzen der konventionellen oralen Gabe von Microgrammquantitäten synthetischer Nicht-Nahrstoff-Substanzen an Tiere, speziell wenn diese Substanzen Lipophile sind und hohe Toxizität oder biologische Aktivität haben. Die Techniken sind vereinbar mit Zeitablaufstudien und sind speziell brauchbar für Studien, die nicht absorbierbare Markierungsstoffe verwenden. Die Geräte für das Auspressen der halbfesten Mageningesta des Spenders oder der simulierten Mahlzeiten werden beschrieben. Der Grad und die Dynamik der Absorption eines lipophilen Carbamates waren viel langsamer und verzögert nach einem Ingestaaustausch als nach einer konventionellen Gabe durch eine Ösophagealkanulle, während das Ausmaß des Metabolismus scheinbar größer war nach der Ingestaaustauschgabe.

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A SIMPLE AND RAPID METHOD FOR ASSAYING CYTOTOXICITY

T. F. M. FERGUSON and C. PROTTEY

Environmental Safety Division, Unilever Research Colworth/Welwyn, Colworth House, Sharnbrook, Beds., England

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Abstract—A procedure has been developed in which fibroblasts are harvested from guinea-pig kidney and grown to confluency. Assay tubes containing graded concentrations of substances under examination are inoculated with portions of these fibroblasts and DNA synthesis is measured by means of [^{3}H]thymidine uptake. Cytotoxicity is observed as an inhibition of DNA synthesis. This assay procedure was shown to be sensitive to three different cytotoxic agents, namely hydroxyurea, which is a metabolic inhibitor, sodium lauryl sulphate, a compound which lyses cells, and Rose Bengal, a phototoxic agent. It could be used to investigate the effect of a wide range of compounds that might come into contact with living cells.

INTRODUCTION

There are many references to the use of cells *in vitro* for the toxicity testing of drugs, additives, carcinogens and other chemicals, including the papers by Dawson & Dryden (1969), Freeman, Murtishaw & Knox (1970) and Metcalfe (1971). Such methods have involved the maintenance of cells in culture and the subsequent addition of the agents under examination, the toxicity of which have then been evaluated by measuring effects upon some parameter of cellular vitality. For example, cellular replication has been studied by counting cells entering mitosis, morphological changes in cells have been determined by microscopic examination of changes in cytoplasmic granularity or nuclear pyknosis, by loss of adhesion to the culture vessels or by cytolysis, and metabolism has been investigated by measuring DNA, RNA or protein formation or respiration. These assays are rapid and reproducible and large numbers may be performed at the same time. Measurement of DNA synthesis in rapidly proliferating lines of cells is a very popular system for assaying cellular viability, for example in cell-kinetics studies (Burger, Bombik, Brockenridge & Sheppard, 1972; Chopra & Flaxman, 1972; Schaer, Ramseier & Schindler, 1971). Tritium-labelled thymidine (^{3}HTdR) of very high specific activity is a cheap cellular tracer, it is exclusively incorporated into DNA, and the latter is easily extracted from the cells.

This paper describes a simple assay procedure for cytotoxicity using fibroblasts *in vitro* and measuring the incorporation of ^{3}HTdR into DNA. The effects of three known cytotoxic compounds are used as examples, namely hydroxyurea, a known inhibitor of DNA synthesis (Adams & Lindsay, 1967), sodium lauryl sulphate, which has a cytolytic effect (Putnam, 1948) and Rose Bengal, which is phototoxic (Allison, Magnus & Young), being toxic to cells when irradiated with tungsten light. The assay system for cytotoxicity described below may be used for testing a wide range of compounds for their potential hazard to living systems.

EXPERIMENTAL

Materials. Trypsin, from hog pancreas, was obtained from Koch-Light Laboratories, Colnbrook, Bucks. Eagle's medium M.E.M. was obtained as a concentrate (× 10) from Wellcome Reagents Ltd., Beckenham, Kent. Falcon flasks were obtained from Biocult, Ayr, Scotland. [6–^{3}H]Thymidine was obtained from the Radiochemical Centre, Amersham, Bucks., as a sterile aqueous solution which was diluted with sterile water to give 10 μCi/0·1 ml (5·0 Ci/m-mol). Hydroxyurea was obtained from Sigma Chemical Co. Ltd., London, and Rose Bengal from Hopkins and Williams, Chadwell Heath, Essex.

Preparation and culture of fibroblasts. Guinea-pig fibroblasts were prepared from guinea-pig kidneys using trypsin. The kidneys were aseptically removed from two freshly killed male guinea-pigs (300 g body weight) and the membrane sheathing was removed. The kidneys were finely chopped and were agitated in a 0·25% solution of trypsin in phosphate-buffered saline (pH 7·4) for 30 min at 37 C. The action of the trypsin was arrested by the addition of an equal volume of guinea-pig serum and the cell suspension was filtered through three layers of gauze to remove the tissue fragments. The cells were harvested by centrifugation at 200 **g** and were resuspended in Eagle's medium (Eagle, 1959) containing 20% guinea-pig serum and were maintained at 37°C in two plastics Falcon flasks. After 24 hr the medium was replaced by decantation, which removed most of the other contaminating cells (endothelial and blood cells). Fibroblasts which attached to the walls of the Falcon flasks rapidly proliferated. Media were replaced every 3–4 days and conditions of strict asepsis were always observed. All culture vessels were flushed with 5% CO_2 in air and tightly stoppered after each media change. Generally it was found that after 15 days the Falcon flasks contained confluent cultures of fibroblasts, representing between 1·8 and 2·4 × 10^6 cells.

Measurement of DNA synthesis in actively growing fibroblast cultures. Confluent cultures of fibroblasts in Falcon flasks were sub-cultured by incubating with 0·5% trypsin for 5–10 min to detach and disaggregate the cells. The action of the trypsin was stopped by adding an equal volume of serum. The cells were then harvested by gentle centrifugation and resuspended in fresh Eagle's medium without serum. The final cell density was usually between 1 and 2 × 10^5 cells/0·2 ml. Stoppered glass centrifuge tubes were then inoculated with 0·2 ml aliquots, the complete 1·0 ml incubation system comprising 10 μCi [6–^{3}H]thymidine (equivalent to 2 nmoles), 20% guinea-pig serum and Eagle's medium. Cells were incubated at 37°C for 17 hr, at which time cultures were rapidly cooled to 4 C and centrifuged gently to precipitate the cells. The radioactive medium was decanted and discarded. The cell pellets were resuspended in Eagle's medium without serum, but fortified with 0·2 mM unlabelled thymidine (100 times the concentration of radioactive thymidine originally used) to wash out residual labelled thymidine. After centrifugation the medium was decanted and the cell pellets were extracted three times with 1 ml ice-cold 5% trichloracetic acid, followed by a single extraction with 1 ml ethanol. These extracts were also discarded. The cell-pellet residues were finally extracted three times with 1 ml 5% trichloracetic acid at 70°C to remove the labelled DNA. Aliquots of the combined hot acid extracts were then counted in a liquid scintillation spectrometer (with efficiencies for tritium of about 7·5%) to determine the level of synthesis.

Effect of Rose Bengal. All operations involving Rose Bengal, except the tungsten-light irradiation, were performed under yellow safety light. Culture tubes containing various levels of Rose Bengal were inoculated with cells and then exposed to tungsten light at a distance of 5·0 cm for either 5 or 15 min. The light source used was a Crompton 100 W

single-core tungsten-filament lamp, which produced an incident dose rate of 1·1 mW/cm^2 over the spectral range for Rose Bengal absorption (450–600 nm). Then the tubes were pulsed with 10 μCi [^{3}H]thymidine and incubated in the dark for 17 hr. Radioactive DNA was then extracted exactly as described above.

Design of study. The effect of varying the standard culture conditions was examined, in respect of the cell concentration, serum level, incubation time and mass of thymidine added to each incubation, and the applicability of the assay was demonstrated by tests on hydroxyurea, sodium lauryl sulphate and Rose Bengal.

RESULTS

Factors affecting DNA synthesis in guinea-pig fibroblasts in vitro

Cell concentration. Figure 1 illustrates the effect of varying the number of cells incubated. The level of DNA synthesis reached a plateau at 1·68 × 10^5 cells, presumably due either to exhaustion of one of the cofactors or to contact inhibition of the fibroblasts.

Serum level. At a fixed level of cells of 1·68 × 10^5/incubation, guinea-pig serum levels were varied between 0 and 20% (Fig. 2). No variations were seen over the 5–20% range. However, the rate of DNA synthesis in the absence of serum was only 45% of that in the presence of serum.

Incubation time. Initially an incubation period of 17 hr was chosen, as this represented a convenient overnight interval. Although DNA synthesis (S phase) represents only a fraction of the total cell cycle, it was thought that a lengthy incubation was required for two reasons. First, prior to entering S phase, the fibroblasts would require to adhere to the culture vessels, and this would take several hours. Secondly, it was unlikely that the fibroblasts would be in synchrony, and a lengthy incubation period would therefore allow most, if not all, of the fibroblasts to synthesize DNA. A series of incubations was set up, each containing 10 μCi ^{3}HTdR, 20% guinea-pig serum and 1·22 × 10^5 fibroblasts. The tubes were pulsed with thymidine for various lengths of time over a 24-hr period, as shown in Table 1. It is seen that thymidine was not incorporated uniformly into DNA throughout this period. During the first 6 hr nearly 50% of the total synthesis occurred and during

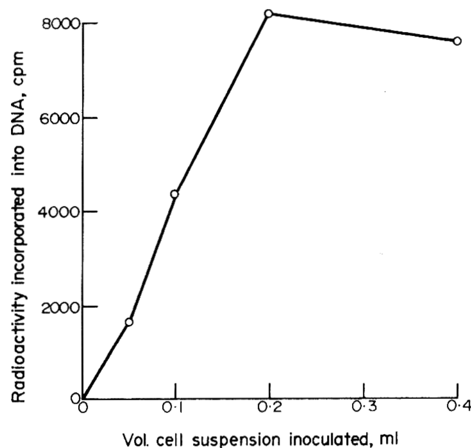

Fig. 1. Cell concentration curve. Each incubation contained 10 μCi ^{3}HTdR and 20% guinea-pig serum and 0·1 ml of the cell suspension contained 84,000 fibroblasts. Incubation was for 17 hr at 37°C.

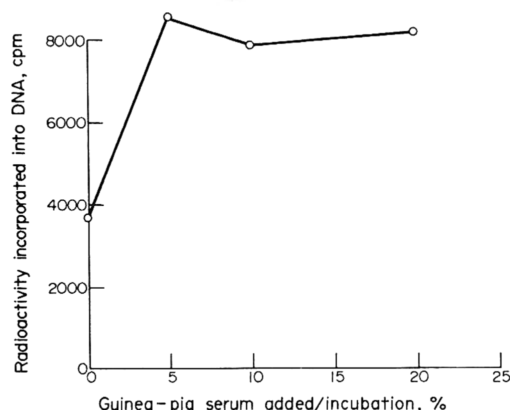

Fig. 2. Serum concentration curve. Each incubation contained 10 μCi ^{3}HTdR, 168.000 fibroblasts and a variable amount of serum. Incubation was for 17 hr at 37 C.

the first 12 hr almost 66% of the total. These data suggest that little could be gained by prolonging the length of incubation and, therefore, for the sake of convenience, all incubations were left overnight for 17 hr.

Mass of thymidine. As the specific activity of the labelled thymidine used was high, the actual mass added for each incubation was very low (2·0 nmoles). In order to check whether thymidine was rate-limiting upon the synthesis of DNA, various amounts of unlabelled thymidine were added, and the resultant degree of DNA synthesis is shown in Fig. 3. It can be seen that tripling the thymidine concentration only increased DNA synthesis by 10%. In all subsequent incubations, therefore, the level of ^{3}HTdR added was fixed at 10 μCi, that is 2 nmoles.

Demonstrations of the assay with known cytotoxic agents

Effect of hydroxyurea. A series of tubes, each containing 20% serum, 10 μCi [6-^{3}H]thymidine and 1·68 × 10^5 cells were incubated with various amounts of hydroxyurea. The results (Fig. 4) show that 1 mM levels cause more than an 80% inhibition in DNA synthesis, which is complete at 10 nM. Thus, the assay system is sensitive to a known chemical inhibitor of DNA production.

Effect of sodium lauryl sulphate. By virtue of its strong surface activity, sodium lauryl sulphate is a haemolysin, and various concentrations of this compound were tested under

Table 1. *Effect of incubation time upon DNA synthesis in fibroblasts*

Pulse time (hr)				
Start	Finish	Duration of pulse (hr)	Radioactivity in DNA (cpm)	Percentage of incorporation during different parts of 24 hr period
0	24	24	12,489	100
0	12	12	7971	63·8
0	6	6	5961	47·7
6	12	6	3513	28·2
6	24	18	7314	58·6
12	24	12	3015	24·2

Each incubation contained 122,000 fibroblasts, 10 μCi ^{3}HTdR and 20% serum. All incubations were of 24 hr duration, but the ^{3}HTdR was added at various times as indicated.

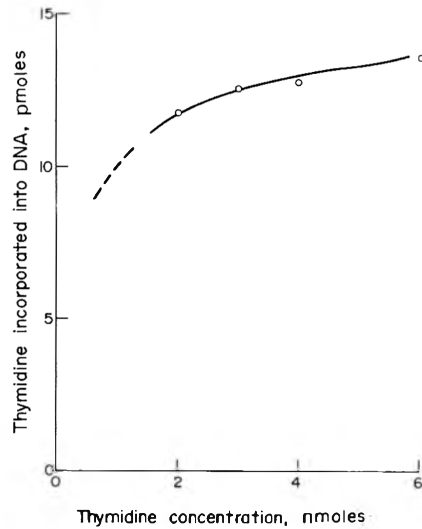

Fig. 3. Thymidine concentration curve. Each incubation contained 168,000 fibroblasts. 20% guinea-pig serum and 10 μCi thymidine, but various levels of unlabelled thymidine were also added. Incubation was for 17 hr at 37°C.

conditions similar to those used for hydroxyurea, except that serum was omitted from all incubations, because the compound may bind tightly to protein, thereby reducing its lytic properties (Putnam, 1948). The results are shown in Fig. 5. In the absence of serum the level of radioactivity incorporated into DNA was less than half of that seen in its presence (cf Figs 2 & 4). At 0·01 mM sodium lauryl sulphate there was a marked stimulation of DNA labelling, but this was lost at higher concentrations,. At 0·1 mM sodium lauryl sulphate the inhibition of synthesis was greater than 90% and at 0·5 mM inhibition was total. The assay procedure thus demonstrates that agents which may interfere with cellular metabolism due to their surface-active properties may be examined by following their effect upon DNA in cultured fibroblasts.

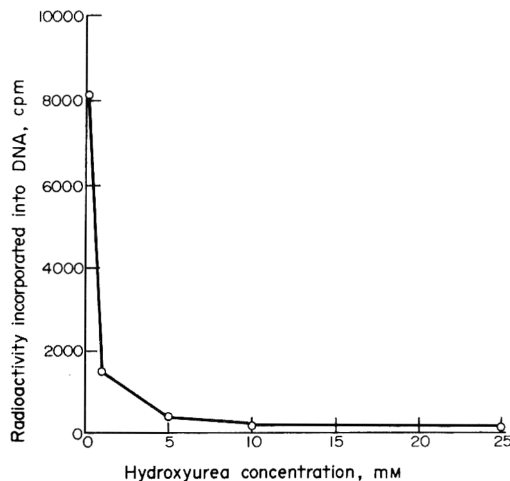

Fig. 4. Hydroxyurea concentration curve. Each incubation contained 10 μCi ^{3}HTdR and 168,000 fibroblasts in 20% guinea-pig serum. Incubation was for 17 hr at 37 C.

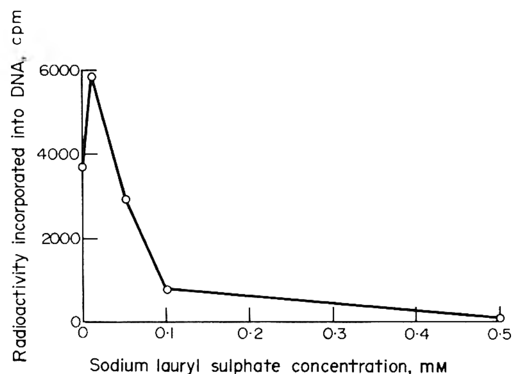

Fig. 5. Sodium lauryl sulphate concentration curve. Each incubation contained 10 μCi ^{3}HTdR and 168,000 fibroblasts, but no serum. Incubation was for 17 hr at 37 C.

Effect of Rose Bengal. Three types of incubation were set up. To one series, various amounts of Rose Bengal were added but they were not irradiated, so that the intrinsic toxicity of the compound could be measured. The other two groups of incubations were similar but were irradiated with tungsten light for 5 min and for 15 min respectively. The results (Table 2) show that the effect of Rose Bengal in the absence of irradiation caused more than 50% inhibition at the 50 μg/ml level, but this was not seen at the 5 μg/ml level. However, when cultures containing Rose Bengal were irradiated, the degree of inhibition of DNA synthesis was much greater. Whereas 5 μg/ml had no effect in unirradiated cells, after irradiation for either 5 or 15 min DNA synthesis was reduced by one half. In the presence of 50 μg/ml, these levels were reduced even more drastically. In the absence of Rose Bengal, irradiation for 5 min caused about 25% inhibition in DNA synthesis, a finding that cannot be explained. While the number of duplicates performed was insufficient for statistical analysis, the fact that this inhibition was not observed after 15-min irradiation suggests that its appearance after 5 min was due to experimental variation.

Table 2. *Effect of Rose Bengal, in the presence and absence of tungsten light, upon DNA synthesis in fibroblasts*

Amount of Rose Bengal added (μg/ml)	Radioactivity incorporated into DNA (cpm)		
Duration of irradiation (min)...	0	5	15
0	1906	1476	1728
5	1872	739	819
50	880	293	140

Each culture contained 135,000 fibroblasts but no serum. Incubations were for 17 hr at 37 C in the dark, immediately following irradiation (where used) and addition of 10 μCi^{3} HTdR.

DISCUSSION

The cytotoxicity assay described utilizes rapidly proliferating fibroblasts *in vitro*. DNA synthesis was chosen as the parameter for cellular vitality as cells will invariably duplicate their nuclear DNA during the S phase of the cell cycle which precedes mitosis. Measurement of [^{3}H]DNA synthesis from ^{3}HTdR is easy and rapid compared with, say,

counting mitotic figures or cell numbers. Preliminary studies were made to ensure that an appropriate pulse time for DNA labelling was chosen, 17 hr finally being used for convenience. This interval would allow for most of the cells that were going to divide to synthesize DNA, but would, presumably, be too short for the complete cell cycle to take place. Chopra & Flaxman (1972) have calculated this to be about 60 hr *in vitro*, so the probability of the fibroblasts dividing more than once during 17 hr *in vitro* is very low. Despite the simplicity of this assay, one is actually measuring a complex function of the cell, which may be subject to inhibition by many different factors (changes in structural integrity and conformation, depletion of requisite co-factors for DNA synthesis, inhibition of respiration and energy production). This method may be more meaningful as a measure of cytotoxicity than relying, for example, upon evaluation of structure by microscopy (which would be subjective and time consuming) or the measurement of cellular enzymes (phosphatases, dehydrogenase), which may be active in intact and lysed cells alike.

To demonstrate the versatility of the assay method, we have chosen three different types of agents deleterious to cells *in vitro*. Hydroxyurea is a metabolic inhibitor of DNA synthesis exclusively, interfering with ribonucleotide reductase but not affecting RNA and protein synthesis (Adams & Lindsay, 1967). Its effect upon the cultured fibroblasts was complete at a level of 10 mM. As the action of this compound is reversible, other techniques for measuring its cytotoxic action, relying for example upon morphological examination or cellular enzyme determination, would be worthless.

Sodium lauryl sulphate is known to lyse cells. The assay procedure demonstrated that DNA synthesis in isolated cells was very sensitive to the effect of solutions of this compound. At very low concentrations (0·01 mM) there was a marked stimulation of DNA labelling. This was unexpected, and may have been due to an enhanced penetration rate of ^{3}HTdR into the fibroblasts by modification of the plasma membrane, or to the effect of the compound itself upon the enzymes of DNA synthesis. At higher levels of sodium lauryl sulphate, however, DNA synthesis was totally inhibited. Under the latter conditions the fibroblasts, when examined by light microscopy, showed signs of lysis.

The assay system was also tested with a known phototoxic compound. Rose Bengal was chosen as it is not taken up by the cells but binds to the plasma membrane (Allison *et al.* 1966). Following light irradiation, oxygen in an excited energy state is produced, with consequent deleterious effects upon the cells. It was found that Rose Bengal, although reasonably toxic to fibroblasts in its own right at 50 μg/ml, almost completely inhibited DNA synthesis after irradiation with tungsten light, thus demonstrating the sensitivity of the assay system.

This assay procedure has been shown to be sensitive to three very different cytotoxic agents. It is not unreasonable to suppose that it could be used to investigate the effect of a wider range of compounds that may come in contact with living cells.

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Une méthode simple et rapide pour déceler la cytotoxicité

Résumé—On a mis au point un procédé consistant à recueillir des fibroblastes du rein du cobaye et à les cultiver jusqu'à confluence. On inocule de portions de ces fibroblastes des tubes à essais contenant des concentrations échelonnées des substances à étudier et on mesure la synthèse de l'ADN d'après l'absorption de le [^{3}H]thymidine. La cytotoxicité se manifeste par une inhibition de cette synthèse. On a démontré que cette méthode de dépistage est sensible à trois agents cytotoxiques différents, en l'occurrence l'hydroxyurée, qui est un inhibiteur du métabolisme, le sulfate de lauryle sodé, qui provoque la lyse des cellules, et le Rose Bengale, agent phototoxique. La méthode pourrait servir à étudier les effets de nombreux produits susceptibles d'entrer en contact avec des cellules vivantes.

Ein einfaches und schnelles Verfahren für Cytotoxizitätproben

Zusammenfassung—Ein Verfahren wurde entwickelt, durch das Fibroblasten von Meerschweinchennieren gewonnen werden und zum Verwachsen gezüchtet werden. Teströhrchen mit stufenweiser Konzentration von zu bestimmenden Substanzen werden geimpft mit Portionen dieser Fibroblasten, und die DNS-Synthese wird gemessen mit Hilfe der [^{3}H]Thymidin-Aufnahme. Cytotoxizität wird festgestellt als eine Hemmung der DNS-Synthese. Es wurde gezeigt, daß diese Bestimmungsmethode auf drei verschiedene cytotoxische Substanzen ansprach, nämlich Hydroxyharnstoff, ein Abbauhemmstoff, Natriumlaurylsulfat, eine Verbindung, die Zellen lysiert, und Rose Bengal, ein Phototoxicum. Dies kann verwendet werden, um den Effekt eines weiten Felds von Verbindungen, die in Kontakt mit lebenden Zellen kommen könnten, zu untersuchen.

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SHORT PAPERS

EFFECTS OF BUTYLATED HYDROXYTOLUENE ALONE OR WITH DIETHYLNITROSAMINE IN MICE

N. K. CLAPP, R. L. TYNDALL*, R. B. CUMMING and J. A. OTTEN

Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830, USA

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Summary—Mice (18 months old) given butylated hydroxytoluene (BHT) at a dietary level of 0·75% for 16 months had 63·6% of lung tumours compared with 24·0% in controls. Mice given dietary BHT as well as diethylnitrosamine (DENA) in the drinking-water (average total intake 330 mg DENA/kg) had more lung tumours per mouse (4·0) than did the controls or those given DENA alone or BHT alone (1·4–2·2). The incidence of reticulum-cell sarcomas in mice killed at 12 months of age in the group treated with BHT plus DENA exceeded control values (52·6 and 22·7%, respectively), while the incidence in groups receiving DENA alone or BHT alone was not significantly different from that in controls. At 18 months, the incidence of reticulum-cell sarcoma in both groups given BHT was significantly less than in the groups not receiving BHT. At 18 months of age, a higher percentage of squamous-cell carcinomas was seen in the forestomach of mice given BHT plus DENA than in mice receiving DENA alone. The production of cysts within the liver parenchyma after DENA treatment also appeared to be potentiated by BHT treatment.

Introduction

Food antioxidants such as butylated hydroxytoluene (BHT) have generally been considered harmless with regard to general toxicity (Deichmann, Clemmer, Rakoczy & Bianchine, 1955), teratogenicity (Clegg, 1965), and mutagenicity (Epstein & Shafner, 1968). However, we recently reported hyperplasia of hepatic bile ducts in mice following long-term administration of BHT (Clapp, Tyndall & Cumming, 1973). The ultimate course taken by this lesion has not yet been determined, but its extent and severity consitute a source of concern.

It has been shown previously (Cumming & Walton, 1973) that prior administration of BHT in the diet drastically modified the acute lethal toxicity of diethylnitrosamine (DENA), as well as a number of other effects reported by various authors and reviewed by Cumming & Walton (1973). This report is an account of a pilot experiment involving the administration of BHT with or without the potent carcinogen, DENA, in mice.

Experimental

Animals. Male BALB/c mice were used in this study and were housed in conventional laboratory facilities with restricted human traffic into the mouse cells; husbandry procedures were designed to minimize outside contamination of the mice. Before the start of the experiment, sera from the mice were tested for the presence of antibodies to common murine viruses (Microbiological Associates, Bethesda, Md) with negative results; the mice were likewise grossly and histologically free from ecto- and endoparasites.

* Present address: Medical Division, Oak Ridge Associated Universities, Oak Ridge, Tennessee 37830, USA.

Treatment. Mice were divided alternately into four groups of approximately 50 each, all of which had free access to food (Purina Laboratory Chow, Ralston Purina, St. Louis, Mo.) and water. BHT (0·75%) was added to the food of one group, DENA (Eastman Organic Chemicals, Rochester, N.Y.) was added to the drinking-water of another group at a concentration of 4 mg/100 ml for 7 wk (the average total dose consumed by the mice being approximately 330 mg/kg body weight), a third group received both BHT and DENA, and the fourth (control) group was given untreated food and water. BHT treatment was started when the mice were 8 wk old and continued until death, while DENA treatment began at 11 wk of age and continued for 7 wk. A few mice were killed from each group at 2–3-month intervals to determine whether or not they were developing tumours. About two-thirds of each group of mice were killed at 12 months of age, when some early deaths were observed, and the rest at 18 months of age, when most of the DENA-treated mice were reaching a stage of ill-health and death was imminent.

After death, the lungs were inflated *in situ* with a mixture of 450 ml 95% alcohol, 50 ml formalin and 25 ml glacial acetic acid. They were preserved in this solution and then cleared, by a modification of the technique of Spalteholz (Emmel & Cowdry, 1964), and were finally preserved in oil of wintergreen. Tumour nodules were observed under a dissecting microscope and recorded. The remaining tissues were preserved in Zenker-formol and embedded in paraffin, sections being stained with hematoxylin and eosin or by special staining techniques, as required.

Results

At the dose levels used, DENA consumption caused ruffled hair and a failure to gain weight, in contrast to controls, while BHT had no noticeable effect on food consumption or body growth. A few mice died between the ages of 12 and 18 months but cannibalism and rapid body deterioration after death prevented consistent and complete autopsies on these mice.

The results of the observations on lung tumours are shown in Table 1. In mice killed at 12 months of age, only DENA-treated animals had an incidence of lung tumours (45·4%)

Table 1. *Incidence of lung tumours in BALB/c mice given BHT, DENA or both*

Treatment		No. of mice	No. of tumours	No. of tumour-bearing mice	Incidence‡ (%)	No. of tumours/ no. of tumour-positive mice
BHT*	DENA†					
				Mice killed at 12 months of age		
0	0	50	10	9	18·0	1·1
0	+	33	21	15	45·4	1·4
+	+	20	9	7	35·0	1·3
+	0	19	2	2	10·5	1·0
				Mice killed at 18 months of age		
0	0	25	11	6	24·0	1·8
0	+	6	13	6	100	2·2
+	+	10	40	10	100	4·0
+	0	11	10	7	63·6	1·4

*Given at a level of 0·75% in the feed from the time the mice were 8 wk old until they were killed.
†Given in drinking-water (4 mg/100 ml) for 7 wk, starting when the mice were 11 wk old, to provide a total dose of approximately 330 mg DENA/kg body weight.
‡$\frac{\text{No. of tumour-bearing mice}}{\text{Total no. of mice killed}} \times 100$.

different ($P < 0.01$) from that of the controls (18·0%). In contrast, at 18 months, mice given DENA alone or DENA with BHT had a 100% incidence of lung tumours, while 63·6% of those given BHT alone had lung tumours. These values are all significantly higher than the 24·0% incidence seen in controls ($P < 0.001$ and < 0.025, respectively). While the number of tumours observed in each tumour-bearing mouse did not differ between the groups at 12 months, at 18 months the DENA plus BHT group had 4·0 tumours per tumour-bearing mouse compared with the 1·4–2·2 tumours per tumour-bearing mouse in the other three groups. The tumours observed in treated and untreated mice were papillary adenomas, which commonly develop in numerous mouse strains.

The incidence of reticulum-cell sarcoma and of stomach and liver changes is presented in Table 2. At 12 months, the incidence of reticulum-cell sarcoma in the group given BHT plus DENA was greater than that in the control group ($P < 0.025$), while the incidence after DENA alone or BHT alone did not differ significantly from that of the controls. At 18 months, the incidence of reticulum-cell sarcoma in groups receiving BHT, with or without DENA, was lower than that in the controls. This was statistically suggestive with BHT plus DENA ($P < 0.10$) and significant ($P < 0.05$) with BHT alone. If the two groups receiving BHT are combined, and compared with the two not receiving BHT, the lower incidence of leukaemia in the former is seen to be highly significant ($P < 0.005$). DENA alone does not alter the incidence of reticulum-cell sarcoma. Papillomas and squamous-cell carcinomas of the forestomach were approximately equal in incidence at 12 months. But despite the small numbers of mice at 18 months, there was a shift from equal distribution of tumour type in the group receiving only DENA to a predominance of squamous-cell carcinomas in the BHT-DENA group; this suggests potentiation of the DENA effect upon the forestomach of the BALB/c mouse. In addition to the bile-duct hyperplasia previously reported in these same mice (Clapp *et al.* 1973), a potentiation by BHT of the production of hepatic cysts by DENA was suggested by the increased incidence of cysts produced in BHT-DENA animals. Both DENA-treated groups killed at 12 months had significantly more cysts than the controls ($P < 0.001$ in both cases) and these two treated groups were also statistically different from each other ($P < 0.025$). At 18 months the differences were not as great, since the incidence in DENA-treated animals had reached the same level as that seen in the BHT-DENA mice. Liver tumours were not seen in any group.

Discussion

The protection against the acute toxicity of DENA by prefeeding with BHT was attributed to an enzyme-induction mechanism (Cumming & Walton, 1973). Magour & Nievel (1971) reported that BHT induced the activity of DENA de-ethylase in rat-liver microsomes, and it has been suggested that this enzyme is essential for the activation of DENA to a carcinogenic metabolite (Druckrey, Preussman, Schmähl, and Müller, 1961). Thus enzyme induction may constitute a common mechanism for protecting the animal from acute toxic effects of DENA on the one hand while potentiating the carcinogenic capacity of DENA on the other. In the data reported here, potentiation is seen in the induction of reticulum-cell sarcomas at 12 months, and is suggested by the difference in tumour type in the stomach, by the numbers of tumours per tumour-bearing mouse, and by the incidence of cyst formation in the liver. BHT increased the incidence of lung tumours and caused hyperplasia of the hepatic bile ducts (Clapp *et al.* 1973). The lack of bile-duct hyperplasia in mice killed at 18 months may have resulted from premature mortality of animals

Table 2. *Incidence of reticulum-cell sarcoma, stomach tumours and liver changes in BALB/c mice receiving BHT, DENA or both*

Treatment		No. of mice	Incidence of reticulum-cell sarcomas‡ (%)	Incidence of stomach tumours			Incidence of liver lesions	
BHT*	DENA†			Papillomas‡ (%)	Squamous-cell carcinoma‡ (%)	Total‡ (%)	Cyst‡ (%)	Bile-duct hyperplasia‡ (%)
Mice killed at 12 months of age								
0	0	44	22·7	4·5	2·3	6·8	2·3	0
0	+	28	35·7	42·8	42·8	85·7	32·1	0
+	+	19	52·6	31·6	47·4	78·9	68·4	0
+	0	18	38·9	11·1	0	11·1	0	33·3
Mice killed at 18 months of age								
0	0	25	56·0	4·0	0	4·0	0	0
0	+	7	71·4	42·8	57·1	100	71·4	0
+	+	10	20·0	10·0	80·0	90·0	80·0	0
+	0	8	12·5	0	12·5	12·5	25·0	0

*Given at a level of 0·75% in the feed from the time the mice were 8 wk old until they were killed.
†Given in drinking-water (4 mg/100 ml) for 7 wk, starting when the mice were 11 wk old, to provide a total dose of approximately 330 mg DENA/kg body weight.
‡ $\frac{\text{No. of mice bearing the tumour or lesion}}{\text{Total no. of mice killed}} \times 100$

with this lesion. The lower incidence of reticulum-cell sarcoma seen at 18 months contrasts with the effects observed at 12 months and may also have been the result of population selection. Studies in progress are designed to elucidate the presumed premature mortality and the possible beneficial effect of BHT.

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THE LACK OF SIGNIFICANT ABSORPTION OF METHYLCELLULOSE, VISCOSITY 3300 CP, FROM THE GASTROINTESTINAL TRACT FOLLOWING SINGLE AND MULTIPLE ORAL DOSES TO THE RAT

W. H. BRAUN, J. C. RAMSEY and P. J. GEHRING

Chemical Biology Research, Dow Chemical USA, Midland, Michigan 48640, USA

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Summary—[^{14}C]Methylcellulose labelled in the methoxyl group and with a viscosity of 3300 cP was administered to one group of six rats as a single oral dose and to another group of six rats in five daily doses, the doses in each case being approximately 500 mg/kg/day. During the 48 hr following administration of the single dose, 102·2% of the total dose of ^{14}C activity was eliminated in the faeces. No radioactivity was detected in the expired air. Less than 0·1% of the original dose of ^{14}C was found in the urine, selected tissues and remaining carcass. No accumulation of ^{14}C activity was detected in the body or in selected tissues after multiple dosing. It is concluded that following oral administration to rats, high-viscosity methylcellulose is not absorbed and is rapidly cleared through the body via the faeces.

Introduction

Methylcellulose is a polymeric carbohydrate derivative with the general formula shown in Fig. 1. It is used as a thickener, binder, emulsifier, stabilizer and colloidal suspending agent. As such, it can be found in salad dressings, pie fillings, baked goods, dietetic foods and many other products consumed by man. Toxicological studies reported by McCollister, Kociba & McCollister (1973) and others (Bauer & Lehman, 1951; Deichmann & Witherup, 1943; Knight, Hodge, Samsel, DeLap & McCollister, 1952) attest to the safety of methylcellulose. However, a review of the literature revealed no information about the fate of methylcellulose following oral ingestion. Therefore, the objective of this study was to determine the degree of absorption of a particular high-viscosity methylcellulose (3300 cP) upon ingestion by rats. Initially, absorption was evaluated following a single oral dose. Subsequently, repeated daily doses were administered to enhance the possibility of detecting a small degree of absorption.

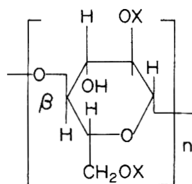

Fig. 1. General formula of methycellulose (X = H or CH_3).

Experimental

Material. [^{14}C]Methylcellulose (3300 cP), labelled in the methoxyl group, was synthesized by Dr. Wen Cheng of The Dow Chemical Company. This sample met the USP speci-

fications for high-viscosity methylcellulose (*United States Pharmacopeia*, 1970), as well as the specifications of the *Food Chemicals Codex* (1972) and of the Joint FAO/WHO Expert Committee on Food Additives (1964 & 1967). The specific activity was 104·4 μCi/g methylcellulose.

Dosage. For administration to rats, a 3·5% (w/v) solution of [^{14}C]methylcellulose in distilled water was prepared. Syringes (5 ml) fitted with feeding needles were used to administer an amount of this solution providing a dose of approximately 500 mg methylcellulose/kg to each rat. The exact quantity of ^{14}C, as well as the weight of material administered, was determined by weighing the syringes before and after dosing.

Animals. The weight ranges of the Sprague-Dawley Spartan-strain rats used in this study were 192–250 and 185–215 g for males and females, respectively. In the single-dose study, three rats of each sex were given one oral dose of [^{14}C]methylcellulose following a 6-hr fasting period. In the repeated-dose study, the same dose was administered daily for 5 days to three rats of each sex following 6-hr fasting. Following the administration of each dose, food and water were freely available.

Single-dose study. The rats were housed in glass metabolism cages designed for the separate collection of urine, faeces and CO_2 in expired air. Airflow through the cages was maintained at 500 ml/min, and the cages were kept in an air-conditioned room with a light-dark cycle of alternating 12-hr periods. Urine and faecal samples were collected at 6-hr intervals for the first 24 hr, and at 12-hr intervals thereafter. To trap CO_2, the air leaving each cage was bubbled sequentially through traps containing 140 and 40 ml 5 *M*-2-aminoethanol in 2-methoxyethanol. These traps were changed at 6-hr intervals for the first 24 hr following administration of [^{14}C]methylcellulose, and were analysed for ^{14}C activity. Since no significant amount of ^{14}C could be detected in these samples, expired CO_2 was not collected beyond 24 hr. The rats were killed by decapitation 96 hr after administration of the single oral dose. The carcasses were skinned and the hearts, livers, lungs, kidneys and gastro-intestinal tracts were removed. Samples of excreta, tissues and carcasses and of the water used to rinse the metabolism cages were stored frozen until analysed for ^{14}C.

Repeated-dose study. The rats were housed in metal metabolism cages designed for the separate collection of urine and faeces. Samples of excreta were collected at 24-hr intervals. The rats were killed 24 hr after the final dose, and the carcasses and tissue samples were handled as described above.

Radioactivity determinations. All determinations of ^{14}C activity were carried out in a Nuclear Chicago Mark II Liquid Scintillation System using external standard ratios to determine quench correction. The radioactivity in the urine was determined by counting a 250 μl aliquot in 1 ml water and 15 ml Aquasol®. Aqueous homogenates of the carcasses, livers, kidneys, lungs and gastro-intestinal tracts were prepared. Aliquots (150 mg) of these homogenates were oxidized in a Beckman Biological Material Oxidizer. The resulting CO_2 was trapped in 5 M-2-aminoethanol in 2-methoxyethanol and subsequently counted in a solution containing 3·5 g PPO and 0·8 g POPOP/litre 2-methoxyethanol–toluene (1:1, v/v). Aliquots (5 ml) of the expired CO_2 traps were added to 15 ml 2-methoxyethanol–toluene (1:1, v/v) containing 4 g PPO and 0·1 g POPOP/litre. The detection limit for this study was defined as twice the background dpm. All samples with less than twice the background dpm were assigned nil radioactivity.

®Aquasol Universal LSC Cocktail was used as received from New England Nuclear Corp.

Results

Table 1 gives the percentage of the total radioactivity recovered in the faeces of each rat given a single oral dose of approximately 500 mg/kg [^{14}C]methylcellulose. Within 72 hr, 102·2 ± 2·7% (± standard deviation) was excreted via the faeces, while less than 0·1% was found in the urine. No radioactivity was detected in the expired CO_2 collected during the first 24 hr following administration. The heart, kidney, gastro-intestinal tract, liver, lung, carcass, skin and hair and cage washings contained less than 0·1% of the administered radioactivity.

Table 1. *Excretion of radioactivity in the faeces following administration of a single oral dose of [^{14}C]methylcellulose to rats*

Rat no.	Time (hr)...	Faecal excretion of ^{14}C (% of dose) 6	12	18	24	36	48	60	72	0–72
					Males					
1		0·01	74·51	20·34	0·71	0·23	nil	nil	nil	95·8
2		0·01	46·82	43·41	5·43	9·15	nil	nil	nil	104·8
3		0·01	71·69	27·55	2·22	3·02	nil	nil	nil	104·5
					Females					
1		0·02	48·65	47·61	5·02	1·24	0·11	nil	nil	102·7
2		0·01	79·35	16·18	6·83	1·87	nil	0·08	nil	104·3
3		0·03	48·55	47·09	2·23	3·27	nil	nil	nil	101·2
										Mean.... 102·2 ± 2·7*

*Mean ± standard deviation.

Repeating the dose of approximately 500 mg/kg every 24 hr for 5 days did not increase the ^{14}C activity in the heart, kidney, liver, lung, carcass and skin. The total amount found in these tissues was still less than 0·1% of the total dose. The radioactivity in the urine of these rats expressed as a percentage of the total dose is shown in Table 2. The significant levels of ^{14}C activity occasionally detected in the urine were attributed to faecal contamination.

Table 2. *Radioactivity in the urine following repeated oral doses of [^{14}C]methylcellulose in rats*

Rat no.	Time* (hr)...	Urinary excretion of ^{14}C (% of total dose) 24	48	72	96	120	Total (%)
			Males				
4		nil	nil	nil	nil	nil	nil
5		nil	nil	nil	nil	nil	nil
6		nil	nil	nil	nil	nil	nil
			Females				
4		nil	0·185†	nil	0·089	0·457†	0·775†
5		nil	nil	nil	nil	nil	nil
6		nil	nil	nil	nil	0·067	0·106†

*Time after first dose.
†Faeces observed in urine bottle.

Discussion

From the results presented here, it may be concluded that little, if any, [^{14}C]methylcellulose with a viscosity of 3300 cP is absorbed following ingestion. These results are not surprising, because the average molecular weight of this material is about 77,000. Small

amounts (less than 0·1%) of the administered ^{14}C activity were found in the carcasses and urine. This indication of minute absorption may have resulted from absorption of ^{14}C-labelled fragments of small molecular weight formed during synthesis or split off in the gastro-intestinal tract.

These findings, together with those of previous toxicological studies (Bauer & Lehman, 1951; Deichmann & Witherup, 1943; Knight *et al.* 1952; McCollister *et al.* 1973), support the conclusion that the use of this type of methylcellulose for the purposes mentioned in the introduction is not hazardous.

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ABSORPTION OF PESTICIDAL CARBAMATES FROM PERFUSED INTESTINAL LOOPS IN CONSCIOUS SWINE

J. C. PEKAS

United States Department of Agriculture, Agricultural Research Service, Metabolism and Radiation Research Laboratory, Fargo, North Dakota 58102, USA

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Summary—Five carbamate pesticides (carbyne, zectran, baygon, mobam and carbaryl) dissolved in isotonic saline in a concentration of 10^{-6} were perfused in sequence through an isolated intestinal loop of a miniature pig. The disappearance half-times were approximately 30 min, although the rate of absorption varied somewhat with the structure of the carbamate. The perfusion system used was constructed entirely of teflon and the results obtained reflected the true intestinal absorption of the carbamates.

Introduction

In a previous study of the intestinal absorption of carbamate pesticides by perfusion of isolated loops of intestine, the lipophilic substrates were rapidly taken up by the latex and polyvinyl chloride tubing and catheters of the perfusion equipment (Pekas, 1972). In this study, the absorption of five pesticidal carbamates was re-examined using an improved perfusion system.

Experimental

The original perfusion system (Pekas, 1974) was improved by replacing the latex and polyvinyl chloride tubing with teflon tubing and by replacing the latex catheters in the intestinal loop with teflon substitutes machined from teflon rods. Also, a pump with an all-teflon pump head (Fluorocarbon, Anaheim, CA, Model DPA-150) replaced the peristaltic pump.

Five ^{14}C-labelled carbamates (see Table 1) were each dissolved in isotonic saline to a concentration of 10^{-6} M. Solution of the carbamates was verified by ^{14}C assay of the medium.

A miniature pig (USDA, Beltsville, Md) was trained and prepared with an isolated loop of terminal ileum (approximately 60 cm long) at 10 months of age (Pekas, 1974). The perfusions were conducted between 40 and 45 days after surgery. Each carbamate-containing medium was equilibrated with the apparatus and perfused through the isolated loop of intestine in the sequence shown in Table 1. Samples of the perfusion medium were taken from the reservoir-mixing flask at 0, 5, 20, 40 and 60 min of intestinal perfusion, and the change in ^{14}C concentration was measured by liquid scintillation. Total recovery of the unabsorbed ^{14}C after the perfusion was measured, as in a previous study with ^{14}C-glucose (J. C. Pekas, unpublished observations 1973). Saline rinses of the apparatus and intestinal loop were used (Pekas, 1974) to overcome limitations imposed on the absorption measurements because of concentration shifts resulting from water absorption or secretion.

Table 1. *Identity and radioactive labelling of perfused carbamates and sequence of intestinal perfusion*

Carbamate*		Position of ^{14}C label	Intestinal perfusion sequence
Common name	Chemical structure		
Carbyne	4-Chloro-2-butynyl *N*-(3-chlorophenyl) carbamate	[1-^{14}C]Butynyl	Blank perfusion†, 1 & 2
Zectran	4-Dimethylamino-3,5-xylyl *N*-methylcarbamate	[^{14}C]Carbonyl	3
Baygon	*O*-Isopropoxyphenyl *N*-methylcarbamate	[1,3-^{14}C]Isopropoxy	4
Mobam	4-Benzothienyl *N*-methyl carbamate	[4,7-^{14}C]Benzothienyl	5
Carbaryl	1-Naphthyl *N*-methyl carbamate	[^{14}C]Carbonyl	6

*Each carbamate was dissolved in 100 ml isotonic saline to give a concentration of 1×10^{-6} M. Solution of the ^{14}C carbamate was verified by ^{14}C assay.
†In the blank perfusion, the two catheters (identical to the catheters used in the intestinal loops) were joined through a glass union.

Results and Discussion

The logarithm of the ^{14}C concentration is plotted as a function of perfusion time in Fig. 1.

Included in Fig. 1 are the results of a blank perfusion in which carbyne was continuously recycled through the perfusion system as in the intestinal perfusions, except that the catheters were connected by a glass union. Carbyne was selected for the blank perfusion because it was retained by tubing and catheters to the greatest extent in the previous study

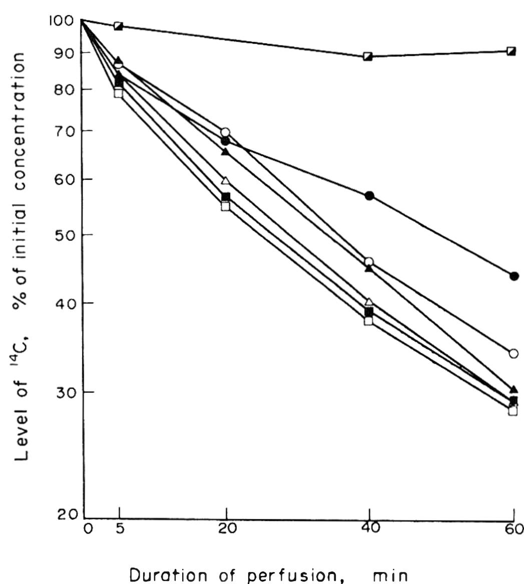

Fig. 1. Logarithmic curves of ^{14}C disappearance over 1 hr during intestinal perfusion of media containing ^{14}C-labelled carbyne (◪, blank perfusion; □, perfusion no. 1; ■, perfusion no. 2), zectran (○), baygon (●), mobam (△) or carbaryl (▲), each in a concentration of 10^{-6} M.

(Pekas, 1972). The slight disappearance of [^{14}C]carbyne during the blank perfusion is in sharp contrast to the rapid disappearance when latex and polyvinyl chloride tubing were used.

Although the plotted logarithmic ^{14}C-disappearance curves did not fit perfect first-order kinetics, the time required for the ^{14}C concentration to decrease to half of the initial concentration was estimated from the plotted line. These estimates of the disappearance half-time ($T_{0.5}$) ranged from approximately 25 min for carbyne to 50 min for baygon. Disappearance half-times ranged between 30 and 35 min for zectran, mobam and carbaryl. The quantities of ^{14}C absorbed were estimated more precisely by subtracting from the initial quantity the quantity of ^{14}C recovered in the perfusion fluid and saline rinses. These recovery values, along with the percentage decrease in the ^{14}C concentrations, are shown in Table 2. The tendency for the absorption rate estimated from the concentration change

Table 2. *Absorption of* [^{14}*C*]*carbamates by two criteria*

		^{14}C absorption in 1 hr (% of initial concn) estimated from	
Carbamate	Perfusion sequence	Concentration change*	Quantitative recovery†
Carbyne	Blank	5·0	3·1 (1·2)‡
	1	71·2	67·6
	2	70·5	66·0
Zectran	3	66·3	65·7
Baygon	4	56·2	54·9
Mobam	5	70·4	64·1
Carbaryl	6	69·5	67·1

*Estimated from percentage decrease in ^{14}C concentration.
†Estimated from 100 − % recovery of ^{14}C.
‡Value in parentheses was corrected for the ^{14}C recovered (1·9%) by methanol extraction of the perfusion apparatus after the blank perfusion (98·8% total recovery).

to exceed that estimated from the ^{14}C recovered suggests either that water was secreted into the intestinal loop and diluted the ^{14}C concentration or that the saline rinse recovered excess ^{14}C by "washout" of tissue carbamates or radiolabelled metabolites. The relative rates of absorption of the five carbamates were approximately the same by each of these criteria.

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CARCINOGENICITY OF SYNTHETIC AFLATOXIN M_1 IN RATS

G. N. WOGAN and S. PAGLIALUNGA

Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA

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Summary—Synthetic, racemic aflatoxin M_1 administered to male Fischer rats by stomach tube in 40 doses totalling 1 mg/rat induced hepatocellular carcinoma in one rat of a group of 29 killed 100 wk after dosing. Natural aflatoxin B_1 at the same dose induced liver tumours in 9/9 rats after 53 wk. The hydroxylated derivative, therefore, has a much lower carcinogenic potency than the parent substance.

Introduction

Aflatoxin M_1 (4-hydroxyaflatoxin B_1) was originally identified in the milk of cows and rats fed aflatoxin-containing rations (Allcroft & Carnaghan, 1962 & 1963) and is found in the urine of rats, sheep and monkeys dosed with aflatoxin B_1 (Allcroft, Rogers, Lewis, Nabney & Best, 1966; Dalezios, Wogan & Weinreb, 1971; Holzapfel, Steyn & Purchase, 1966). Humans ingesting diets contaminated with aflatoxin B_1 also excrete a small proportion of the ingested toxin in the form of M_1 (Campbell, Caedo, Bulatao-Jayme, Salamat & Engel, 1970). The metabolic capacity for producing this hydroxylated derivative therefore seems to be widely distributed in animals.

Comparatively little is known about the acute and chronic toxicity of M_1 in animals, owing to the limited availability of the natural compound, which must be isolated from biological sources. Purchase (1967) and Holzapfel *et al.* (1966) reported that its LD_{50} value in ducklings was comparable to that of B_1 and that similar liver lesions were produced by the two toxins. In the only previously reported carcinogenicity evaluation, Sinnhuber, Lee, Wales, Landers & Keyl (1970) found that M_1 was carcinogenic to rainbow trout but was only about one third as potent as B_1 in inducing liver tumours when fed continuously at a level of 4 ppb in the diet.

Further experiments became possible with the availability of milligram quantities of synthetic aflatoxin M_1 (Büchi & Weinreb, 1969). Laboratory synthesis of this compound yields a racemic mixture of two isomers, as is the case with synthetic B_1 (Büchi, Foulkes, Kurono, Mitchell & Schneider, 1967) while naturally occurring B_1 consists of a single isomer (Asao, Büchi, Abdel-Kader, Chang, Wick & Wogan, 1965). Structures of these compounds are shown in Fig. 1.

In an earlier series of experiments, we compared in rat liver the acute toxicity and biochemical effects of synthetic aflatoxins M_1 and B_1 with those of natural B_1 (Pong & Wogan, 1971). The effects of aflatoxin M_1 were qualitatively similar to those of B_1, and the two synthetic compounds were of approximately equal potency. Both were less potent than natural B_1, suggesting that only one isomer in the racemic mixtures was active. At that time, we initiated an experiment to determine whether synthetic M_1 of the same lot

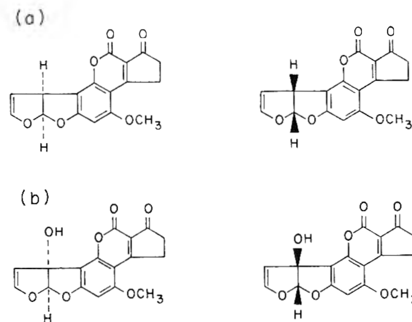

Fig. 1. Structures of the enantiomeric forms of aflatoxins B_1 (a) and M_1 (b). In each case the natural form is on the left.

would be carcinogenic to rats and to compare its potency with that of natural B_1. We report here the results of that experiment.

Experimental

Materials. Natural aflatoxin B_1 was isolated and purified by previously described methods (Asao *et al.* 1965). Synthetic, racemic aflatoxin M_1 was generously provided by Prof. George Büchi of the Department of Chemistry, M.I.T. The compound contained less than 5% impurities, judged by spectral and chromatographic properties (Büchi & Weinreb, 1969).

Animals and treatment. Weanling male Fischer rats were starved for 6 hr and were then dosed by intubation with 0·5 ml of a freshly prepared suspension of aflatoxin in distilled water. Food was offered 1 hr later. Each animal was dosed 5 days/wk for eight consecutive weeks and held thereafter without treatment. The animals were housed singly in wire cages and were fed a semi-purified diet (Wogan & Newberne, 1967). Thirty rats were treated with aflatoxin M_1 and ten with aflatoxin B_1, each animal receiving 25 μg/day and therefore a total dose of 1 mg. Fifteen rats were dosed with distilled water only. The experiment was terminated when animals died or began to show clinical deterioration, at which time all animals in the group were killed. Each rat was autopsied, and tissues were subjected to histopathological examination (Newberne & Wogan, 1968).

Results

The results are summarized in Table 1. All rats treated with aflatoxin B_1 developed hepatocellular carcinomas. The earliest tumour observed was present in a rat that died

Table 1. *Comparative carcinogenicity of aflatoxins M_1 and B_1*

Treatment	Length of observation (wk)	Incidence of liver pathology*			Time of detection of first tumour (wk)
		Hyperplasia	Transitional cells	Carcinoma	
Control	100	0/12	0/12	0/12	—
Aflatoxin B_1	53	0/9	1/9	9/9†	47
Aflatoxin M_1	100	4/29	4/29	1/29	96

*In animals surviving longer than 47 wk.
†Metastases were found in the lungs of three of these animals.

after 47 wk, and all the remaining animals, killed after 53 wk because of weight loss, also bore tumours.

Only one rat dosed with aflatoxin M_1 developed a hepatocellular carcinoma; this animal was killed after 96 wk. The remaining animals in this group were killed after 100 wk, and of these eight had liver lesions which we regarded as early or advanced preneoplastic lesions (hyperplasia or transitional cells, respectively), on the basis of earlier experience with aflatoxin responses in this strain of rat (Newberne & Wogan, 1968). The controls showed no significant liver pathology, and the only tumours observed in other tissues were interstitial cell tumours of the testes, which occur spontaneously in high incidence in this rat strain and were unrelated to aflatoxin treatment.

Discussion

The potency of aflatoxin B_1 as a hepatocarcinogen for rats is well illustrated by this experiment. With respect to tumour incidence and time of appearance, these results agree well with previous observations (Wogan, 1973). When evaluated under identical experimental conditions, aflatoxin M_1 clearly had a much lower carcinogenic potency. Even though the effective dose of M_1 was probably only half that of B_1 (assuming only one isomer of the racemic mixture to be biologically active), tumour induction capacity was far less than that previously observed with equivalent doses of B_1. In the only other published comparison of the carcinogenic activity of these two toxins, natural M_1 was also found to be less potent than natural B_1 as a carcinogen for the rainbow trout (Sinnhuber *et al.* 1970). However, the difference in potency was much less than in the present case.

Of particular interest are the differences between acute and chronic responses to aflatoxin M_1 in the rat. In our previous studies on the acute effects of aflatoxin M_1 (carried out with the same batch of the synthetic material and at the same time as the present experiment was initiated), synthetic M_1 produced responses qualitatively similar to those of synthetic or natural B_1 in several experimental parameters. These included lethality, electron-microscopic changes, precursor incorporation into nuclear RNA, and nuclear RNA/DNA ratios in the liver. These findings were taken to indicate that the two toxins may act through similar mechanisms in producing toxic and subcellular effects. Furthermore, in these acute responses, synthetic M_1 and B_1 were of apparently equal potency.

In contrast, the present findings indicate that M_1 is much less potent than B_1 as a carcinogen for rat liver. Aflatoxin M_1 may therefore represent a useful compound for future experiments designed to differentiate cytotoxic from transformational events, a distinction important to the study of the mechanisms underlying aflatoxin carcinogenesis.

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MONOGRAPHS

Monographs on Fragrance Raw Materials*

D. L. J. OPDYKE

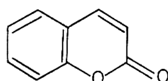
Research Institute for Fragrance Materials, Inc., P.O. Box 1152, Englewood Cliffs, New Jersey 07632, USA

(*Received 7 December 1973*)

COUMARIN

Synonyms: 2-Oxo-1,2-benzopyran; 1,2-benzopyrone; *cis-o*-coumaric acid lactone; coumarinic anhydride; tonka bean camphor.

Structure:



Description and physical properties: EOA Spec. no. 201.
Occurrence: Found in many plants and essential oils such as cassia, melilot, orchid, lavender and balsam of Peru (Späth, 1937; Gildemeister & Hoffman, 1966).
Preparation: From salicylaldehyde by suitable chemical reaction and purification (Bedoukian, 1967).
Uses: In public use since the 1900s. Use in fragrances in the USA amounts to about 250,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·03	0·003	0·015	0·3
Maximum	0·2	0·02	0·1	0·8

Status

The FDA has prohibited the use of coumarin in food (21 CFR 121.106).

Biological data

Acute toxicity. The acute oral LD_{50} was reported to be 196 mg/kg in mice (Kitagawa & Iwaki, 1963), 293–680 mg/kg in rats (Hazleton, Tusing, Zeitlin, Thiessen & Murer, 1956; Jenner, Hagan, Taylor, Cook & Fitzhugh, 1964) and 202 mg/kg in guinea-pigs (Jenner *et al.* 1964). The sc LD_{50} was reported as 310–342 mg/kg in mice (Kitagawa & Iwaki, 1963).

Chronic toxicity (*oral route*). In a 90-day rat study, coumarin at 50 or 250 ppm in the diet had no effect on weight gain, efficiency of food utilization or organ pathology, but a dietary level of 2500 ppm impaired food efficiency and produced liver enlargement and liver damage (Hazleton *et al.* 1956). In a 2-yr rat study, no effects were seen with 1000 ppm

*The latest of the previous sets of these monographs appeared in *Food and Cosmetics Toxicology* 1973, **11**, 1011.

coumarin in the diet, but growth retardation and liver damage (bile-duct proliferation, cholangiofibrosis and focal necrosis) were seen with 2500 and 5000 ppm fed for 2 yr and also with 10,000 ppm fed for up to 8 wk (Hagan, Hansen, Fitzhugh, Jenner, Jones, Taylor, Long, Nelson & Brouwer, 1967). In another 2-yr rat study, feeding of 5000 ppm for 18 months was reported to produce bile-duct carcinomas, predominantly in males; extrahepatic metastasis was low (Bär & Griepentrog, 1967). The diagnosis of bile-duct carcinomas has been questioned however (*Food and Cosmetics Toxicology*, 1969).

In a 3-wk dog study, eight daily oral doses of 100 mg/kg produced liver damage and kidney changes compatible with a bile nephrosis (Hazleton *et al.* 1956).

In a 1-yr dog study, daily oral doses of 25, 50 and 100 mg/kg body weight given for 130–330, 35–277 and 9–16 days, respectively, caused emaciation, jaundice, liver damage (focal necrosis, fibrosis, and bile-duct proliferation) and pathological changes in the spleen, bone marrow and gall bladder, but no effect was seen with 10 mg/kg/day given for 297–350 days (Hagan *et al.* 1967).

Chronic toxicity (dermal route). A single application of 15% coumarin in acetone to mouse skin failed to produce epidermal hyperplasia within 3 days of dosage and no tumour-initiating activity was seen after either a single dose of 45 mg coumarin (15% in acetone) or a total dose of 150 mg coumarin (one dose of 10% in acetone followed by 12 weekly doses of 3·3% in acetone) to mouse skin followed in both cases by treatment with the tumour promoter, croton oil (Roe & Salaman, 1955).

Chronic toxicity (sc route). Twice-weekly sc injections of 2 mg coumarin in 0·5 ml arachis oil given for 65 wk to rats did not induce sarcomas at the injection site over 2 yr (Dickens & Jones, 1965).

Teratogenicity. The offspring of mice fed dietary levels of 0·05–0·25% coumarin on days 6–17 of pregnancy exhibited no malformations but increased stillbirths and delayed ossification were seen at the 0·25% level and increased mortality up to 3 wk of life was seen at all levels (Roll & Bär, 1967).

Sensitization. A maximization test (Kligman, 1966) carried out on 25 human volunteers using an 8% concentration in petrolatum produced no sensitization reactions (Greif, 1967).

Percutaneous absorption. Rabbits dosed dermally or orally with coumarin showed a similar pattern in the urinary excretion of coumarin metabolites (Pekker & Schäfer, 1969).

Metabolism. There is a striking difference in the metabolism of coumarin between man and other mammalian species. In man, coumarin is metabolized mainly to 7-hydroxycoumarin (Shilling, Crampton & Longland, 1969) but this metabolic transformation assumes lesser importance in the rat (Kaighen & Williams, 1961; Van Sumere & Teuchy, 1971) and rabbit (Kaighen & Williams, 1961), in which two species the excretion of 2-hydroxyphenylacetic acid is more marked than in man.

In man, 68–92% of an oral dose of coumarin is excreted in the urine as 7-hydroxycoumarin and 1–6% as 2-hydroxyphenylacetic acid (Shilling *et al.* 1969).

In the rat, about 55% of an oral dose of [3-^{14}C]coumarin is excreted in the urine, 3% of the dose as hydroxycoumarins, including 7-hydroxycoumarin, and 20% as 2-hydroxyphenylacetic acid; about 36% of the dose is eliminated in the faeces, partly as 2-hydroxyphenylacetic acid (Kaighen & Williams, 1961). Feuer, Golberg & Gibson (1966) found that 48 hr after administration of an oral dose of [3-^{14}C]coumarin to rats, 70% of the dose was excreted in the urine and 10% in the faeces. The major metabolites in 24-hr urine were 2-hydroxyphenylacetic acid and 2-hydroxyphenyllactic acid. In a more recent study in rats (Van Sumere & Teuchy, 1971), 37% of an ip dose of [2-^{14}C]coumarin was excreted in the

urine, 30% in the expired air and 14% in the faeces. Metabolites identified in the urine included 5-, 7- and 8-hydroxycoumarins, *o*-coumaric acid, melilotic acid (2-hydroxyphenylpropionic acid) and 2-hydroxyphenylacetic acid together with unchanged coumarin, but only 0·7% of the dose was excreted as 7-hydroxycoumarin.

In the rabbit, benzene-ring hydroxylation and conjugation was more marked than in the rat and 90% of an oral dose of [3-^{14}C]coumarin was excreted in the urine as 3-hydroxycoumarin (21·7% of the dose), 7-hydroxycoumarin (12%), 4-, 5-, 6- and 8-hydroxycoumarins (0·4–3·4%), 2-hydroxyphenylacetic acid (20%) and 2-hydroxyphenyllactic acid (3%) (Kaighen & Williams, 1961). Following application of coumarin as a 5% ointment to the shaved skin of rabbits, the urinary metabolites included mainly 7-hydroxycoumarin and smaller amounts of 3- and 4-hydroxycoumarin, 2-hydroxyphenylacetic acid, 6,7-dihydroxycoumarin and unchanged coumarin, as judged by the intensity of the thin-layer chromatographic spots (Pekker & Schäfer, 1969). However the claim for 7-hydroxycoumarin as a major urinary metabolite following oral or dermal dosage of coumarin to rabbits needs to be confirmed (*Food and Cosmetics Toxicology*, 1970).

Other species such as the ferret, guinea-pig and mouse metabolize coumarin to 3-, 5-, 7- and 8-hydroxycoumarins, which are excreted in the urine (Mead, Smith & Williams, 1958).

On incubation with rat-liver microsomes, [3-^{14}C]coumarin is transformed into 3- and 7-hydroxycoumarins, 2-hydroxyphenylacetic acid and 2-hydroxyphenyllactic acid (Gibbs, Janakidevi & Feuer, 1971).

Biochemical assays on liver microsomes of various species have revealed coumarin-7-hydroxylase activity in rabbits, guinea-pigs, coypu, cats and pigeons, but not in mice or rats (Creaven, Parke & Williams, 1965) but more recently coumarin 7-hydroxylase has been demonstrated in rat-liver microsomes although its activity was lower than that of coumarin 3-hydroxylase (Feuer, 1970; Gibbs *et al.* 1971). Human liver can also 7-hydroxylate coumarin *in vitro* (*Food and Cosmetics Toxicology*, 1966).

The species difference in the metabolism of coumarin requires important toxicological consideration. Coumarin and other hepatotoxic agents inhibit liver glucose-6-phosphatase and this effect is regarded as being indicative of liver damage (Feuer, Golberg & Le Pelley, 1965a,b). 2-Hydroxyphenylacetic acid strongly inhibits rat-liver glucose-6-phosphatase *in vitro* and to a lesser extent *in vivo*, whereas neither coumarin nor 7-hydroxycoumarin (the major metabolite in man) causes inhibition *in vitro* (Feuer *et al.* 1966). It is thus conceivable that the hepatotoxicity of coumarin in the rat is attributable to 2-hydroxyphenylacetic acid, the major urinary metabolite in the rat but minor metabolite in man. Consequently man could be less susceptible than the rat to the hepatotoxic action of coumarin.

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p-CRESOL

Synonyms: 4-Hydroxytoluene; 1-methyl-4-hydroxybenzene.
Structure: $CH_3{\cdot}C_6H_4{\cdot}OH$.
Description and physical properties: White crystals with a phenolic odour (*Merck Index*, 1968).
Occurrence: Has been found in a score of essential oils including ylang ylang and oil of jasmine (Gildemeister & Hoffman, 1966).
Preparation: By alkali fusion of *p*-toluenesulphonic acid (Arctander, 1969).
Uses: In public use since the 1930s. Use in fragrances in the USA amounts to less than 2000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·005	0·001	0·001	0·04
Maximum	0·2	0·02	0·005	0·4

Analytical data: Gas chromatogram, RIFM no. 72–110; infra-red curve, RIFM no. 72–110.

Status

p-Cresol was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1970) included *p*-cresol in the list of artificial flavouring substances not admissible at present.

Biological data

Acute toxicity. The acute oral LD_{50} was reported as 1·8 g/kg in the rat (*Merck Index*, 1968). The acute dermal LD_{50} in rabbits was reported as 3·6 (2·67–4·86) g/kg (Denine, 1973).

Irritation. *p*-Cresol applied full strength on intact or abraded rabbit skin was irritating (Denine, 1973). *p*-Cresol tested at 4% in petrolatum produced no irritation after a 48-hr closed-patch test in 25 human subjects (Kligman, 1972).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at 4% concentration in petrolatum and produced no sensitization reactions (Kligman, 1972).

Threshold Limit Value. The TLV for *p*-cresol has been set at 5 ppm, at which level prolonged use may cause reddening and itching of the skin and, in time, dermatitis, eczema and even ulceration. Inhalation of the vapour has caused headache, nausea and vomiting, and tremor (American Conference of Governmental Industrial Hygienists, 1970).

Metabolism. *p*-Cresol is oxidized at the methyl group in both dogs and rabbits to yield *p*-hydroxybenzoic acid. In the rabbit up to 10% of oral doses of 0·25–0·5 g is excreted as free and conjugated *p*-hydroxybenzoic acid (Williams, 1959).

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p-CRESYL ACETATE

Synonyms: *p*-Tolyl acetate; 4-methylbenzoic acid methyl ester.
Structure: $CH_3{\cdot}C_6H_4{\cdot}CO_2{\cdot}CH_3$
Description and physical properties: EOA Spec. no. 223.
Occurrence: Found in oils of cananga, wallflower and ylang ylang (Gildemeister & Hoffman, 1966).
Preparation: By acetylation of *p*-cresol.
Uses: In public use since the 1920s. Use in fragrances in the USA amounts to less than 1000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·02	0·002	0·01	0·12
Maximum	0·1	0·01	0·05	0·4

Analytical data: Gas chromatogram, RIFM no. 72–233; infra-red curve, RIFM no. 72–233.

Status

p-Cresyl acetate was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1970) included *p*-cresyl acetate in the list of admissible artificial flavouring substances at a level of 4 ppm. The *Food Chemicals Codex* (1972) has a monograph on *p*-cresyl acetate.

Biological data

Acute toxicity. The acute oral LD_{50} in rats was reported as 1·9 (1·12–3·23) g/kg (Denine, 1973). The acute dermal LD_{50} in rabbits was reported as 2·1 (1·24–3·57) g/kg (Denine, 1973).

Irritation. *p*-Cresyl acetate applied full strength on intact or abraded rabbit skin produced no irritation (Denine, 1973). *p*-Cresyl acetate tested at 4% in petrolatum produced no irritation after a 48-hr closed-patch test in 25 human subjects (Kligman, 1972).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at 4% concentration in petrolatum and produced no sensitization reactions (Kligman, 1972).

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p-CRESYL METHYL ETHER

Synonyms: Methyl *p*-cresol; *p*-methylanisole; 4-methylphenol methyl ether; 4-methyl-1-methoxybenzene.
Structure: $CH_3{\cdot}C_6H_4{\cdot}OCH_3$.
Description and physical properties: EOA Spec. no. 224.
Occurrence: Found in oil of ylang ylang, cananga and others (Gildemeister & Hoffman, 1966).
Preparation: By methylation of *p*-cresol.
Uses: In public use since the 1920s. Use in fragrances in the USA amounts to less than 10,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·03	0·003	0·01	0·2
Maximum	0·15	0·015	0·05	0·2

Analytical data: Gas chromatogram, RIFM no. 72–193; infra-red curve, RIFM no. 72–193.

Status

p-Cresyl methyl ether was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1970) included *p*-cresyl methyl ether in the list of admissible artificial flavouring substances at a level of 5 ppm. The *Food Chemicals Codex* (1972) has a monograph on *p*-cresyl methyl ether.

Biological data

Acute toxicity. The acute oral LD_{50} in rats was reported as 1·92 (1·51–2·45) g/kg (Hart, 1971). The acute dermal LD_{50} in rabbits was reported as > 5 g/kg (Hart, 1971).

Irritation. *p*-Cresyl methyl ether applied full strength on intact or abraded rabbit skin was moderately irritating (Hart, 1971). *p*-Cresyl methyl ether tested at 2% in petrolatum produced no irritation after a 48-hr closed-patch test in 25 human subjects (Kligman, 1971).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 human volunteers. The material was tested at a concentration of 2% in petrolatum and produced no sensitization reactions (Kligman, 1971).

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CUMINALDEHYDE

Synonyms: Cuminic aldehyde; *p*-isopropylbenzaldehyde; 4-isopropylbenzaldehyde.
Structure: $CH_3 \cdot CH(CH_3) \cdot C_6H_4 \cdot CHO$.
Description and physical properties: *Givaudan Index* (1961).
Occurrence: Found in at least 50 essential oils such as cumin, Eucalyptus species, cinnamon, boldo and rue, and as the main constituent in oil of *Pectis papposa* Harn and Gray (Gildemeister & Hoffman, 1963).
Preparation: From *p*-isopropylbenzyl chloride and hexamethylenetetramine (Arctander, 1969).
Uses: In public use since the 1930s. Use in fragrances in the USA amounts to less than 3000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·002	0·05
Maximum	0·05	0·05	0·02	0·4

Analytical data: Gas chromatogram, RIFM no. 72–114; infra-red curve, RIFM no. 72–114.

Status

Cuminaldehyde was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1970) included cuminaldehyde in the list of admissible artificial flavouring substances, at 15 ppm.

Biological data

Acute toxicity. The acute oral LD_{50} was reported as 1·39 g/kg in the rat (Jenner, Hagan, Taylor, Cook & Fitzhugh, 1964). The acute dermal LD_{50} in rabbits was reported as 2·8 (2·24–3·50) g/kg (Denine, 1973).

Irritation. Cuminaldehyde applied undiluted to the backs of hairless mice produced no irritating effects (Urbach & Forbes, 1972), but was irritating when applied full strength to intact or abraded rabbit skin (Denine, 1973). Cuminaldehyde tested at 4% in petrolatum produced no irritation after a 48-hr closed-patch test in 25 human subjects (Kligman, 1972).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at 4% concentration in petrolatum and produced no sensitization reactions (Kligman, 1972).

Phototoxicity. No phototoxic effects were reported for cuminaldehyde (Urbach & Forbes, 1972).

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CYCLAMEN ALDEHYDE

Synonyms: *p*-Isopropyl-α-methylhydrocinnamaldehyde; 2-methyl-3-(*p*-isopropylphenyl)-propionaldehyde.
Structure: $CH_3{\cdot}CH(CH_3){\cdot}C_6H_4{\cdot}CH_2{\cdot}CH(CH_3){\cdot}CHO$.
Description and physical properties: EOA Spec. no. 149.
Occurrence: Apparently has not been reported to occur in nature.
Preparation: By the condensation of cuminic aldehyde and propionaldehyde followed by hydrogenation in the presence of a catalyst (Bedoukian, 1967).
Uses: In public use since the 1920s. Use in fragrances in the USA amounts to about 150,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·02	0·02	0·01	0·12
Maximum	0·2	0·03	0·03	0·3

Analytical data: Gas chromatogram, RIFM no. 70–65; infra-red curve, RIFM no. 70–65.

Status

Cyclamen aldehyde was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1970) included cyclamen aldehyde in the list of admissible artificial flavouring substances, at a level of 1 ppm. The *Food Chemicals Codex* (1972) has a monograph on cyclamen aldehyde.

Biological data

Acute toxicity. The acute oral LD_{50} value in rats was reported as 3·81 g/kg (Jenner, Hagan, Taylor, Cook & Fitzhugh, 1964).

Irritation. Cyclamen aldehyde tested at 3% in petrolatum produced a mild irritation after a 48-hr closed-patch test in 25 human subjects (Kligman, 1971).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 human volunteers. The material was tested at a concentration of 3% in petrolatum and produced no sensitization reactions (Kligman, 1971).

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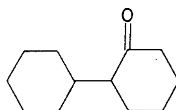
2-CYCLOHEXYL CYCLOHEXANONE

Synonyms: Bicyclohexanone; 2-cyclohexyl cyclohexan-1-one.
Structure:



Description and physical properties: A colourless liquid with a comparatively sweet odour (Arctander, 1969).
Occurrence: Apparently has not been reported to occur in nature.
Preparation: By self-condensation of cyclohexanone followed by reduction.
Uses: Use in fragrances in the USA amounts to about 35,000 lb/yr.
Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·05	0·01	0·10	0·1
Maximum	0·30	0·03	0·15	2·0

Analytical data: Gas chromatogram, RIFM no. 72–116; infra-red curve, RIFM no. 72–116.

Status

Cyclohexyl cyclohexanone is not listed by the Council of Europe (1970), the *Food Chemicals Codex* (1972), FEMA (1965) or the FDA.

Biological data

Acute toxicity. The acute oral LD_{50} was reported as >5 g/kg in the rat (Denine, 1973). The acute dermal LD_{50} was reported as >5 g/kg in the rabbit (Denine, 1973).

Irritation. Cyclohexyl cyclohexanone applied full strength to intact or abraded rabbit skin was not irritating (Denine, 1973). Tested at 20% in petrolatum, it produced no irritation after a 48-hr closed-patch test in 25 human subjects (Kligman, 1972).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at 20% concentration in petrolatum and produced no sensitization reactions (Kligman, 1972).

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p-CYMENE

Synonyms: Cymene; *p*-methyl-isopropylbenzene; 4-isopropyl-1-methylbenzene.
Structure: $CH_3{\cdot}C_6H_4{\cdot}CH(CH_3){\cdot}CH_3$.
Description and physical properties: A colourless mobile liquid with a 'gassy', kerosene-like odour (Arctander, 1969).
Occurrence: Found in nearly 100 volatile oils, including lemon, sage, thyme, coriander, star anise and cinnamon (Gildemeister & Hoffman, 1960).
Preparation: By catalytic disproportionation of dipentene (Arctander, 1969). It can also be obtained by dehydration of camphor and is an important by-product in the sulphite process for paper manufacture (Gerarde, 1960).
Uses: In public use since the 1920s. Use in fragrances in the USA amounts to less than 9000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·01	0·1
Maximum	0·1	0·01	0·1	0·4

Analytical data: Gas chromatogram, RIFM no. 72–117; infra-red curve, RIFM no. 72–117.

Status

p-Cymene was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1970) included *p*-cymene in the list of artificial flavouring substances not admissible at present. Browning (1965) provided an extensive monograph on *p*-cymene.

Biological data

Acute toxicity. The acute oral LD_{50} in rats was reported as 4·75 g/kg (Jenner, Hagan, Taylor, Cook & Fitzhugh, 1964). The lethal dose by ip administration is 2·162 g/kg in the guinea-pig (Chassevant & Garnier, 1903). The acute dermal LD_{50} in rabbits was reported as >5 g/kg (Moreno, 1973).

Skin absorption. *p*-Cymene is well absorbed through the skin. In studies with ^{14}C-labelled *p*-cymene, the penetration observed was 254 $\mu g/cm^2$ in 60 min (Wepierre, Cohen & Valette, 1968). Absorption by the skin is more rapid than with toluene, *p*-xylene or ethylbenzene (Valette & Cavier, 1954).

Irritation. *p*-Cymene applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was moderately irritating (Moreno, 1973). Tested at 4% in petrolatum, it produced no irritation after a 48-hr closed-patch test in 25 human subjects (Kligman, 1972). *p*-Cymene is reported to be a primary skin irritant; contact with the undiluted liquid can produce erythema, dryness and defatting, the intensity depending on the dose and duration of contact (Gerarde, 1960). In an effort to explore the possible effects of vehicle and concentration on cutaneous irritation the Kligman & Wooding (1967) method was used to test the following formulations, applied under occlusion daily for 10 days to the same spot on the backs of ten subjects: Diethyl phthalate; diethyl phthalate containing 0·4% *p*-cymene; diethyl phthalate containing 4% *p*-cymene; petrolatum; petrolatum containing 0·4% *p*-cymene; petrolatum containing 4% *p*-cymene. There were no instances of marginal irritation and no differences between the groups (Kligman, 1973).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at 4% concentration in petrolatum and produced no sensitization reactions (Kligman, 1972).

Threshold Limit Value. The TLV for *p*-cymene has been set at 100–200 ppm (by analogy with toluene), at which level it may be expected to have irritative and narcotic properties (*Handbook of Organic Industrial Solvents*, 1961).

Metabolism. In this compound both an isopropyl and a methyl group occur together and the available evidence shows that only the methyl group is oxidized, cumic acid (*p*-isopropylbenzoic acid) and its conjugate (cuminuric acid) being the main metabolites in dogs and in sheep (Williams, 1959). Following inhalation, only a small part is excreted unchanged, the remainder being oxidized to water-soluble metabolites. As early as 1873, Ziegler suggested that the readily oxidized propyl side-chain formed a –COOH group (Browning, 1965). The ultimate product in the case of dogs and sheep is cumic acid, which is probably excreted as a conjugate with glycine (Gerarde, 1960).

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CYSTE ABSOLUTE

Synonym: Ciste absolute.
Description and physical properties: A greenish alcoholic extract of the plant *Cistus ladaniferus* with a sweet amber-like odour (Naves & Mazuyer, 1947).
Occurrence: Found in the leaves, stems and flowering tops of the plant *Cistus ladaniferus* L. (Fam. Cistaceae) (Naves & Mazuyer, 1947).
Preparation: By alcoholic extraction of the concrete from the plant (Naves & Mazuyer, 1947).
Uses: In public use since the 1940s. Use in fragrances in the USA amounts to less than 1000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·01	0·1
Maximum	0·1	0·01	0·04	0·4

Analytical data: Infra-red curve, RIFM no. 72–118.

Status

The FDA approves cyste absolute for food use (21 CFR 121.1163). The Council of Europe (1970) listed cyste absolute (*Cistus incanus* Ladaniferus) in the list of substances, spices and seasonings whose use is deemed admissible, with a possible limitation of the active principle in the final product.

Biological data

Acute toxicity. The acute oral LD_{50} was reported as >5 g/kg in the rat (Shelanski & Moldovan, 1973). The acute dermal LD_{50} was reported as >5 g/kg in the rabbit (Shelanski & Moldovan, 1973).

Irritation. Undiluted cyste absolute applied to the backs of hairless mice produced no irritating effects (Urbach & Forbes, 1973). It was also non-irritating when applied full strength to intact or abraded rabbit skin (Shelanski & Moldovan, 1973). Tested at 4% in petrolatum, it produced no irritation after a 48-hr closed-patch test in 25 human subjects (Kligman, 1973).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at 4% concentration in petrolatum and produced no sensitization reactions (Kligman, 1973).

Phototoxicity. No phototoxic effects were reported for cyste absolute (Urbach & Forbes, 1973).

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DECYLENIC ALCOHOL

Synonyms: 9-Decenol-1; ω-decenol.

Structure: $CH_2{:}CH{\cdot}[CH_2]_7{\cdot}CH_2OH$.

Description and physical properties: A colourless oily liquid with a rose-like odour (Arctander, 1969).

Occurrence: Apparently has not been reported to occur in nature.

Preparation: From 1,10-decamethylene glycol (Arctander, 1969).

Uses: In public use since the 1950s. Use in fragrances in the USA amounts to less than 20,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·03	0·005	0·01	0·05
Maximum	0·09	0·009	0·04	0·2

Analytical data: Gas chromatogram, RIFM no. 71–73; infra-red curve, RIFM no. 71–73.

Status

Decylenic alcohol is not included in the listings of the FDA, FEMA (1965) or the Council of Europe (1970) or in the *Food Chemicals Codex* (1972).

Biological data

Acute toxicity. Both the acute oral LD_{50} value in rats and the acute dermal LD_{50} value in rabbits exceeded 5 g/kg (Moreno, 1972).

Irritation. Decylenic alcohol applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was moderately irritating (Moreno, 1972). Tested at 2% in petrolatum, it produced no irritation after a 48-hr closed-patch test in 25 human subjects (Kligman, 1972).

Sensitization. A maximization test (Kligman, 1966) was carried out on 25 human volunteers. The material was tested at a concentration of 2% in petrolatum and produced no sensitization reactions (Kligman, 1972).

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Fd Cosmet. Toxicol. Vol. 12, pp. 407–408. Pergamon Press 1974. Printed in Great Britain

Review Section

REVIEWS OF RECENT PUBLICATIONS

Evaluation of Mercury, Lead, Cadmium and the Food Additives Amaranth, Diethylpyrocarbonate, and Octyl Gallate. Joint FAO/WHO Expert Committee on Food Additives. WHO/Food Add./72.4. p.84. £0.75. (Also issued as *F.A.O. Nutr. Mtg Rep. Ser.* no. 51A. Rome, 1972).

The summaries of data presented in the Sixteenth Report of the Joint FAO/WHO Expert Committee on Food Additives (*Cited in F.C.T.* 1973, **11,** 255) have been considerably amplified in this important publication, with citation of the appropriate references and an explanation of the rationale for the recommended limitations on intakes.

In the separate monographs on the three heavy metals, emphasis has been placed on toxicological and metabolic data derived from man rather than animals. This is particularly so in the case of cadmium. Other topics reviewed are occurrence in the environment, uses, methods of analysis, levels in foods and, in the case of mercury, transformation from the inorganic to the organic form.

It is interesting to discover the ingenious methods by which a provisional tolerable weekly intake (PTWI) has been derived for each metal. For mercury, a direct relationship between human intake and toxic effects could not be established, but the relationship between intake and levels in hair and blood could be correlated with levels found in the victims of methylmercury poisoning. The Committee estimated that a minimum daily intake of 0·3 mg (mainly of methylmercury) over prolonged periods was required to cause poisoning, and on this basis established a PTWI of 0·3 mg total mercury, with a maximum of 0·2 mg mercury as methylmercury. A safety factor somewhat less than ten is thus involved, and it is pointed out that this PTWI is currently being exceeded by some fish-eating minorities.

In the case of lead, the Committee has adopted what appears to be a curiously circular argument, estimating the level of absorption from all sources as 1 μg/kg/day, of which up to 20 μg could be absorbed from air; for a 70-kg adult this would leave up to 10 μg to be contributed by water and up to 40 μg by food. Since not more than 10% of ingested lead is thought to be absorbed, the PTWI was set at 3 mg for an adult. This provisional figure may in fact be justified on the basis that normal intake levels are not associated with clinical signs of lead poisoning. In its report, the Committee recognizes that any increase in levels in water or air will reduce the tolerable level in food, and identifies atmospheric lead as the source most accessible to action in support of the long-term objective of reducing the total body burden.

The cadmium evaluation, which owes a large debt to the excellent book by L. Friberg, M. Piscator and G. Nordberg (*ibid* 1972, **10,** 559), cites a value of 200 ppm cadmium as a critical level in the renal cortex, and assumes an absorption rate of 5% and a daily excretion of 0·005% of the body load. The PTWI of 400–500 μg is based on these assumptions and is intended to produce a cadmium level in the kidney of not more than 50 ppm, a

value which is commonly exceeded in Japan. The Committee calls for standardization of analytical methods and for further data on cadmium concentrations in common foodstuffs and whole diets, a need already met in the UK by the recent publication *Survey of Cadmium in Food* (*ibid* 1974, **12**, 139).

The WHO report also re-evaluates amaranth, diethyl pyrocarbonate and *n*-octyl gallate in the light of recent studies, incorporating the data presented in previous reports in the monographs on the first two compounds. Results of recent work on the effects on reproduction and carcinogenicity of amaranth are regarded as difficult to interpret at this stage, and the previously allocated unconditional acceptable daily intake (ADI) of 0–1·5 mg/kg (*ibid* 1967, **5**, 789) has been changed to a temporary ADI of 0–0·75 mg/kg. Although the basis for this is not clearly stated, it is apparently derived from a no-effect level of 7·5 mg/kg established in a rat reproduction study with application of a ten-fold safety factor. It is interesting to note that the FDA, using the same data as a basis for proposing tolerance levels in food, applied the same safety factor but judged the no-effect level to be 15 mg/kg (*Food Chemical News* 1972, **14**(16), 13).

For diethyl pyrocarbonate, recent work on urethane formation has been reviewed, and it is significant that a no-effect level for this carcinogen has been accepted by the Committee. Since soft drinks treated with diethyl pyrocarbonate do not contain more than 10 μg urethane/litre, use in such beverages is considered justified, but not use in wines, in which much higher levels of urethane may be formed.

The monograph on octyl gallate summarizes feeding and reproduction studies conducted since the last evaluation of this additive (*Cited in F.C.T.* 1965, **3**, 812). These have confirmed the no-effect level of 100 mg/kg, established in previous rat feeding studies and used as the basis of the ADIs of up to 0·2 mg/kg (unconditional) and up to 0·5 mg/kg (conditional) published earlier. However, because octyl gallate can evoke sensitization reactions in the buccal mucosa, its use in beer or in other beverages consumed in large amounts is considered unacceptable.

As always the Committee is to be praised for its painstaking collection and evaluation of toxicological data. The substances here reviewed must have presented a greater challenge than most, because the data were far from clear-cut and considerable ingenuity was required in the estimation of intakes which should be tolerable by man.

Fd Cosmet. Toxicol. Vol. 12. pp. 409–416. Pergamon Press 1974. Printed in Great Britain

BOOK REVIEWS

Residue Reviews. Residues of Pesticides and Other Contaminants in the Total Environment. Vol. 45. Edited by F. A. Gunther. Springer-Verlag, Berlin, 1973. pp. vii + 186. DM 43.80.

As all Journal addicts will know, the regular volumes of *Residue Reviews* present an invaluable and integrated picture of recent advances in the field of agricultural chemicals found as food contaminants. The latest offering in this useful series exemplifies the wide range of subject matter, distilled from a host of papers in recent years, on pesticide residues in the environment.

The opening article of Volume 45 centres on an area of pesticides research often neglected in the past, namely the adverse effect of pesticides on the soil fauna. It is pointed out that many pesticide-sensitive animal species living in soil, not least the homely earthworm, are essential either for the degradation of withered leaves and other vegetation into soil constituents or for controlling pest populations. While insecticides are directly harmful to numerous types of invertebrates, herbicides tend to play their part by attacking the vegetation which provides both food and home for many animal species. Paradoxically the more persistent insecticide residues in soil do not always have a greater influence on animal numbers than transient pesticide residues. Clearly, the lethality of a pesticide is a function of both its persistence and its toxicity. A chemical like D-D mixture, which persists for only a few weeks after use as a soil fumigant may, by virtue of its high toxicity, decrease the numbers of mites and other animals for 2 years or more, whereas chlorfenvinphos which may persist in the soil for up to a year or so, affects animal numbers for only about the same period.

This contribution also deals with the uptake of sublethal residues into the tissues of soil invertebrates. Earthworms and molluscs, for example, accumulate to varying extents the residues of some organochlorine insecticides, including DDT, dieldrin and heptachlor, but there is little evidence that organophosphorus compounds are accumulated. In a general conclusion, the authors show that the relationship between dose and effect for a given pesticide tends to be logarithmic, so that excessively large doses of relatively toxic pesticides would be needed to kill a large proportion of the total soil fauna.

The second contribution in this volume considers the roles of the various groups of experts appointed jointly by FAO and WHO to provide toxicological evaluations of both food additives and pesticide residues. Since all food constituents can be toxic in large quantities, it is essential that acceptable daily intakes (ADIs) are defined for each additive, and this has been done for some 400 food additives and nearly 100 pesticides over a period of 12 years. The elaboration of a meaningful ADI for an additive necessitates the estimation of daily intakes of specific foods, values which are based, in part, on average food-consumption data. This approach led to a tragic underestimation in connexion with the use of cobalt in beer, since the acceptable limit for this metal was based on an average beer consumption of 4 litres/day by heavy drinkers, and this amount proved to be significantly exceeded by some, in whom a combination of this and other factors led to the development of cardiomyopathy. Actual intakes for the vast majority of food additives and pesticide residues are, however, well below the potential figures calculated by the accepted method

and the estimates of consumer risk thus err on the side of safety. The annual FAO/WHO reports constitute invaluable guides to the safety-in-use of the growing number of deliberate and involuntary food additives.

The third contribution provides a summary of post-war research on the degradation of ten common pesticides of varying chemical structure under aquatic conditions. Each compound is discussed in relation to its chemical, photochemical and microbial degradation. The authors report a general paucity of data on the detailed kinetics of these reactions and stress the importance of further work in this field. Clearly, any prediction of the fate and environmental effects of pesticides depends primarily on identification of the products and the relative rate of each degradative reaction.

There follows a discussion of the relative merits of microbiological bioassay methods for detecting dressings on pesticide-treated seeds. Such techniques have been in common use since the war, and have the advantages of low cost, simplicity and a fair degree of accuracy and sensitivity. The standard method involves placing the seeds in a solid agar medium previously inoculated with an appropriate micro-organism. As the test organisms grow, the dressing diffuses from the seed into the medium and regions of growth inhibition develop around each seed.

It is fitting that the final contribution to this volume should be concerned with the essential theme of all *Residue Reviews*, namely the biological effects of pesticides on life forms other than the target organism. More specifically, this paper is concerned with the indirect effects of herbicides, various types of which have been found to increase the incidence or severity or diseases caused in crop plants by viruses, fungi and nematodes. Four possible mechanisms of such action are postulated, together with methods of controlling the increase in diseases.

Overall, this volume presents a lucid account of work in several areas that have been relatively neglected in the past. As usual there are some 30 pages of valuable references and a useful subject index.

Protein Deficiency and Pesticide Toxicity. By E. M. Boyd. Charles C. Thomas. Springfield. Ill. 1972. pp. x + 468. $29.50.

The deleterious effects of a protein-deficient diet present a major problem to many of the developing countries and to a substantial proportion of the world's population. Technology has provided an impressive array of materials, in the form of insecticides, herbicides and fungicides, which by virtue of their selective toxicity have contributed towards an improvement in food production in impoverished societies. Their benefit, however, has not been without cost in terms of human hazard.

The work described in this book was undertaken by Professor Boyd in response to requests from both WHO and the Food and Drug Directorate in Ottawa for information concerning the toxicity of insecticides, fungicides and herbicides in countries where the diet is normally low in protein. Experiments were conducted to estimate and characterize the acute and long-term oral toxicity of a variety of materials in rats fed on normal and protein-deficient diets.

The results obtained and their implications are discussed in this publication, which is divided into seven sections. Part I, entitled "Kwashiorkorgenic diets" provides a basis for subsequent chapters by presenting a wide-ranging discussion on the effects of protein-defi-

ciency in man and the rat and on the effect of different diets and low body weights on the calculation of toxic doses of chemical agents in animals. Pilot studies led to the selection of three standard low-protein diets for the planned toxicity tests on pesticides in rats. These were a diet containing no protein, one containing 3·0–3·5% casein and one containing 9% casein, the latter level being about one-third of the optimal dietary level of casein and allowing animals to grow at about one-third of the optimal rate. The "toxic" effects of these three diets are described in some detail.

The next five sections deal systematically with the oral toxicity of 16 different herbicides, fungicides and organochlorine, organophosphorus and carbamate insecticides when given in a single dose, and of DDT and captan also when given in 100 daily doses, to rats maintained on a protein-deficient diet. Each compound is considered separately and the clinical signs, histopathology and organ-weight changes associated with its administration to protein-deprived rats are compared with its effects in rats maintained on a diet adequate in protein. It was found that whereas protein deficiency enhanced the toxicity of some materials, such as DDT and captan, other materials did not follow this pattern. Dimethoate, for example, was no more toxic in rats fed a 3·5% casein diet than in controls fed a 26% casein diet. A comparison of the toxicities of the various pesticides in association with the different types of diet is made in the closing section.

Each chapter ends with a useful paragraph in which the conclusions to be drawn from the data presented are outlined. The book contains a number of photomicrographs, although these tend to be of poor quality, and concludes with a detailed author and subject index.

The work presented demonstrates that protein-deficient diets may augment the toxicity of a variety of pesticides in rats. This raises the possible question of an augmented hazard to man when these materials are used in countries where low-protein diets are the rule rather than the exception. How far the results obtained in rats may be extrapolated to the human situation poses a problem that remains to be resolved.

Packaging and the Law. Edited by F. A. Paine. Newnes–Butterworths, London, 1973. pp. 104. £2.20.

This publication represents one item in a series entitled *Fundamentals of Packaging*, which the publishers are producing in conjunction with the UK Institute of Packaging. The aim of the series is to provide, in "a convenient set of reference books information on such fundamental topics as marketing and distribution, quality assurance, legal requirements, environmental and mechanical protection, and packaging evaluation".

No branch of law or set of statutes in the UK may be conveniently classified as the "law on packaging". The particular regulations affecting packaging are diverse and are to be found embedded in, for example, principles of contract, the Sale of Goods Act, the Trade Descriptions Act, transport legislation, weights and measures laws, the Food and Drugs Act, statutes on poisons and medicines and laws on copyright, trade marks and design. The material contained in this book outlines the legal requirements for packaging imposed by all these aspects of the law.

Food-packaging standards and safety are, of course, under the overall control of the Food and Drugs Act 1955, which is designed to ensure that no food injurious to health is sold for human consumption. Since some manufacturers seem to be unaware of the lack

of legal requirements specifically relating to materials intended for food-contact use and the consequent relative importance to food packaging of regulations concerned primarily with direct food additives. the section on this aspect of the problem may provide some useful clarification.

It is suggested that the marketing expert, the packaging manager, the company secretary and the accountant will find information of considerable value in this series. Judging from the subject matter contained in the eight chapters so far available, it seems likely that this handy little series will succeed in its aims.

International Encyclopedia of Pharmacology and Therapeutics. Section 71, Vol. I. Pharmacology and Toxicology of Naturally Occurring Toxins. Edited by Helena Rašková. Pergamon Press, Oxford, 1971. pp. xxix + 299. £7.50.

This book, the partner of a second volume already reviewed in these pages (*Cited in F.C.T.* 1973, **11**, 889), divides its attentions between bacterial toxins (Part I) and snake venoms (Part II).

In the first part, the Editor of these volumes and her co-author, K. Mašek, draw on their extensive research experience in the field of bacterial exotoxins, especially those produced by Staphylococci, to provide a comprehensive review of the pharmacodynamics of staphylococcal, streptococcal and clostridial exotoxins. There are also chapters on *Corynebacterium diphtheriae*, *Shigella dysenteriae* and *Bordetella pertussis*. The general features of intoxication by these exotoxins in man and various laboratory animals are covered and details are given of the pharmacodynamics and mode of action of the toxins.

Only one chapter is devoted to endotoxins, a bias justified in the authors' view by the relatively extensive literature on this subject already published. Nevertheless, a more detailed treatment of this aspect by authors having as extensive a knowledge of endotoxins as the present authors have of exotoxins would have yielded a more balanced and useful review.

The second part of the book, on the pharmacology of snake venoms, is confined to the venoms produced by members of the Proteroglyphae and Solenoglyphae families, which encompass the sea snakes, cobras and vipers. The authors, S. B. and Olga B. Henriques, describe briefly the clinical aspects of envenomation and then give a detailed account of the chemical composition of the venoms. The remaining chapters provide an interesting and useful survey of the pharmacological and pathological effects of snake venoms.

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

Attention was drawn to the photosensitivity hazard of salicylanilides in 1960 and 1961 by the removal from the market of soaps containing 3,3',4',5-tetrachlorosalicylanilide. This book deals with the discovery of the photoallergic properties of these compounds and the way in which responses are mediated. It is in two main parts, the first being a literature survey and the second consisting of a report on experiments conducted by the first author in preparation for his M.D. thesis.

The first part of the book is particularly to be recommended to those new to the subject. It combines a simple introduction to photo-excitation with an excellent review of photo-

sensitivity. The second part describes separately ten experiments, four on biophysical aspects and six on immunological aspects of salicylanilide photosensitivity. The clarity of this part could have been improved by a short statement of the aims at the beginning of each experiment instead of a direct plunge into the experimental method. The book concludes with a short summary of the experimental findings and a brief discussion.

Although it gives rise to the feeling that some of the experimental detail might have been better dealt with in individual papers, this book, which includes a list of 365 references, fulfils a need for more information in the fairly new field of photosensitivity.

Progress in Experimental Tumor Research. Vol. 16. Pathology of the Syrian Hamster. Edited by F. Homburger. S. Karger, Basel, 1972. pp. xvi + 637. £20.70.

In view of the number of animal species already available for experimental work, one might think that the emergence of yet another is unnecessary. Few species, however, combine the advantages of small size, relatively short lifespan and a prolific reproduction rate. In fact, before the appearance of the Syrian hamster on the scene there were only two species which effectively combined these qualities, namely the rat and mouse.

For any experimental species, background data on the nutritional requirements, susceptibility to infectious diseases, degenerative conditions associated with the process of ageing and tumour incidence are indispensable if experimental findings are to be interpreted intelligently. This sort of information is available for both the rat and the mouse, but hitherto there has been little information on the hamster. The book named above fills important gaps in our knowledge of the biology of this small laboratory animal.

The contributors are all pioneers. Some developed techniques concerned with animal husbandry; others studied the lesions produced by chemicals, infective agents or naturally occurring disease; even dental pathology is given some degree of attention.

From the point of view of the oncologist, two parts are of outstanding importance. One is a chapter dealing with tumour viruses and their effect on the hamster, while another section outlines the information available in a tumour registry for neoplasms reported in the Syrian hamster. It is interesting to note from another chapter that the Syrian hamster is susceptible to tumour induction by the carcinogens that have already been shown to be active in other species. Equally interesting and important is the fact that viruses do not appear to induce tumours as readily in the hamster as in the mouse. Neoplasms are the principal response to the injection of viruses, but naturally occurring infections do not appear to be an important factor in the production of tumours. This inevitably provokes speculation as to whether the hamster would not make a good substitute for the mouse in this area of research.

This book is noteworthy for bringing together a wealth of information, which otherwise would not readily be accessible to most of the workers eager to explore the suitability of the hamster for use in the safety testing of food additives, cosmetics and other chemicals.

Clinical Immunobiology. Vol. 1. Edited by F. H. Bach and R. A. Good. Academic Press, New York, 1972. pp. xiv + 296. £6.

Some of man's most recalcitrant and bizarre diseases are now recognized as having an immunological basis. In their preface to the book named above, the Editors state that

the erstwhile "cloak of black magic" approach of clinicians to allergic diseases, for example, is being replaced by "scientific understanding". The cynic, recollecting articles written by some eminent immunologists, may be excused for wondering whether scientific understanding and black magic are all that different. His cynicism is likely to undergo some form of immunological suppression, however, if he is fortunate enough to read this book, which deals with a difficult subject clearly and concisely, using the deceptive simplicity of style and exposition that is achieved only by experts thoroughly conversant both with their own subject and with the art of communicating knowledge.

The Editors are to be congratulated on their selection of 17 authors whose 12 articles on different aspects of clinical immunobiology exhibit a highly desirable uniformity of approach and, in all cases, a comparable clarity of thought and ability to summarize and co-ordinate experimental research and clinical experience and observation. Compendia of articles of this type usually comprise a very mixed bag of excellent, mediocre and frankly bad contributions. This does not apply to this book, which proceeds smoothly and elegantly from an admirably lucid account of structure–function relations in the lymphoid system to equally good articles on immunoglobulins, cellular immunity, transplantation immunology, immunological tolerance, inflammation, immunogenetics, immunosuppression and tumour immunology, finishing with two excellent summaries of the current basic concept of underlying mechanisms in allergy and immunological-deficiency disease.

Obviously there must be gaps in a 295-page book covering a very wide field. Experimental immunologists might look for more discussion on the role of the macrophage or on interspecies differences in immunological responses—or, in general, for more detailed information relating to their own particular fields of interest. Similarly, the clinician might detect a definite slant towards immunological problems involved in transplantation, a comparatively esoteric field of medicine, and look for a rather fuller discussion of more mundane problems such as allergic alveolitis, food allergy or allergic reactions to various drugs. In fairness it must be said, however, that many of these aspects are dealt with adequately in other publications, and this volume has the merit of dealing succinctly with some important and difficult problems, such as immunological tolerance and the climate in which auto-immune disease can develop, questions which are not dealt with elsewhere in a form readily acceptable or available to most students of medicine. Finally, this is only the first volume of a proposed series. It is to be hoped that the Editors will maintain the very high standard that they have set and will enlarge their field, so that subsequent volumes will deal with other aspects of immunobiology of interest not only to medical and veterinary clinicians but also to experimentalists of various disciplines working in closely allied fields.

Macrophages and Cellular Immunity. Edited by A. I. Laskin and H. Lechevalier. CRC Monoscience Series. Butterworths, London, 1972. pp. vi + 123. £6.50.

Before the evolution of vertebrates and their development of humoral defence mechanisms, the macrophage was the original host component capable of isolating, surrounding, ingesting and otherwise eliminating or neutralizing foreign particles or cells. It remains an important factor in the defence of the vertebrate body. Since its importance was recognized by Metchnikoff, it has been studied less intensively than antibodies because the latter are more amenable to *in vitro* studies. Nevertheless, increasing experience in the

maintenance of macrophages in a functional state *in vitro* is greatly facilitating their study.

A review of "Host-parasite interactions with macrophages in culture" by M. Solotorovsky and L. S. F. Soderberg is one of the four articles in this book. It provides a useful background discussion of various cultural procedures and results which, while not without their limitations, can be compared with *in vivo* experimental findings. This would perhaps have made a more suitable opening article for the book than the discussion of the role of the macrophage in defence against *Cryptococcus neoformans*, which occupies that place. While of interest in its own right, this particular contribution, by H. H. Gadebusch, deals with a specific example which is somewhat restrictive in comparison with the wider aspects of the macrophage's function.

A. A. Gottlieb and S. R. Waldman summarize the possible functions of macrophages in immunity in general, and D. S. Nelson considers their role as effectors of cell-mediated immunity in particular. Macrophages, in their primitive role as scavengers of foreign material, ingest and degrade antigens and thus play an important role in the way in which these are presented to the lymphocytes. They can concentrate antigen on their surface, where it may be available for certain critical lymphocytes and may be associated with the induction of tolerance to some antigens and with the stimulation of other immune responses. Antigens similarly concentrated within the cell and attached to a ribonucleoprotein moiety may be slowly released and "condition" lymphocytes, a process which possibly forms an important step in the development of delayed responses to bacterial and tissue antigens. The control that macrophages appear to exert on the rate of DNA synthesis and lymphoid-cell proliferation may serve to control the rate and extent of the immune response and may perhaps exert a control also on neoplastic cells.

The importance of macrophages has probably been underestimated. Although their value in resisting bacterial, viral and protozoal infections is well recognized, their role in the destruction of autologous normal cells, tumour cells and allogenic cells is only just beginning to be appreciated. There is not, as yet, a great deal of precise information about the interaction between lymphocytes and macrophages in cell-mediated immunity, but it is becoming clear that while the principal performers in cell-mediated immune responses are lymphocytes, the performers in the reactions that dispose of invading organisms or offending cells are macrophages.

The three main review articles in this book provide an excellent summary of the present knowledge of macrophages and their functions and offer a good starting point for anyone wishing to acquire more detailed information or to do further research in this field.

BOOKS RECEIVED FOR REVIEW

Enzyme Nomenclature. Recommendations (1972) of the International Union of Pure and Applied Chemistry and the International Union of Biochemistry. Prepared by the Commission on Biochemical Nomenclature. Elsevier Scientific Publishing Co., Amsterdam, 1973. pp. 443. $5.80 (approx.).

Carbohydrate Chemistry—VI (Madison 1972). Plenary lectures presented at the VIth International Symposium on Carbohydrate Chemistry held at Madison, USA, 14–18 August 1972. Edited by W. M. Doane. Butterworths, London, 1973. pp. 78. £2.35.

Residue Reviews. Residues of Pesticides and Other Contaminants in the Total Environment. Vol. 47. Edited by F. A. Gunther. Springer–Verlag, Berlin, 1973. pp. vii + 198. DM 48.10.

Isolated Liver Perfusion and its Applications. Edited by I. Bartošek, A. Guaitani and L. L. Miller. Raven Press, New York, 1973. Distributed by North-Holland Publishing Co., Amsterdam. pp. xvii + 283. $21.20 (approx.).

Fd Cosmet. Toxicol. Vol. 12, pp. 417–423. Pergamon Press 1974. Printed in Great Britain

Information Section

ARTICLES OF GENERAL INTEREST

QUESTIONS OF SAFROLE METABOLISM AND CARCINOGENICITY

Safrole is a weak hepatocarcinogen in the rat, producing malignant tumours when fed at dietary levels of 0·5% and above (*Cited in F.C.T.* 1963, **1**, 107; *ibid* 1964, **2**, 86). In mice, hepatomas have been induced by its long-term oral administration (Innes *et al. J. natn. Cancer Inst.* 1969, **42**, 1101) and by its sc injection during infancy (Epstein *et al. Toxic. appl. Pharmac.* 1970, **16**, 321), although in the latter case only the males were susceptible. The end-products of safrole metabolism in the rat and guinea-pig are Mannich bases, probably formed after initial oxidation of safrole to allyl alcohol and another conjugated alcohol (*Cited in F.C.T.* 1973, **11**, 325). Two recently published papers identify this second alcohol as 1′-hydroxysafrole (1-OHS) and indicate that it is far more hepatocarcinogenic than is safrole itself. An ester of 1-OHS, 1′-acetoxysafrole (1-AcOS) is also shown to be a potent alkylating agent and carcinogen.

In a study by Borchert *et al.* (*Cancer Res.* 1973, **33**, 575) the urine of various species given safrole or 1-OHS orally or ip yielded 1-OHS in all cases after incubation with β-glucuronidase. Rats, hamsters and guinea-pigs excreted similar proportions (1–3·5%) of an ip dose of 300 mg safrole/kg as 1-OHS, but in mice the yield of 1-OHS was much higher (33%). When safrole was given at 0·5% in the diet, the proportion excreted as 1-OHS was halved in mice but increased in rats to 5–10% during the first 18 days, with a decline to 3–4% thereafter. About 40% of 1-OHS was excreted unchanged when the compound was given ip (100 or 200 mg/kg) or orally (0·55% in the diet) to rats or ip to mice, but mice excreted only 6% of an oral dose of 1-OHS unchanged. Pretreatment with phenobarbitone in the drinking-water or 3-methylcholanthrene ip caused a ten-fold increase in the amount of 1-OHS excreted by rats from safrole given ip. This effect was not seen in rats given safrole orally or in other species, neither did such pretreatment affect 1-OHS excretion after its administration to rats. Species differences were apparently not due to differences in the relative amounts of biliary and urinary excretion, since little, if any, 1-OHS was found in the bile and the proportion excreted in the urine could not be increased by biliary ligation.

Analogy with other carcinogens suggested that the ester, 1-AcOS, rather than 1-OHS might be the ultimate carcinogenic metabolite of safrole. 1-AcOS was confirmed as an electrophilic reagent by its reactions with methionine (to form the thioether, 3′-methylmercaptoisosafrole, and smaller amounts of 1′-methoxysafrole) and with guanosine monophosphate (to form three products, the major one of which after degradation to the nucleoside and acetylation was identified as *O*-6-(isosafrol-3′-yl)-*N*-2-acetylguanosine-2′,3′,5′-triacetate). Further evidence was provided by a demonstration of its reaction with the nucleosides guanosine and adenosine, and probably to a small extent with cytidine, but apparently not with uridine or thymidine. Under the same conditions, 1-OHS, 3′-hydroxyisosafrole and 3′-acetoxyisosafrole showed no significant reactions with methionine or with

guanosine or its monophosphate, suggesting that unlike 1-AcOS, they were not likely to be ultimate carcinogenic metabolites of safrole.

Another paper by the same group (Borchert *et al. ibid* 1973, **33**, 590) reports a very high incidence of hepatocellular carcinomas, and a 33–50% incidence of forestomach papillomas, in male rats fed a dietary level of 0·55% 1-OHS for 8·5–10 months and killed at 12 or 18 months. In contrast, only two of 48 rats fed 0·5% safrole for the same period had liver carcinomas on termination, and none had stomach papillomas. The livers of those fed 1-OHS, unlike those fed safrole, were necrotic and fibrotic. Weight gain in both groups was retarded, but the survival rate up to the time when tumours developed was high. Rats fed 0·41% 1-AcOS (equivalent to 0·3% safrole) showed a similar retardation of weight gain but poor survival. Only one hepatoma was identified in this group at 12 months, but all those surviving more than 6 months had multiple stomach papillomas, accompanied in two cases by squamous-cell carcinomas.

A different pattern of response was observed in male mice fed 0·4 or 0·5% dietary levels of safrole, or 0·44 or 0·55% dietary levels of 1-OHS for 13 months and killed 3 months later. The incidence of liver tumours in these mice was 27% in those given safrole and only 17% in those given 1-OHS, but survival in the latter group was low. On the other hand, interscapular sarcomas (mostly angiosarcomas) developed in 20 of the 65 mice fed 1-OHS but only in two of the 80 fed safrole and in one of 50 controls. 1-AcOS proved highly toxic to mice and there was an increased mortality even at dietary levels of 0·05 or 0·03%, but no tumours were evident in the survivors. Liver tumours were far more common in male mice injected sc with a total dose of 9·5 μmoles safrole, 1-OHS or 1-AcOS, given in four injections between 1 and 21 days of age and sacrificed at 12–14 months, the liver tumour incidence being 40% from treatment with safrole, 84% from 1-OHS and 82% from 1-AcOS (as compared with 8% in controls). Female mice injected according to the same schedule were far less sensitive, showing a low incidence of hepatic tumours only in response to 1-OHS (16%) and 1-AcOS (9%).

When adult male rats were given, over a 10-week-period, 20 sc injections each of 18·6 μmoles safrole, 1-OHS or 1-AcOS, or several other safrole derivatives, local tumours developed 12–18 months after the first injection in response to 1-AcOS (30% incidence), 1-OHS (8%) and 3-bromoisosafrole (11%), but not in response to safrole, 3′-hydroxyisosafrole, 3′-acetoxyisosafrole, isosafrole, 3′-methoxyisosafrole or 1′-methoxysafrole. Liver tumours were also seen in one rat in each of the groups given 1-OHS, 1-AcOS or 3′-acetoxyisosafrole. However, the incidence of skin papillomas in female mice given twice-weekly topical applications of 1·8 μmoles safrole, 1-OHS, 1-AcOS or 1′-methoxysafrole, or the corresponding 2′,3′-dihydrosafrole derivatives, for 7 weeks, followed by twice-weekly applications of phorbol-12,13-didecanoate, was no greater than that in mice given the tumour promoter alone.

These findings were taken to indicate that 1-OHS is a proximate carcinogenic metabolite of safrole and that 1-AcOS may be the ultimate carcinogenic metabolite. An attempt to ascertain whether the latter compound is formed *in vivo* from safrole is now underway, as are several other related investigations into the mechanism of safrole carcinogenicity.

[C. C. Rostron, BIBRA]

CONTINUING UNCERTAINTIES OVER 2,4,5-T

The major arguments about the herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) have centred on the question of its possible teratogenicity. A few years ago (*Cited in F.C.T.*

1971. **9,** 152) it was argued that the teratogenic effects apparently induced by 2,4,5-T might in fact be attributable to its contamination with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin ('dioxin'). It soon became clear, however, that this was an oversimplification and that dioxin could not be held entirely responsible for the malformations observed experimentally. It was demonstrated (*ibid* 1972, **10,** 722) that the sc treatment of pregnant mice either with dioxin or with 2,4,5-T containing less than 0·05 ppm dioxin caused both cleft palate and kidney abnormalities, although in rats sc doses of dioxin produced kidney abnormalities but no cleft palate and 2,4,5-T given orally in doses up to 80 mg/kg/day caused no malformations. Subsequently evidence was presented that doses of 100 or 150 mg 2,4,5-T/kg/day given orally to rats, again on days 6–15 of pregnancy, reduced foetal weight significantly and increased the incidence of foetal deaths and skeletal abnormalities, although the dioxin content of the sample was less than 0·5 ppm (*ibid* 1973, **11,** 512).

The latter study also demonstrated foetal damage, similar to that produced by DDT, following the treatment of pregnant rats with 2,4-dichlorophenoxyacetic acid (2,4-D) in oral doses of 100 or 150 mg/kg/day. Another comparison of the effects of 2,4-D and 2,4,5-T was made in pregnant hamsters by Collins & Williams (*Bull. env. Contam. & Toxicol.* (*U.S.*) 1971, **6,** 559). Daily oral doses of the compounds given on days 6–10 of pregnancy clearly demonstrated the greater toxicity and teratogenicity of 2,4,5-T. Commercial-grade 2,4-D in doses of 20–100 mg/kg/day was associated with occasional abnormalities (usually fused ribs), a decrease in foetal viability and an increased incidence of haemorrhages of the gastro-intestinal tract in liveborn offspring, but the effects were not clearly dose-related. In contrast, 2,4,5-T free from detectable dioxin markedly increased foetal mortality and, generally, the incidence of gastro-intestinal haemorrhage and malformations, when given in doses of 100 mg/kg/day. There were, however, wide variations between different commercial samples of 2,4,5-T, even when these were free from dioxin contamination. The incidence of foetal abnormalities was clearly related to dose level in animals given 2,4,5-T contaminated with dioxin, and in these groups the main malformations were absence of eyelids and delayed cranial ossification. Contamination with dioxin increased the levels of embryonic mortality and haemorrhage incidence associated with 2,4,5-T doses of 40 mg/kg or more, and foetal viability was decreased by these samples in a dose-related manner.

The effects of commercial samples of 2,4-D and 2,4,5-T have also been studied in mice (Båge *et al. Acta pharmac. tox.* 1973, **32,** 408). On days 6–14 of pregnancy, NMRI mice were given sc injections either of the butoxyethyl ester of 2,4,5-T or of a mixture of 2,4-D and 2,4,5-T, both formulations containing less than 1 ppm dioxin and related compounds. The test materials were dissolved in dimethylsulphoxide and given in doses of 50 or 110 mg/kg/day. Both preparations were teratogenic and embryotoxic at the higher dosage level, foetal mortality and the incidence of cleft palate, rib and vertebral malformation and sc haemorrhages being increased and foetal growth being considerably reduced. There was only a slight increase in the frequency of renal malformations, however, and cystic kidneys were not found. Gastro-intestinal haemorrhage was also absent. At the lower dosage level, the 2,4,5-T ester had a more marked effect than the 2,4,5-T/2,4-D mixture.

Neubert & Dillmann (*Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* 1972, **272,** 243) reported on the induction of cleft palate in NMRI mice by 2,4,5-T and/or dioxin dissolved in rapeseed oil and given by gavage. They tested three samples of 2,4,5-T (containing less than 0·02 ppm, 0·05 ppm and an unknown quantity of dioxin, respectively), together with the butyl ester of 2,4,5-T, 2,4,5-trichlorophenol and pure dioxin. A significant incidence of

cleft palate occurred with doses of 2,4,5-T above 20 mg/kg/day, given on days 6–15 of pregnancy, while 10 mg/kg/day reduced foetal weight without affecting mortality. Similar effects were seen with the butyl ester. Single doses of 150–300 mg/kg increased the incidence of cleft palate, particularly when given on day 12 or 13 of pregnancy. Oral doses of 1 μg dioxin/kg/day given on days 6–15 also induced cleft palate, while 4–5 μg/kg/day increased foetal mortality. Dioxin given in a single dose of 20–50 μg/kg between day 7 and 13 induced cleft palate without increasing mortality, the peak response occurring with dosage on day 11.

Neuberg & Dillmann (*loc. cit.*) found some evidence of potentiation of teratogenic effect when 2,4,5-T and dioxin were administered together, but a clear demonstration of this with 2,4,5-T doses of 30–60 mg/kg required a dioxin concentration of at least 1·5 ppm. The presence of 2,4,5-trichlorophenol had no discernible effect on the teratogenicity of 2,4,5-T.

The disposition and elimination of [1-^{14}C]2,4-D and [1-^{14}C]2,4,5-T in the pregnant mouse following a single iv injection have been shown by Lindquist & Ullberg (*Experientia* 1971, **27,** 1439) to differ substantially and to throw some light on the difference in the teratogenicity of these two compounds. The labelled dose of 2,4,5-T was injected either 8 days after mating, with whole-body autoradiography following after 4 hours, or 9 days after mating, with autoradiography after 24 hours, or in late pregnancy, with autoradiography at 5 and 20 minutes, 1, 4, 16, 24 and 48 hours and 4 days afterwards. Labelled 2,4-D was injected late in pregnancy and the animals were killed for autoradiography after 5 and 20 minutes and 1, 4 and 24 hours. In view of the known stability of the compounds in the body, ^{14}C activity was taken as an indication of the concentration of the unchanged compound in a tissue.

No significant concentration of 2,4,5-T appeared in the embryo when a dose was given early in pregnancy; in the dams the kidneys and visceral yolk sac were the only organs that held higher concentrations than the blood. When the injection was given later in pregnancy, the yolk-sac concentration of 2,4,5-T exceeded the concentration in the blood and serous fluids as early as 5 minutes after treatment. Further accumulation and retention in the yolk sac was evident between 20 minutes and 48 hours after the injection, while the concentration elsewhere slowly decreased. The other exception to the generally uniform distribution of 2,4,5-T was the brain, in which the concentration remained low throughout. 2,4,5-T given late in pregnancy passed the placenta slowly until concentrations in foetal and maternal tissues were comparable. There was no specific site of accumulation in the foetus, the highest level of activity being found in the blood. 2,4-D disappeared much more rapidly from the treated animals; it passed to the foetus but showed only a slight tendency to accumulate in the visceral yolk sac and was eliminated from all tissues, including the yolk sac, within 24 hours of injection.

Fang *et al.* (*Toxic. appl. Pharmac.* 1973, **24,** 555) have shown that there is no significant difference between pregnant and non-pregnant rats in the rate of their elimination of [1-^{14}C]2,4,5-T. Following gavage administration of a dose in the range of 0·17–41 mg/kg, some 75% of the radioactivity was excreted in the urine and 8% in the faeces during the first 24 hours. No radioactivity was found in the expired air, but it was present in all tissues and particularly in the kidney. Tissue levels reached a peak 6–12 hours after dosing and then declined rapidly. Activity was also detected in the foetuses and in the milk. It was estimated that the mean biological half-life of 2,4,5-T in the tissues was much greater in newborn rats than in adults (97 hours compared with 3·4 hours).

While transplacental passage of a compound does not necessarily imply an ability to exert some teratogenic effect, it does provide an appropriate situation for the exercise of any such potential. Millard *et al.* (*Biochem. biophys. Acta* 1973, **308,** 230) have tackled from the biochemical angle the old question of the actual capacity of 2,4,5-T and its contaminant, dioxin, to induce teratogenic effects. This group has studied *in vitro* the effect of these two compounds on three enzymes concerned with DNA synthesis and cell division, namely the ribonucleotide reductase, DNA polymerase and thymidine kinase of developing rat brain. 2,4,5-T was found to be a potent inhibitor of ribonucleotide reductase in homogenates of brains of 16-day rat embryos, but had no effect on the other two enzymes even at the highest concentration tested (10^{-4} M). The reductase inhibition was partially reversed by addition of Fe^{2+}. Ribonucleotide reductase was shown, by the same assay method, to be similarly inhibited by treatment of the dam on day 14 of pregnancy with an sc dose of 50 mg 2,4,5-T/kg. This dose also increased the number of non-viable embryos in the litters taken at 16 days. Dioxin also inhibited ribonucleotide reductase *in vitro* but to a much less marked degree, only a 25% inhibition of activity being effected by a 10^{-4} M concentration compared with 87 and 42% inhibitions by 2,4,5-T in concentrations of 10^{-4} and 10^{-6} M, respectively. The inhibitory potency of 2,4,5-T was more closely comparable with that of hexachlorophene, which was included in the *in vitro* study, but 10^{-4} M-hexachlorophene also inhibited DNA polymerase activity by 85%.

The ability of 2,4,5-T to inhibit under these conditions the activity of ribonucleotide reductase, which has been suggested as a rate-limiting step in DNA synthesis, provides a limited biochemical background which may be relevant to some of the observed effects of this herbicide. It is clear that the whole question of 2,4,5-T is a complex one, and the deliberations of the long-awaited US public hearing, now scheduled for April 1974, will be followed with interest.

[P. Cooper, BIBRA]

MORE STUDIES ON NTA

We recently reported on the chequered history of nitrilotriacetic acid (NTA) and its proposed substitution for polyphosphates in detergents (*Cited in F.C.T.* 1973, **11,** 674). At that time, it had been shown to be absorbed and cleared rapidly by animals (with some deposition in bone), to have no promoting effect on the toxic or teratogenic effects of mercury or cadmium and to have no innate teratogenic effect itself.

Studies on the metabolic fate of NTA in various animal species have now been followed, appropriately, by a metabolic study in man (Budny & Arnold, *Toxic. appl. Pharmac.* 1973, **25,** 48). Eight male volunteers were each given an oral dose of 10 mg ^{14}C-labelled NTA in a gelatin capsule after a 10-hour fast. Ingestion of some fluid but no food was allowed for the following 2 hours. Blood samples were taken at frequent intervals and all urine and faeces were collected up to 120 hours after administration of the dose. In addition, expired carbon dioxide was collected from half of the men, continuously during the first 6 hours after dosing and then for 15-minute periods at 8, 12, 24, 30, 48 and 72 hours.

The faeces accounted for 77% of the administered dose, out of a total mean recovery of 89%. This mean recovery included one very low figure, thought to be due to a lost faeces sample, so the actual faecal and total recoveries were greater than this. Some 12% of administered radioactivity was excreted in the urine, with less than 0·1% appearing in the

expired air. Most of the absorbed [^{14}C]NTA—about 87%—appeared in the urine within the first 24 hours. A further 8 and 2·5% appeared in the urine on days 2 and 3, respectively, after which only traces were found. Peak blood levels occurred at about 2 hours after dosing with mean values of 6·5 ng/g in the serum and 2 ng/g in the cells. Some 10 hours later, serum levels were approaching zero. Analyses using thin-layer chromatography and reverse isotope dilution techniques gave no indication of any biotransformation and identified over 96% of the urinary radioactivity as NTA.

The blood level and urinary excretion patterns and the absence of metabolic degradation of absorbed NTA in man thus closely parallel the findings reported in the dog and rat (*Cited in F.C.T.* 1971, **9**, 888; *ibid* 1973, **11**, 674). The degree of absorption from the human gastro-intestinal tract is, however, much smaller than that found in the experimental animals. Absorption in these volunteers was, in fact, only about 25% of that in the other species. The possibility of enterohepatic circulation in man is considered unlikely, since the earlier studies showed that this occurs in neither the rat nor dog. The inter-species similarities in the metabolic fate of NTA, coupled with the relatively low level of absorption in man, encourage confidence in the extrapolation of results of toxicity studies in the rat and dog to the assessment of human hazard.

Because of the readiness with which NTA will form strong complexes with divalent metal ions, Michael & Wakim (*Toxic. appl. Pharmac.* 1973, **24**, 519) have examined its effect on the absorption, excretion and physiological function of zinc (Zn), calcium (Ca), iron, magnesium, copper, phosphorus, sodium and potassium.

Rats were fed a diet containing 2% NTA for 30 days, following which period the animals were kept in metabolism cages for 4 days, faeces and urine being collected for metal-ion analyses after acclimatization for 1 day. Similar analyses were carried out on blood collected after decapitation. Increases in urinary pH and urinary Zn excretion were accompanied by a decrease in faecal excretion of Zn. There was also some increase in the urinary excretion of Ca and sodium, together with a decrease in faecal Ca and phosphorus. Higher Zn levels were found in the serum of female rats, but there were no significant changes in the serum levels of other metals.

A 91-day study was carried out on groups of six rats fed a diet containing 10, 18 or 25 ppm Zn and levels of 0, 0·03, 0·15 or 0·50% (w/w) NTA. Animals on the 10 ppm Zn diets and no NTA showed reductions in weight gains and feed efficiency, and similar reductions occurred with the higher Zn diets supplemented with 0·5% NTA. Addition of 0·15% NTA to the 10 ppm Zn diet led to some improvement in the growth rate. NTA increased Zn absorption, and also caused a dose-related increase in Ca absorption. The Zn content of bone was related to both the Zn and NTA levels of the diet, but these had no significant effect on the weight or ash content of the tibia.

Enzyme studies of the effect of NTA on the activity of carbonic anhydrase (a Zn-containing enzyme) in the liver and kidneys showed no inhibition of the enzyme, and no consistent effect was found on serum alkaline-phosphatase activity in weanling rats fed NTA for 2–4 weeks.

Previous studies (Williams & Mills, *Br. J. Nutr.* 1970, **24**, 989) had shown that Zn absorption was directly related to the Zn content of the diet, and dietary levels of less than 12 ppm Zn caused reduced growth. As 0·15% NTA improved the growth of animals receiving only 10 ppm Zn, it appeared that NTA enhanced Zn absorption. This was supported by haematology studies carried out at the end of the 91-day study. Moreover, by causing a redistribution of Zn in the body, as indicated by the finding of reduced Zn levels in the

testes and kidneys of rats given NTA-supplemented diets containing 10 or 18 ppm Zn, NTA may make Zn more available for biological processes in the body. The rise in urinary pH associated with NTA feeding did not seem to be associated with carbonic-anhydrase inhibition, and was possibly a direct effect of the increased alkaline load in the body resulting from the feeding of NTA as the trisodium salt. Although enhanced Ca absorption during NTA feeding was indicated, there were no physiological findings to support this idea. The mineral content of the tibia was not affected, nor was there any significant effect on the activity of serum alkaline phosphatase, an enzyme associated with bone metabolism.

The probable intake of NTA from the water supplies by a 70-kg man is estimated to be 1·4 μg Na_3NTA/kg/day on the basis of work by G. Calvin and G. K. Ashforth (*Water Res.*, in press). The 0·03% dietary level of NTA used in this study was equivalent to an intake of 10–30 mg NTA/kg/day by the rats. From this, the authors submit that 1·4 μg Na_3NTA/kg/day would have no effect on mineral metabolism.

Another problem giving rise to concern over the use of NTA has been its possible conversion to a nitrosamine (*Cited in F.C.T.* 1971, **9,** 889). Lijinsky *et al.* (*J. natn. Cancer Inst.* 1973, **50,** 1061) have investigated this possibility. Groups of 15 male and 15 female MRC rats were given 20 ml of a solution containing 0·5% NTA or 0·5% iminodiacetic acid (IDA) with and without 0·2% Na nitrite or 0·5% Na nitrate in the drinking-water for 5 days/week for 84 weeks. A positive control group was given 0·2% nitrosoiminodiacetic acid (NIDA), the expected product of nitrosation *in vivo*, 5 days/week for 72 weeks. The authors found no signs of toxic effect during the treatment period, most animals surviving until the end of the experiment. Unfortunately, an unexpectedly high and unexplained incidence of tumours was found in all groups, including the controls. Their distribution and type suggested that neither NTA and nitrite nor NIDA was carcinogenic. It is not impossible, however, that a study on a larger scale might reveal a tumorigenic effect of NTA, too slight to have been detected in the present small study.

[F. A. Charlesworth, BIBRA]

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TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS

COLOURING MATTERS

2697. Orange RN gets a black mark

Olsen, P. & Hansen, E. (1973). Bile duct proliferation in pigs fed the food colour Orange RN. *Acta pharmac. tox.* **32,** 314.

A BIBRA short-term feeding study has established that the minimum no-untoward-effect level of Orange RN (C.I. (1956) no. 15970) in rats was 60 ppm of the diet (equivalent to an intake of about 3 mg/kg/day), while effects of doubtful toxicological significance (borderline Heinz-body production, methaemoglobinaemia, reticulocytosis, splenic enlargement and increased splenic iron) first appeared with a dietary level of 600 ppm (Gaunt *et al. Fd Cosmet. Toxicol.* 1971, **9,** 619). More severe but similar effects were produced by a diet containing 6000 ppm Orange RN.

A feeding study in pigs has now been reported. Dietary levels of Orange RN providing daily doses of 10, 40 or 160 mg/kg for 3 months led to transient pallor and changes in the colour and consistency of faeces. Pigs fed 40 mg/kg/day showed slight liver fibrosis, and 160 mg/kg/day produced severe interstitial fibrosis of the liver with multiple foci of nodular parenchymal hyperplasia. Both at 40 and 160 mg/kg/day there were consistent findings of increased interstitial connective tissue in the liver. All groups fed Orange RN showed a proliferation of the epithelial cells of the bile ductules. This was dose-related in intensity. While such proliferation may accompany liver cirrhosis, it may be presumed to represent a selective toxic effect of the substance on the bile-duct epithelium. Since no such pathological change was recorded in the feeding study in rats mentioned above or in a longer one carried out in rats and mice (*Cited in F.C.T.* 1969, **7,** 680) it would appear that the liver of the pig may be more sensitive to the effects of Orange RN than are the livers of small rodents.

FLAVOURINGS, SOLVENTS AND SWEETENERS

2698. Methanol levels in chronic alcoholism

Pieper, W. A. & Skeen, Marianne J. (1973). Changes in blood methanol concentrations in chimpanzees during periods of chronic ethanol ingestion. *Biochem. Pharmac.* **22,** 163.

Accumulation of endogenously produced methanol has been reported in man following a 10–15 day bout of ethanol ingestion sufficient to maintain blood-ethanol concentrations above about 20 mg/100 ml (Majchrowicz & Mendelson, *J. Pharmac. exp. Ther.* 1971, **179,** 293).

In young chimpanzees (*Pan troglodytes*) given increasing daily doses of ethanol for 6–14 wk, blood-methanol concentrations began to rise when the ethanol concentration continually exceeded 10–20 mg/100 ml. This occurred when the daily ethanol intake exceeded 5–7 g/kg, the amount which could be metabolized and eliminated within 24 hr. Under these conditions methanol concentrations in the blood increased for 4–5 days to a plateau

value, remaining at elevated though fluctuating levels until blood ethanol levels fell below 60–15 mg/100 ml. When the ethanol level continued to fall, the methanol concentration fell at a rate which was determined by the ethanol elimination rate.

This finding suggests that the accumulation of methanol in the blood results from competitive inhibition of methanol oxidation by the ethanol. Whether ethanol achieves this end by inhibiting the oxidation of methanol by alcohol dehydrogenase, or by interference with some alternative enzyme system such as a catalase peroxidative system or hepatic microsomal oxidizing system, remains unknown.

[Such a consideration obviously has an important bearing on the efficacy of treating methanol poisoning in man by iv infusion of ethanol.]

2699. Nephrotoxicity of *S*-dichlorovinyl-L-cysteine

Stonard, M. D. (1973). Further studies on the site and mechanism of action of *S*-(1,2-dichlorovinyl)-L-cysteine and *S*-(1,2 dichlorovinyl)-3-mercaptopropionic acid in rat liver. *Biochem. Pharmac.* **22**, 1329.

S-(1,2-Dichlorovinyl)-L-cysteine (DCVC) is a nephrotoxic agent that may be formed during food processing by reaction of trichloroethylene, used for solvent extractions, with a food component (*Cited in F.C.T.* 1966, **4**, 226). Evidence from *in vitro* experiments suggests that the compound interferes with kidney functioning by impairing mitochondrial respiration in this tissue (Parker, *Fd Cosmet. Toxicol.* 1965, **3**, 75), and more recently Stonard & Parker (*Biochem. Pharmac.* 1971, **20**, 2417) identified the site of action as the intramitochondrial 2-oxoacid dehydrogenase systems.

Further investigation of the inhibitory mechanism has been carried out by Stonard (cited above), who also compared the effect of DCVC with that of *S*-(1,2-dichlorovinyl)-3-mercaptopropionic acid (DCVMP), another inhibitor of mitochondrial respiration. The fact that 2-oxoacid dehydrogenases are multi-enzyme complexes possessing identical cofactor requirements was used to study the possible components of these enzyme complexes, with which DCVC and DCVMP might combine. The enzymes involved were lipoyl dehydrogenase (LD), the only enzyme common to both pyruvate and 2-oxoglutarate dehydrogenase systems, and glutathione reductase (GR), an extramitochondrial enzyme which catalyses similar reactions and has a similar active-site configuration.

DCVC pre-incubated with the supernatant fraction from disrupted mitochondria inhibited the activity of LD derived from isolated rat-liver mitochondria. No such inhibition occurred with DCVC added directly to the assay system and DCVMP caused only slight inhibition of LD either with or without pre-incubation. This indicated that, for DCVC, conversion to an active metabolite was a prerequisite of LD inhibition. Similar results were obtained in connexion with the inhibition of GR in the rat-liver cytosol. The cytosol of rat liver has a greater capacity than the liver mitochondria for breaking down DCVC to pyruvate and ammonia, but the inhibition of LD was found to be more powerful than that of GR, a fact possibly related to the affinity of the thiovinyl moiety of DCVC for the glutathione present in the cytosol. Both of these flavoprotein enzymes accept one electron per flavin moiety from the respective hydrogen donors, NADH or NADPH, the other electron going to a redox-active disulphide group. The authors therefore suggest that a DCVC metabolite (possibly 1,2-dichlorovinyl mercaptan) interacts, with loss of chlorine atoms, with the moieties common to both enzymes, flavin-adenine dinucleotide and the redox-active disulphide.

AGRICULTURAL CHEMICALS

2700. Filling in the paraquat picture

Murray, R. E. & Gibson, J. E. (1972). A comparative study of paraquat intoxication in rats, guinea pigs and monkeys. *Expl mol. Path.* **17**, 317.

Sinow, J. & Wei, E. (1973). Ocular toxicity of paraquat. *Bull. env. Contam. & Toxicol.* (*U.S.*) **9**, 163.

The LD_{50}s of paraquat given intragastrically to rats, guinea-pigs and monkeys were 126, 22 and 50 mg/kg, respectively. The animals showed anorexia, adipsia, diarrhoea, hyperpnoea, dyspnoea and tachycardia, and all became hypoxic. Doses exceeding 63 mg paraquat/kg killed monkeys within 1–2 days, after a convulsive phase. Monkeys given 50–53 mg/kg became dyspnoeic prior to death, while those given 30–40 mg/kg were dyspnoeic for 1–5 days and subsequently developed pulmonary fibrosis. In all species the primary toxic lesion appeared in the lungs, with areas of focal necrosis affecting the gastro-intestinal tract, liver and kidneys. Animals dying within 7 days of dosing showed acute pulmonary effects, including haemorrhage, oedema and congestion. Survivors among the rats and monkeys, but not the guinea-pigs, showed interstitial lung fibrosis, which developed after 7–10 days. The pulmonary fibrosis characteristic of paraquat poisoning in man (*Cited in F.C.T.* 1972, **10**, 703) is thus paralleled in rats and monkeys, but not in guinea-pigs.

Splashes of paraquat in the eye have been reported to cause initial pain and redness and later corneal opacity (*ibid* 1970, **8**, 597). The second paper cited above gives details of the effect of various concentrations on the rabbit eye. Different dilutions of a concentrate containing 242 mg paraquat/ml were introduced in 0·2 ml volumes into the eyes of rabbits, and the reaction was observed after 12 hr and again at daily intervals for 20 days. Severe conjunctival reactions followed applications of the 6·25 and 12·5% dilutions (15 and 30 mg/ml), the stronger solution occasionally causing slight corneal damage also. Conjunctival vessels became reddened, the lids were swollen and partially closed, and there was a mucopurulent discharge followed by diffuse corneal opacification. The 25 and 50% dilutions of the concentrate caused congestion and swelling of the iris, intensified corneal opacification and a pannus reaction. The 50% dilution produced severe corneal opacification, iritis and conjunctivitis. All rabbits receiving 0·2 ml of the undiluted concentrate in a single eye and one given the 50% dilution in both eyes died within 6 days, but apparently not from fibrotic lung changes.

PROCESSING AND PACKAGING CONTAMINANTS

2701. Milk irradiation: No mutagenesis in rodents

Renner, H. W., Grünewald, Th. u. Ehrenberg-Kieckebusch, W. (1973). Mutagenitätsprüfung bestrahlter Lebensmittel mit dem "dominant lethal test". *Hum. Genet.* **18**, 155.

The International Food Irradiation Project has commented on difficulties encountered in testing for mutagenicity. A report has now appeared on a reproduction test in rats and mice. This study included the statistical evaluation of five variables studied in the dominant lethal test for mutagenicity.

Five generations of rats totalling 716 animals and one generation of 750 mice were fed for at least 8 wk on a diet containing 35% whole-milk powder, which had been irradiated

with 4·5 Mrad. There were no differences in fertility, duration of pregnancy, litter size, weaning index, sex ratio of litters or incidence of foetal malformations between the test groups of rats and controls given a non-irradiated diet. A careful statistical analysis of data on the numbers of corpora lutea, pre- and post-implantation losses, total implantations and foetal survival failed to reveal any discrepancies that might be attributed to embryotoxic or mutagenic effects of the treated diet. In mice, the 35%-irradiated diet produced no detectable effect on these five parameters, and other mice given a totally irradiated diet similarly showed no evidence of adverse effects.

Although the irradiated diets had a relatively high content of free radicals, they were not shown in this study to have any adverse effects on the reproductive capacity of either rats or mice.

THE CHEMICAL ENVIRONMENT

2702. Increased cancer incidence in nickel workers

Pedersen, E., Hogetveit, A. C. & Andersen, A. (1973). Cancer of respiratory organs among workers at a nickel refinery in Norway. *Int. J. Cancer* **12**, 32.

In general agreement with observations made in a nickel refinery in Wales (*Cited in F.C.T.* 1972, **10**, 113), this investigation has revealed a greatly increased incidence of cancer of the respiratory organs in men employed at a nickel refinery in Norway. Production in the refinery (established in 1910) was largely discontinued between 1940 and 1945 because of the second World War. Since 1945, and particularly since 1950, major changes in the production process have greatly reduced the exposure of workers to dust and fumes.

From 1953 to 1971, 67 cases of respiratory cancer (48 lung, 14 nasal and five laryngeal) were identified among the 1916 men covered by the study. Half of these occurred among men who had been employed before 1940 and the majority (53) in men who had worked in either the roasting and smelting or electrolysis sections. About 20% of all lung-cancer cases occurring between 1953 and 1971 among males in the district occurred in men who were, or had been, employed at the refinery. It was not found practicable to attempt to correlate this incidence with smoking habits or possible exposure in other occupations.

Six men aged between 49 and 62 yr, who began working in the refinery in the period 1954–1956, developed lung cancer with 4–11 yr. Among men employed before 1954, the interval between employment and the development of lung cancer was generally 15 yr or more, the exceptions being one case after 10 yr and two after 12 yr. There was no obvious explanation for the unusually short latent period in the six later cases. There was no indication of particularly heavy exposure or of significant exposure in previous occupations and the men were either light or non-smokers. So far no lung-cancer cases have been reported among men first employed after 1956.

Of the 14 nasal cancers identified, ten occurred among men employed in the plant for more than 15 yr, and only one occurred in those first employed after 1945. Owing to the long latent induction period for this type of cancer, however, it cannot yet be asserted that a reduction in risk has definitely been achieved. The 14 cases found among the refinery employees accounted for 50% of all sinus cancers found during the follow-up period among the 21,000 males over the age of 40 in the district (Vest-Agder county). Studies in

Norway and elsewhere have provided no evidence that cancer of the nasal sinuses is associated with smoking.

Previous reports have given no indication of an increased risk of laryngeal cancer among nickel workers. The five cases identified in this study, four of which were in men employed after 1945 in the roasting and smelting processes, permit no firm conclusions to be drawn but suggest, in the authors' opinion, that this may be another manifestation of risk due to occupational exposure to nickel.

2703. Excretion of nickel

Ho, W. & Furst, A. (1973). Nickel excretion by rats following a single treatment. *Proc. West. Pharmac. Soc.* **16,** 245.

In addition to the known hazards of nickel (Ni) associated specifically with its inhalation (see previous item; *Cited in F.C.T.* 1973, **11,** 1145) or with skin contact in sensitive persons (*ibid* 1972, **10,** 726), workers have warned of effects associated either with excessive levels of Ni in the diet (*ibid* 1969, **7,** 693) or, in young animals, with Ni deficiency. The acceptable range of levels for the ingestion of this element has yet, however, to be established. The present study was designed to examine the absorption, persistence and fate of various amounts of ingested Ni, and to consider the toxicological significance of these findings.

Female rats were treated once intragastrically with a solution of ^{63}Ni-labelled nickelous chloride, containing 1 μCi ^{63}Ni (equivalent to 1·8 μg of solid). Other rats were given, with the labelled dose, unlabelled nickelous chloride equivalent to 4, 16 or 64 mg Ni/kg body weight. In a parallel study the Ni solutions were injected ip.

In all cases, the entire oral dose was eliminated within 48 hr, 3–6% being found in the urine and the rest in the faeces. Urinary excretion reached a peak at about 3 hr. In contrast to the orally-treated rats, the animals injected ip excreted most of the Ni in the urine, the maximum rate being reached by about 2 hr. Only 1–2% appeared in the faeces. Although oral doses up to 64 mg/kg caused no apparent ill effects, ip injection of the highest dose killed 60% of the animals.

The results indicate the restricted capacity of the rat intestine for nickel absorption and the rapid elimination of the element from the blood stream via the kidneys. These two factors are likely to preclude the development of toxic responses to ingested doses of a metal that can have adverse effects on metabolic functions if it penetrates into the tissues.

[The average dietary intake of nickel (*Cited in F.C.T.* 1972, **10,** 595) is probably in the region of 10–500 μg Ni/day.]

2704. Trace metals in young blood

Delves, H. T., Clayton, Barbara E. & Bicknell, Joan (1973). Concentration of trace metals in the blood of children. *Br. J. prev. soc. Med.* **27,** 100.

The fact that many of the 20 trace metals identified in human tissue have no known physiological role suggests a possible link between the tissue concentration of certain metals and the extent to which these metals are ingested by man. It is thus of considerable interest to monitor these two parameters and, where possible, to correlate specific toxic effects with high levels of uptake and tissue retention of an apparently non-essential element. The study cited above was limited for practical reasons to the trace-metal analysis

of blood samples from normal children and from those suspected of having lead poisoning. a programme acknowledged by the authors to provide only a qualitative approach to the question of the presence of such metals in the body tissues.

Samples of blood (2 ml) were taken from a control group of children, aged between 2 months and 15 yr, and from a group of comparable age range who were suspected of having lead poisoning. The samples were analysed for lead, copper, zinc, manganese, nickel, cobalt, strontium, chromium, bismuth, iron, lithium and cadmium by a procedure involving sequential solvent extraction (except in the case of chromium) and atomic-absorption spectrophotometry.

The main clinical symptom in the patients comprising the test group was severe pica, usually associated with anaemia, mental retardation or convulsions, and almost half of the samples from the 189 patients had lead concentrations above 30 μg/ml blood, as opposed to only one of 44 samples from the control children. Many of the samples taken from the patients contained significantly higher concentrations of lead, copper, zinc, cadmium, manganese, strontium and chromium than were found in the control samples. On the other hand, zinc and copper, as well as iron, were sometimes present in abnormally low concentrations. It did not prove possible to relate any consistent clinical patterns to the abnormal blood levels of metals other than lead. In some cases, particularly the high levels of chromium, manganese and strontium, it seemed likely that pica had resulted in excessive ingestion of these metals as well as lead.

[The results presented in this paper are difficult to interpret because they do not take into account the possible effects of the nutritional and health status of individual patients. Within the recognized limitations of blood analyses, it would be of interest to determine blood levels of the various metals as a function of their intake in food, and in patients with defined clinical symptoms. The authors themselves suggest the inclusion of metabolic balance determinations in a prospective study of these metals in children with pica but without any history of subnormality.]

2705. Pneumoconiosis in the guinea-pig

Sharan, R. K. (1973). Studies on simple pneumoconiosis in guinea-pigs by coal-dust inhalation. *J. Path.* **111,** 31.

We recently reported on a symposium concerned with the many aspects of the problem of coal workers pneumoconiosis (*Cited in F.C.T.* 1973, **11,** 1129) and in particular we considered the reports on the pathological changes resulting from the prolonged inhalation of coal-dust and its consequent accumulation in substantial amounts in the lungs. The early stages of the pneumoconiotic condition have now been reproduced in the guinea-pig following exhaustive studies, by the same author, on the acinar morphology of the normal guinea-pig lung (Sharan, *Indian J. Chest Dis.* 1972, **14,** 15).

Guinea-pigs were exposed when 3–6 months old to bituminous coal-dust on 4 hr/day for 6 days/wk. The atmospheric concentration of the coal-dust was between 1500 and 1900 particles/ml. The experiment lasted for 3 yr, during which time groups of three or six animals were exposed for periods ranging from 1 day to 2 yr, although in the longest exposed group only one of the intended six guinea-pigs survived the full 720 days, the others having died from a variety of causes. The lung lesion produced was found to be essentially a fully developed dust-focus formed in the air sac of the acinus, but no dust deposits were seen

around the respiratory bronchioles. The dust accumulations gradually underwent organization and shrinkage, leading to dilatation of the alveolar ducts and subsequent rupture of the walls to produce an emphysematous condition.

These observations support the explanation suggested by Duguid & Lambert (*J. Path. Bact.* 1964, **88**, 389) for the development of centrilobular emphysema in coal-miners' lungs.

2706. Tissue levels of hexane following inhalation

Böhlen, P., Schlunegger, U. P. & Läuppi, E. (1973). Uptake and distribution of hexane in rat tissues. *Toxic. appl. Pharmac.* **25**, 242.

Hexane has generally been considered a fairly innocuous solvent, but a recent report has associated industrial exposure to hexane with the development of neuropathy (*Cited in F.C.T.* 1973, **11**, 157). However, in this paper the authors are primarily concerned with the uptake and distribution of hexane in the tissues of the rat, using this as a model for investigating the kinetics of exposure to organic solvents used as clinical anaesthetics.

Rats were exposed to 170 g hexane vapour/m^3 in air for 2–10 hr and the hexane concentrations in the tissues were measured by gas-liquid chromatography and studied as a function of the inhalation time. Total lipids in blood and tissues were measured to show whether or not there was a relationship between hexane concentration and the lipid content of a tissue.

The uptake of hexane in the blood, brain, adrenals, kidneys and spleen was found to be functionally dependent on the exposure time up to a saturation level, which was specific for the particular organ and was reached after exposure for 4–5 hr. In contrast, uptake in the liver increased linearly with time of exposure and had not reached saturation level after 10 hr. The reason for this difference appears to lie in the lipid content of the tissues. In organs other than the liver, the levels of tissue lipids were not altered by the hexane treatment and the hexane saturation concentration was directly proportional to the lipid content, but in the liver additional lipid accumulated during hexane exposure, continually changing the affinity of the tissue for the solvent. The association of fatty liver with exposure to anaesthetic solvents is not new. The ratio of hexane concentration to lipid content in the liver was greater than the saturation level of about 4 mg hexane/g lipid in the other organs, because the accumulating additional lipid consisted exclusively of triglycerides, which possess a much higher capacity for forming solutions with organic solvents than do other lipid fractions.

While the hexane concentrations were directly proportional to the lipid content of the brain, adrenals, kidney and spleen, the hexane/lipid ratio in the blood was much higher (25 as opposed to 4 mg hexane/g lipid). It was unlikely that this could be attributed to a greater hexane solubility in blood lipids than in other tissue lipids and the binding of hexane with some other blood components seemed a more probable explanation. Laboratory studies indicated that binding with plasma proteins could make a significant contribution to the solubility of hexane in blood.

NATURAL PRODUCTS

2707. Concanavalin A, the jack bean and the quail

Jayne-Williams, D. J. (1973). Influence of dietary jack beans (*Canavalia ensiformis*) and of concanavalin A on the growth of conventional and gnotobiotic Japanese quail (*Coturnix coturnix japonica*). *Nature New Biology* **243,** 150.

The toxic effects of the raw navy bean to rats are well known (*Cited in F.C.T.* 1966, **4,** 356). Diets containing 50% raw navy beans (*Phaseolus vulgaris*) have also been found to induce progressive loss of weight and hypothermia followed by death in the Japanese quail (*Coturnix coturnix japonica*) (Jayne-Williams & Hewitt, *J. appl. Bact.* 1972, **35,** 331). Germ-free quail given the same diet maintained a normal body temperature and did not show any increased mortality compared with controls. It has been suggested that phytohaemagglutinins (PHA) in beans may inhibit the birds' natural resistance to normally innocuous intestinal bacteria, which pass from the gut lumen into the lymph, blood and liver.

Feeding experiments in quail were carried out using the PHA (concanavalin A) prepared from jack bean (*Canavalia ensiformis*), since this PHA was more readily obtainable in bulk than that from the navy bean. Jack beans are known to cause the death of young rats when fed raw but not when cooked. The same difference was found in the quail, a 50% raw bean diet causing 100% mortality in 3 days, while a diet containing 50% autoclaved bean meal caused no deaths in 14 days. Neither diet was lethal to germ-free birds. In further studies, concanavalin A in sufficient volume to provide an appropriate amount of agglutinating activity was added to autoclaved jack-bean meal and another diet was prepared with a similar amount of autoclaved concanavalin A. The diet containing native concanavalin A killed eight out of ten conventional quail but none of a group of ten gnotobiotic birds. Autoclaved concanavalin A produced no deaths in either group. The evidence indicates therefore that the oral toxicity of the jack bean is present in the same fraction as its haemagglutinating activity, and that it does depend upon the presence of the intestinal flora.

[P. Cooper]

2708. Effect of ochratoxin on glycogen storage

Suzuki, S. & Satoh, T. (1973). Effects of ochratoxin A on tissue glycogen levels in rats. *Jap. J. Pharmac.* **23,** 415.

In common with aflatoxin, the fungal metabolite ochratoxin A has been isolated from strains of both Aspergillus and Penicillium, but the mechanism of its hepatotoxicity has not been investigated as extensively as has that of its notorious associate. In a previous issue (*Cited in F.C.T.* 1969, **7,** 401) evidence was presented suggesting that the toxin may compete with cyclic AMP for the enzyme phosphorylase *b* kinase, to elicit an observed cytoplasmic accumulation of glycogen. The present study was designed to investigate *in vivo* this aspect of ochratoxin A hepatotoxicity in the rat.

A single dose of 15 mg ochratoxin A/kg body weight given orally to either normal or adrenalectomized rats resulted in a marked depletion of hepatic glycogen within 4 hr and an elevation of cardiac glycogen levels within 4–6 hr. In intact rats, the liver glycogen returned to normal in about 5 days, but in the adrenalectomized rats, a dramatic fall ended with death about 7 hr after dosing. In adrenalectomized rats pretreated with hydrocortisone, the effect of the ochratoxin dose paralleled that in the intact rat.

Similar reductions in hepatic glycogen levels have been associated with the administration of aflatoxin B_1 or rubratoxin B to some animal species, though not apparently to rats, in which species ochratoxin A has been associated in previous studies with glycogen accumulation rather than depletion in the liver (Purchase & Theron, *Fd Cosmet. Toxicol.* 1968, **6**, 479; *Cited in F.C.T.* 1969, **7**, 401). The authors mention this discrepancy but offer no explanation for it. They tentatively ascribe their own findings in this study to endocrine imbalance leading to inhibition of glycogen biosynthesis or to increased glycogen breakdown.

COSMETICS, TOILETRIES AND HOUSEHOLD PRODUCTS

2709. Screening of optical brighteners

Griffith, J. F. (1973). Fluorescent whitening agents. Tests for skin-sensitizing potential. *Archs Derm.* **107**, 728.

Optical brighteners in washing powders have from time to time been accused of provoking contact dermatitis (*Cited in F.C.T.* 1972, **10**, 439). This paper is a compilation of results obtained during a 20-yr period in which the screening of a variety of such fluorescent whitening agents (FWA) has been conducted under conditions appropriate to the intended use, except that the test exposures were considerably more severe than those anticipated under use conditions. Altogether 31 compounds were tested, mainly by repeated-insult patch-test procedures (application of nine successive occluded patches for 24-hr periods over a total period of 3 wk followed by a challenge application 2 wk later). In some cases a photosensitization test was also carried out. Usually each compound was tested on 50–80 volunteers, and although these were relatively small groups the authors felt that the degree of insult was sufficiently exaggerated by the use of high concentrations of the FWA and the long periods of exposure to occluded patches to give a meaningful prediction.

Three of the compounds tested gave positive results. They were 1-phenyl-2-(naphtho[2,1-*d*]oxazol-2-yl)ethylene (FWA 18), which has been used in consumer products, 1-(3-chlorophenyl)-3-phenyl-2-pyrazoline (FWA 22) and a mixture of FWA 22 with 1-(3-chlorophenyl)-3-(4-chlorophenyl)-2-pyrazoline (FWA 23). The sensitizing potential of FWA 23 has already been reported (*ibid* 1972, **10**, 439).

Although it is tempting to associate the presence of the chlorophenylpyrazoline moiety in FWAs 22 and 23 and of the oxazole ring system in FWA 18 with the sensitizing activity of these products, other FWAs containing similar structures do not elicit a similar response. Thus the author concludes that the sensitizing properties of a compound must be a function of the whole molecule rather than of any particular substructure within it.

The limited photosensitization tests included in this study provided no evidence to support the contention of a relationship between fluorescence and photosensitization.

2710. Tissue distribution of a new bactericide

Magnusson, B. & Heyden, G. (1973). Autoradiographic studies of ^{14}C-chlorhexidine given orally in mice. *J. periodont. Res.* **8** (Suppl. 12), 49.

In a recent reference to the use of chlorhexidine as a skin bactericide and possible substitute for hexachlorophene (*Cited in F.C.T.* 1973, **11**, 166), we concluded that further attention should be focused on the safety of this compound in such applications. The employ-

ment of these bactericides in mouthwashes, for example, often involves their ingestion, and it is therefore important to study initially the fate of aqueous solutions of chlorhexidine after their oral administration. One such investigation has now been carried out, using young female mice, to determine the distribution of chlorhexidine in the body.

A solution of chlorhexidine diacetate or digluconate uniformly labelled with carbon-14 in the aniline rings, was applied to the back of the mouth, in a single dose equivalent to 0·5 μCi/g body weight. The distribution of radioactivity was studied, using apposition autoradiographic and liquid emulsion micro-autoradiographic techniques, in animals killed after intervals ranging from 15 min to 2 months. Two additional mice given a dose of the diacetate providing 0·14 μCi/g each day for 2 wk were killed 15 min and 1·5 months after the final dose.

Labelled chlorhexidine displayed a marked affinity for the epithelial cells of the alimentary canal and respiratory tract, the greatest accumulation being found in mucous-producing areas. By 6 hr, concentrations in the liver and kidneys were substantial and that in the gall bladder had reached its peak. After 2 wk the bulk of the material had left the body, and consequently levels in the liver and kidneys and alimentary tract were much reduced, although the activity retained in the respiratory tract was still considerable. At the end of 2 months, little activity was retained and most of this was in the upper respiratory tract, with signs of very weak activity in the kidneys and gastric mucosa. No significant differences were found in the distribution of repeated doses.

[This study indicates that chlorhexidine follows the route from ingestion to excretion common to many foreign compounds. It should pave the way for the acquisition of quantitative data on tissue levels and meaningful toxicological studies on the compound.]

2711. Thesaurosis back in the news

Ehrhardt, D. R., Ahn, C. & Sawyers, T. M. (1973). Pulmonary disease associated with the inhalation of cosmetic aerosols. *Chest* **64,** 251.

The phenomenon of pulmonary thesaurosis (storage disease) due to hairspray inhalation has been the source of much controversy since it was first described in 1958 (*Cited in F.C.T.* 1963, **1,** 138). Several subsequent surveys of hairdressers revealed no evidence of the condition (*ibid* 1967, **5,** 590) and experimental animals have proved largely insensitive (*ibid* 1966, **4,** 73) although polyvinylpyrrolidone (PVP), one of the ingredients thought to be a causative agent, has provoked a macrophage response in the lungs of rats (*ibid* 1967, **5,** 591). The indications are that the condition is basically allergic in origin, afflicting only a few unfortunate individuals (*ibid* 1966, **4,** 74; *ibid* 1967, **5,** 275). The present paper brings the number of cases said to have been reported in the literature to 30.

A 43-year-old woman was admitted to hospital with a 6-wk history of fatigue, cough, dyspnoea and high temperature. Diffuse bilateral pulmonary infiltrates were evident on X-ray and spirometry studies revealed severe obstructive and restrictive pulmonary disease. Elevations in sedimentation rate and in serum-globulin levels and a positive histamine skin reaction were found on clinical examination. A biopsy conducted subsequent to prednisone therapy revealed areas of interstitial fibrosis and atelectasis, scant inflammatory cells, areas of hyperplasia in the alveolar lining cells, and numerous macrophages filled with granules staining with periodic acid-Schiff (PAS) reagent. The patient admitted to episodes of "bronchitis" and to daily use of hairspray for the previous 5 yr, with use twice daily for several months before the onset of her present condition. She was advised not to use

hairsprays and her condition gradually improved, the pulmonary infiltrates clearing completely within the following 2 months.

The clinical and pathological findings in this case lent support to the hypersensitivity theory of thesaurosis, but it was unclear whether PVP was the causative agent. The authors recommend that further work should concentrate on definitive methods for demonstrating PVP in lung tissue, since PAS is not a sufficiently specific stain, and on skin tests for demonstrating allergy to PVP or to other cosmetic aerosol constituents.

2712. Hypersensitivity to permanently pressed sheets

Panaccio, F., Montgomery, D. C. & Adam, J. E. (1973). Follicular contact dermatitis due to coloured permanent-pressed sheets. *Can. med. Ass. J.* **109,** 23.

Multi-coloured 50% polyester/50% cotton sheets were the cause of an outbreak of an unusual type of delayed contact dermatitis in over 200 patients in the Ottawa area. The symptoms were very severe itching, often with a burning sensation, and either a generalized skin eruption consisting of excoriated, erythematous, follicular keratotic papules measuring 1–3 mm or, less commonly, a type of lesion involving localized erythematous patches, particularly on the ears. These patches dried to a fine scale and persisted for a shorter time than the generalized eruption, which took weeks or months to disappear.

Initially a diagnosis of fibreglass dermatitis was suspected, but microscopic examination discounted this. It was 7 or 8 months from its first appearance before the outbreak was associated with the use of new sheets of a particular type. Patch testing with various components used in the manufacture of these sheets gave inconclusive results as regards the nature of the allergen, but patch tests to formalin were negative. Some of the patients reacted positively to a monomer used in the permanent-pressing process, and a few to an optical brightener, but none of the dyes used in the sheets was supplied for testing. The onset of the allergic response was found to have occurred at least 10 days after the sheets were first used, and the reaction often persisted for up to 8 wk after their use was discontinued. It appeared in some cases that much laundering of the sheets was required before the allergen was eventually removed.

A possible explanation for the follicular distribution of the dermatitis and its persistence is that sleeping conditions favoured penetration of the allergen into the hair follicles, where it became trapped. The pressure, friction and sweating occurring during sleep could encourage the absorption and penetration of the allergen. The manufacturer of the sheets has now altered the processing techniques so that the outbreak should not be repeated from that source. Unfortunately no details of the identities of the chemicals involved were revealed to assist in the avoidance of other similar outbreaks elsewhere.

BIOCHEMICAL PHARMACOLOGY

2713. Enzyme activities in human liver tissue

Gold, M. S. & Ziegler, D. M. (1973). Dimethylaniline *N*-oxidase and aminopyrine *N*-demethylase activities of human liver tissue. *Xenobiotica* **3,** 179.

The applicability of the human situation of conclusions drawn from animal studies, including the relevance of concepts such as the hundredfold safety factor, is a perennial prob-

lem for the toxicologist. Direct study of human tissues, and particularly of liver tissue, must be an important step in resolving this issue, but it presents technical difficulties. However, a recent demonstration by Schoene *et al.* (*Europ. J. clin. Pharmac.* 1972, **4,** 65) that a number of microsomal-enzyme activities could be measured in small amounts of human hepatic tissue sampled by needle biopsy has paved the way for the study of the metabolism of foreign compounds in homogenates of human liver.

The paper by Gold & Ziegler (cited above) describes experiments on material from a total of 113 surgical and percutaneous needle biopsies of liver tissue from living patients. The *N*-oxidation of *N,N*-dimethylaniline (DMA) and the oxidative demethylation of aminopyrine (AP) were investigated by measuring the rate of formation of the corresponding reaction products, DMA *N*-oxide and formaldehyde, respectively. At its optimum pH (8·4) DMA *N*-oxidase activity was found to be higher than that of AP demethylase at its optimum pH (7·4) in most biopsies. Neither the age nor sex of the patient had any obvious effect on either enzyme activity, although in this connexion it is important to remember that the study was confined to adults, so that the possibility of activity differences in younger patients cannot be dismissed. Enhanced *N*-oxidase activity was observed in patients who had recently been given amphetamines or some tranquillizers, and increased demethylase activity was seen in patients taking barbiturates. Neither enzyme activity appeared to be affected by any specific disease process, although both were markedly reduced in cases of severe damage to the liver cells.

While the high *N*-oxidase/demethylase ratio observed is similar to that found in pig-liver tissue (Bickel, *Xenobiotica* 1971, **1,** 313), it differs significantly from that of rat liver (Das & Ziegler, *Archs Biochem. Biophys.* 1970, **140,** 300). In the light of this study on human tissues, it is suggested that the contribution of *N*-oxidation to the metabolism of amine drugs in man is greater than had previously been conjectured on the basis of the findings in animal studies.

Fd Cosmet. Toxicol. Vol. 12, p. 437. Pergamon Press 1974. Printed in Great Britain

LETTER TO THE EDITOR

DNA ALKYLATION BY NITROSOUREAS

Sir.—In your article 'Mammals. mosses and MNU' (*Food and Cosmetics Toxicology* 1974. **12,** 147) you state that methyl nitrosourea and ethyl nitrosourea given by intraportal injection to adult rats have not been shown to induce tumours in the liver or to produce any alkylation of hepatic nuclear DNA (Lijinsky *et al. Cancer Res.* 1972. **32,** 893). I should like to mention the fact that there is good evidence that these compounds do alkylate DNA of various organs. including the liver. of the adult rat when given by iv injection (Swann & Magee, *Biochem. J.* 1968. **110,** 39; *idem. ibid* 1971. **125,** 841). and that methyl nitrosourea induces hepatomas if given by ip injection to adult rats during the period of restorative hyperplasia following partial hepatectomy (Craddock & Frei, *Br. J. Cancer*, in press).

VALDA M. CRADDOCK.
MRC Toxicology Unit.
Medical Research Council Laboratories.
Woodmansterne Road.
Carshalton, Surrey. England

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FORTHCOMING PAPERS

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

A lifespan study of a polydimethylsiloxane in the mouse. By Margaret G. Cutler, A. J. Collings, Ida S. Kiss and M. Sharratt.

Tumours and hormonal changes produced in rats by subcutaneous injections of linoleic acid hydroperoxide. By Margaret G. Cutler and R. Schneider.

Oral toxicity of tebuthiuron (1-(5-*tert*-butyl-1,3,4-thiadiazol-2-yl)-1,3-dimethylurea) in experimental animals. By G. C. Todd, W. R. Gibson and C. C. Kehr.

Teratogenicity and dominant lethal studies on hexachlorobenzene in rats. By K. S. Khera.

Citrinin mycotoxicosis in beagle dogs. By W. W. Carlton, G. Sansing and G. M. Szczech.

Long-term administration of sterigmatocystin and *Penicillium viridicatum* to mice. By G. M. Zwicker and W. W. Carlton.

Acute intraperitoneal toxicity of ochratoxin A and B derivatives in rainbow trout (*Salmo gairdneri*). By R. C. Doster, R. O. Sinnhuber and N. E. Pawlowski.

Implantation and embryonic survival in rats treated with amaranth during gestation. By K. S. Khera, W. Przybylski and W. P. McKinley. (Short Paper).

Some inter-relationships between vitamin C (L-ascorbic acid) and mercury in the guinea-pig. By Sue Blackstone, R. J. Hurley and R. E. Hughes. (Short Paper).

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

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CORRIGENDA

Volume 12 (1974)

p. 45, line 14: *For* kg/day. In dogs, ChE activity in plasma was depressed by the 3·0, 1·0 and 0·1 mg/kg/day doses, and... *read* kg/day for up to 2 yr. The cholinesterase (ChE) activity of plasma and red cells was depressed in rats...
p. 46, line 29: *For* 0·1 or 3·0 mg/kg *read* 0·1, 1·0 or 3·0 mg/kg...

[Corrected reprints of these two pages are inserted in this issue.]

p. 115, lines 5 and 10 of main text: *For* Hoffman *read* Hoffmann
p. 115, line 10 of main text: *For* Winder *read* Wynder

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Aims and Scope

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

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