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Research Section

A LIFESPAN STUDY OF A POLYDIMETHYLSILOXANE IN THE MOUSE

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(*Received 25 March 1974*)

Abstract—A silicone antifoam agent containing 94% polydimethylsiloxane silicone oil and 6% finely divided silicon dioxide was given in the diet at levels of 0·25 and 2·5% to groups of mice for 76 wk from weaning. Another group of mice received a single sc injection of 0·2 ml of the antifoam at weaning, and a control group was given a single sc injection of 0·2 ml liquid paraffin BP. All surviving mice were killed when 80 wk old. No silicone was detected in the bodies of mice given diets containing 2·5% of the antifoam. Similarly, none was detectable in the liver, kidneys, spleen or perirenal fat of mice given the injection. However, cysts remained at the site of injection throughout the lifespan of many of these animals. There was no increase in the incidence of malignant or benign tumours in the groups of mice receiving the antifoam either in the diet or by injection, and there were no toxic effects that could be ascribed to the administration of silicone. On the other hand, sc injections of liquid paraffin caused an increased incidence and earlier appearance of sc fibromas at the injection site in male mice.

INTRODUCTION

The methylpolysiloxanes are effective antifoam agents. For this reason, they have found use in the processing of certain foods such as bread and cake and in the manufacture of drugs and cosmetics (Fessenden & Fessenden, 1967). Most of the compounds investigated appeared to be biologically inert. No toxic effects were observed after acute or prolonged administration of high levels (Department of Medical Biochemistry and Pharmacology, University of Birmingham, 1967; MacDonald, Lanier & Deichmann, 1960; Rowe, Spencer & Bass, 1950). There was no increase in tumour incidence in rats after a 2-yr feeding study (Rowe *et al.* 1950).

The investigation described here was concerned with an assessment of the carcinogenic risk from these compounds using another species. A silicone antifoam compound containing polydimethylsiloxane silicone oil together with a small amount of silicon dioxide was fed at two dose levels to mice and was also given by sc injection.

EXPERIMENTAL

Material. The silicone antifoam compound was supplied by Midland Silicones Ltd. (now Dow Corning Corp.). It was composed of a mixture of 6% finely divided silicon dioxide and 94% polydimethylsiloxane silicone oil prepared by the process described by Rowe, Spencer & Bass (1948). The polymers in the polydimethylsiloxane silicone oil resembled those of the product known commercially as DC Antifoam A (Dow Corning Corp., 1949),

except that material of low molecular weight had been excluded. The silicone antifoam compound is referred to throughout the rest of this paper as 'silicone'.

Animals. Mice of an outbred strain from the Laboratory Animals Centre, Carshalton, Surrey, were randomly bred under specified-pathogen-free conditions.

Feeding and injection procedures. Groups of 40–50 male and 40–50 female mice received diets containing 0 (control), 0·25 or 2·5% silicone for 76 wk from weaning (4–5 wk of age). The diets consisted basically of a rat cake powder supplied by Heygate and Sons, Ltd., Northampton, and containing approximately 22% protein, 4% oil, 4% fibre, 7% ash and 15% moisture. The required level of silicone was incorporated in the form of a triturate consisting of 10% (w/w) silicone in rat cake powder, and a varying amount (2·5, 2·25 or 0%) of cellulose powder was added to the three diets to balance the different amounts of silicone added. Each diet also contained 3% (w/w) cod-liver oil and 5% (w/w) arachis oil. In the injection study, all the mice received a diet of Thomson's rat cake and were given at weaning a single sc injection of 0·2 ml silicone (test group) or 0·2 ml liquid paraffin BP (control). Animals were caged in groups of five in polypropylene boxes in a room maintained at 20–22°C and were allowed diets and water *ad lib.*

Post-mortem studies. Mice that died or were killed because they became moribund during the experimental period were autopsied and all observed lesions were examined histologically. At wk 80, the surviving mice were killed with ether anaesthesia and autopsied, and microscopic examination was performed on all tissues that appeared abnormal to the naked eye. In addition, sections from the lung, heart, stomach, small intestine, spleen, liver and kidney were examined microscopically in about ten male and ten female 80-wk survivors randomly selected from each group.

Tissue analyses for silicone. Analyses for silicone were carried out on the liver, kidney, spleen and perirenal fat of five mice, 76 wk after sc injection of silicone. Five female and five male mice that had received 2·5% silicone in their diet for 75 wk were transferred to the control diet 8 days before they were autopsied, after which the whole body was analysed for silicone content. The tissues were homogenized in the presence of sodium sulphate and then ground with sand and sodium sulphate until a dry powder was formed. The resulting powders were extracted continuously with benzene for 16 hr in a Soxhlet apparatus. The benzene extract was evaporated to dryness *in vacuo*, the residue was taken up with 10 ml carbon disulphide and its silicone content was determined by examination of the infra-red absorption at 1260 cm^{-1}. The method was capable of detecting 20 μg silicone in 1 g tissue. In the presence of interfering substances, the crude extract was redissolved in 25 ml carbon disulphide and 1 ml of this solution was chromatographed on silica gel using 1 litre benzene–light petroleum as solvent. The solvent was removed from the eluate in a rotary film evaporator and the residue was heated at 60°C and 15 mm pressure for 10 min to remove traces of solvent. The cooled residue was taken up in 10 ml carbon disulphide and examined for silicone as above.

RESULTS

Silicone intake

The mice in these experiments ate approximately 7 g of diet daily and weighed about 30 g during most of the experimental period. The 2·5 and 0·25% silicone diets therefore provided the mice with approximately 5800 and 580 mg silicone/kg/day, respectively. The single dose of 0·2 ml given to the injected animals was equivalent to 201 mg silicone.

Table 1. *Mortality data for groups of mice given 0·25 or 2·5% silicone in the diet or 0·2 ml silicone or liquid paraffin in a single sc injection*

Route of administration	Dietary level or dose of silicone	Initial no. of mice/ group	No. of mice dead at wk							
			10	20	30	40	50	60	70	80
			Males							
In diet	0	50	0	2	2	2	3	5	12	25
	0·25%	49	0	1	3	3	3	4	6	16
	2·5%	50	0	1	1	2	5	5	11	20
Injection (sc)	0†	47	0	0	4	8	12	19	25	30
	0·2 ml	50	3	4	5	6	7	10**	12**	18**
			Females							
In diet	0	50	0	1	1	2	3	6	6	8
	0·25%	48	0	1	1	2	2	2	3	5
	2·5%	48	0	1	2	2	2	3	11	19*
Injection (sc)	0†	50	0	1	2	3	4	5	6	7
	0·2 ml	50	0	0	0	0	1	3	4	9

†This group received an injection of 0·2 ml liquid paraffin.
Values marked with asterisks differ significantly (Student's t test) from the relevant controls: $*P < 0·05$; $**P < 0·01$.

Mortality

The mortality of females given 2·5% silicone in their diet was increased at wk 80 (Table 1). Four of these animals died from the flooding of their cage, however, and the increased mortality ceased to be of statistical significance when these four deaths had been excluded. No increase in mortality was observed in any of the other groups of mice given silicone.

The apparent increase in mortality during wk 40–80 in the group of males given an injection of liquid paraffin (Table 1) was an artefact, as animals were killed after the appearance of sc fibromas so that the lesions could be investigated.

Pathological findings

Non-neoplastic pathological changes found in these mice are summarized in Tables 2 and 3. The only lesions found on microscopic examination but not on macroscopic examination were metaplastic foci and papillary adenomas in 1% of apparently normal lungs and chronic inflammatory changes in 3·9% of apparently normal kidneys and in 1·9% of apparently normal lungs. The principal abnormalities were chronic respiratory disease, chronic inflammatory and degenerative changes in the kidney, proteinaceous plugs in the urinary bladder, hyperplasia of the endometrium, simple cysts of the ovary and superficial ulcers of the stomach. Other abnormalities were scattered throughout the groups in a lower incidence or were confined to one or two groups.

The incidence of superficial ulceration of the stomach was increased in males receiving the 0·25% silicone diet (Table 2), but not in the group receiving the 2·5% silicone diet, and the incidence showed no significant increase when results from males and females were combined. The only other statistically significant difference between the groups receiving dietary silicone and their controls also appeared to be unrelated to the treatment given. This consisted of a reduced incidence of uterine atrophy in females given the 2·5% silicone diet (Table 3).

Table 2. *Non-neoplastic pathological changes in male mice given 0·25 or 2·5% silicone in the diet or a single 0·2 ml sc injection of silicone or liquid paraffin*

Histopathological findings	No. of mice examined...	No. of mice with pathological changes in group given				
		Dietary silicone (% concn)			Injection of	
		0	0·25	2·5	Paraffin	Silicone
		48	48	48	43	47
Stomach						
Superficial ulcers		1	6*	1	0	0
Small intestine						
Superficial ulcers		1	1	1	0	0
Liver						
Chronic degeneration		1	0	1	0	1
Nodular hyperplasia		1	1	1	0	1
Lung						
Chronic respiratory disease		5	5	8	3	3
Kidney						
Chronic pyelonephritis		4	3	6	4	5
Chronic degeneration		2	4	2	4	3
Urinary bladder						
Proteinaceous plug		10	13	15	10	3**
Testis						
Atrophy		0	1	0	2	0
Seminal vesicle						
Retention cyst		0	2	0	3	2
Spleen						
Amyloid degeneration		0	1	0	1	1
Subcutaneous tissue						
Cyst at injection site		—	—	—	1	7*

Values marked with asterisks differ significantly (Student's *t* test) from that of the corresponding control group: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$.

The only statistically significant differences between the groups given silicone or liquid paraffin by injection were a higher percentage of males in the silicone group with cysts at the site of injection and a lower proportion of males in this group with proteinaceous deposits in the urinary bladder.

In Tables 4 and 5 the incidence and types of tumours are recorded. The majority of tumours were lymphosarcomas, papillary adenomas of the lung and subcutaneous fibromas. Other types of tumour occurred in smaller numbers randomly throughout the groups.

There was no increase in the number of malignant or benign tumours in the groups of mice receiving silicone in the diet or by injection, and in some experimental groups the incidence of certain benign tumours was significantly less than in the corresponding controls. The incidence of papillary adenoma in the lung, for example, was reduced in males and females receiving the 0·25% silicone diet and in females given the 2·5% silicone diet, although the incidence was not reduced significantly when compared with that in the control animals in the injection study. These lung tumours were distributed unevenly among the cages of mice, being found in a high proportion of the animals in certain cages and in none of the mice in many of the other cages. Sc fibromas occurred less frequently in males given the silicone injection than in those given the injection of liquid paraffin,

Table 3. *Non-neoplastic pathological changes in female mice given 0·25 or 2·5% silicone in the diet or a single 0·2 ml sc injection of silicone or liquid paraffin*

Histopathological findings	No. of mice examined...	No. of mice with pathological changes in group given				
		Dietary silicone (% concn)			Injection of	
		0	0·25	2·5	Paraffin	Silicone
		48	47	46	49	50
Stomach						
Superficial ulcers		1	2	5	0	0
Liver						
Chronic degeneration		0	0	0	0	0
Nodular hyperplasia		0	0	1	0	0
Lung						
Chronic respiratory disease		2	3	4	3	5
Kidney						
Chronic pyelonephritis		5	6	8	2	2
Chronic degeneration		1	2	4	1	5
Urinary bladder						
Proteinaceous plug		0	1	0	0	0
Ovary						
Simple cysts		5	11	5	7	11
Uterus						
Hyperplastic endometrium		7	7	7	12	16
Atrophy		27	25	16**	4	4
Spleen						
Amyloid degeneration		0	0	0	0	0
Subcutaneous tissue						
Cyst at injection site		—	—	—	10	14

Values marked with asterisks differ significantly (Student's *t* test) from that of the corresponding control group: **$P < 0·01$.

Table 4. *Incidence and types of tumours in male mice given 0·25 or 2·5% silicone in the diet or a single 0·2 ml sc injection of silicone or liquid paraffin*

Site and type of tumour	Effective no.† of mice/group...	No. of mice bearing tumours in group given				
		Dietary silicone (% concn)			Injection of	
		0	0·25	2·5	Paraffin	Silicone
		32	37	31	35	37
Lung						
Papillary adenoma		11 (34)	2 (5)**	5 (16)	3 (8)	3 (8)
Lymphoid tissue						
Lymphosarcoma		2 (6)	2 (5)	0	0	1 (3)
Lymphatic leukaemia		0	0	0	1 (3)	0
Vas deferens						
Squamous carcinoma		0	1 (3)	0	0	0
Subcutaneous tissue						
Fibroma		3 (10)	2 (6)	0	16 (46)	6 (16)**

†No. of animals surviving to 80 wk plus tumour-bearing animals that died or were killed earlier.

Values in parentheses denote the numbers of mice affected expressed as a percentage of the effective no. of mice/group. Values marked with asterisks differ significantly (Student's *t* test) from that of the corresponding control group: **$P < 0·01$.

Table 5. *Incidence and types of tumours in female mice given 0·25 or 2·5% silicone in the diet or a single 0·2 ml sc injection of silicone or liquid paraffin*

Site and type of tumour	Effective no.† of mice/group...	No. of mice bearing tumours in group given				
		Dietary silicone (% concn)			Injection of	
		0	0·25	2·5	Paraffin	Silicone
		42	44	32	48	44
Mammary gland						
Adenoma		0	0	0	1 (2)	0
Lung						
Papillary adenoma		9 (21)	2 (5)**	1 (3)**	8 (17)	5 (11)
Ovary						
Cystadenoma		1 (3)	0	0	0	0
Uterus						
Squamous carcinoma		0	0	0	1 (2)	0
Fibromyoma		0	1 (2)	0	0	2 (5)
Cervix						
Fibroma		0	0	1 (3)	0	1 (2)
Lymphoid tissue						
Lymphosarcoma		1 (2)	0	3 (9)	2 (4)	1 (2)
Reticulum-cell sarcoma		1 (3)	0	0	0	0
Subcutaneous tissue						
Fibroma		0	0	0	1 (2)	2 (5)
	Total no. with tumours...	12 (29)	3 (7)**	5 (15)	10 (21)	10 (23)

†No. of animals surviving to 80 wk plus tumour-bearing animals that died or were killed earlier.
Values in parentheses denote the numbers of mice affected expressed as a percentage of the effective no. of mice/group. Values marked with asterisks differ significantly (Student's *t* test) from that of the corresponding control group: $^{**}P < 0{\cdot}01$.

although the incidence in the former group was similar to that found in the control males of the dietary study (Table 4). In mice given the silicone injection, 50% of the fibromas occurred at the site of injection, whereas 88% of the fibromas were at the injection site in the liquid-paraffin control group. Differences between the control and experimental groups in the overall tumour incidence arose solely from differences between the groups in the proportion of papillary adenomas of the lung or of sc fibromas.

The sc fibromas at the injection site occurred earlier in male mice after the injection of liquid paraffin than after the silicone injection. The first fibroma in the liquid-paraffin group was detected in wk 40 while that in the silicone group was not detected until wk 70, by which time the total in the former group had reached 13. Administration of silicone by either route had no significant effect on the time at which any tumours developed.

Silicone content of the tissues

Analysis of the whole animals did not demonstrate the presence of the silicone component of the administered silicone antifoam compound in ten mice which had been fed a diet containing 2·5% silicone for 75 wk. Similarly, silicone was not detected in samples of liver, kidney, spleen or perirenal fat from five mice given a single sc injection of 0·2 ml silicone 76 wk previously.

DISCUSSION

The many investigators who have studied the effects of methylpolysiloxanes upon living systems agree that little if any damage to organs is detectable after acute or long-term high-level administration (Grasso, Fairweather & Golberg, 1965; MacDonald *et al.* 1960; McNamara, McKay & Quille, 1950; Meals, 1969; Polemann, 1955; Rowe *et al.* 1950). Most of these studies were carried out on the methylpolysiloxanes in Dow Corning Antifoam A or in Dow Corning 200 fluids. No toxic effects followed the feeding of Dow Corning Antifoam A to rats for 2 yr (Rowe *et al.* 1950) and no-ill-effects were observed in dogs that received 3 g Dow Corning Antifoam A/kg for 5 days/wk for 6 months (Child, Paquin & Deichmann, 1951). The biological response following sc, im and ip injections of the polymers was negligible, although elimination was extremely slow (Grasso *et al.* 1965; McGregor, 1960; McNamara *et al.* 1950). Thus, the reported toxicity studies on methylpolysiloxanes closely parallel the findings obtained in mice in the present investigations.

It may be concluded that these silicone antifoam agents carry no detectable carcinogenic hazard at or below the dosage levels employed. No significant toxic effect attributable to these agents was observed in the present study.

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Etude de l'action d'un polydiméthylsiloxane au cours de la vie de la souris

Résumé—On ajoute à la nourriture de groupes de souris, pendant 76 semaines à partir du sevrage, un agent antimoussant siliconé contenant 94% d'huile silicone de polydiméthylsiloxane et 6% de dioxyde de silicone finement divisé. Les taux administrés sont de 0, 25 et 2,5% du régime. Un autre groupe de souris reçoit une seule injection sc de 0,2 ml d'antimoussant au moment du sevrage, et un groupe témoin reçoit une seule injection sc de 0,2 ml de paraffine liquide B.P. Toutes les souris ayant survécu sont tuées quand elles atteignent 80 semaines. Aucun silicone n'a été détecté

dans le corps des souris ayant absorbé les régimes contenant 2,5% d'antimoussant. Parallèlement aucun silicone n'a été trouvé dans le foie, les reins, la rate ou les graisses périrénales des souris ayant reçu l'injection. Cependant des kystes subsistent à l'endroit de l'injection pendant toute la vie de beaucoup de ces animaux. Il n'y a aucun accroissement dans la fréquence des tumeurs malignes ou bénignes dans les groupes des souris qui reçoivent de l'antimoussant soit dans leur régime. soit par injection. et il n'y a aucun effet toxique qui puisse être imputé à l'administration de silicone. D'un autre côté, les injections de paraffine liquide produisent une élévation de la fréquence et une plus grande précocité des fibromes sous-cutanés à l'endroit de l'injection chez les souris mâles.

Eine Lebenszeitstudie von Polydimethylsiloxan an der Maus

Zusammenfassung—Ein Silikon-Antischaummittel, das 94% Polydimethylsiloxan-Silikonöl und 6% fein verteiltes Silikondioxid enthielt, wurde in den Diätspiegeln von 0·25 und 2·5% an Gruppen von Mäusen während 76 Wochen nach der Entwöhnung verabreicht. Eine andere Gruppe von Mäusen erhielt eine einmalige sc Injektion von 0·2 ml des Antischaums bei der Entwöhnung, und eine Kontrollgruppe erhielt eine einmalige Injektion von 0·2 ml "Liquid Paraffin BP". Alle überlebenden Mäuse wurden getötet, als sie 80 Wochen alt waren. Kein Silikon wurde gefunden in den Körpern der Mäuse, deren Nahrung 2·5% des Antischaums enthielt. Gleichfalls war keines auffindbar in der Leber, den Nieren, der Milz oder im perirenalen Fett der Mäuse, die die Injektion erhielten. Jedoch waren Zysten vorhanden an der Injektionsstelle während der Lebenszeit vieler dieser Tiere. Es gab keinen Anstieg im Auftreten von malignen oder benignen Tumoren in den Gruppen der Mäuse, die den Antischaum entweder in der Nahrung oder durch Injektion erhielten, und es gab keine toxischen Effekte, die der Gabe von Silikon zugeschrieben werden konnten. Andererseits erzeugte die sc Injektion von Paraffinum liquidum ein erhöhtes Vorkommen und früheres Auftreten von Fibromen an der Injektionsstelle bei männlichen Mäusen.

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TUMOURS AND HORMONAL CHANGES PRODUCED IN RATS BY SUBCUTANEOUS INJECTIONS OF LINOLEIC ACID HYDROPEROXIDE

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(*Received 10 December 1973*)

Abstract—Linoleate hydroperoxide was given by repeated sc injection to two groups of rats. Those in the first group received a total dose of 200 mg and those in the second group a total dose of 353 mg. No tumours occurred at the site of injection in the treated rats, but the incidence of malignant mammary tumours and of lymphosarcomas was increased among rats in the first group and that of benign mammary tumours and benign pituitary tumours was increased in the second group. These tumours occurred late in the lifespan of the animals.

The incidence of total mammary tumours and of total pituitary tumours increased as the dose of linoleate hydroperoxide was raised, but this increase was due to a greater number of benign rather than of malignant tumours. Enlargement of the pituitary gland, with increases in the proportion of eosinophilic cells in the pars anterior, in body-weight gain and body length, and in the proportion of rats with follicular cysts in their ovaries, also followed the administration of linoleate hydroperoxide. It is suggested that the pituitary and mammary tumours arose as a consequence of an induced hypothalamic disturbance, leading to mammosomatotrophic effects.

INTRODUCTION

Several workers have reported that products such as epoxides and hydroxy fatty acids formed during the heating or oxidation of fats produce injection-site sarcomas in the mouse (Swern, Wieder, McDonough, Meranze & Shimkin, 1970; Van Duuren, Langseth, Orris, Teebor, Nelson & Kuschner, 1966; Van Duuren, Melchionne, Blair, Goldschmidt & Katz, 1971) and an increase of spontaneous tumours after ingestion by rats (Seelkopf & Salfelder, 1962). In an earlier experiment in these laboratories, it was found that an increase in tumour incidence followed the inclusion of oxidized linoleic acid in the diet of rats and mice (Cutler & Schneider, 1973a) and the offspring of rats given this treatment showed an incidence of urogenital malformations higher than that found in the offspring of controls (Cutler & Schneider, 1973b). Application of purified linoleic acid hydroperoxide to the ovaries of mice was followed by an increase of foetal malformations in the progeny.

In the investigations described here, linoleic acid hydroperoxide was given by repeated sc injection to rats and its effects on the tumour incidence and hormonal status of the animals were evaluated.

EXPERIMENTAL

Materials. Antioxidant-free linoleic acid was obtained from Price's Ltd., Bromborough, Cheshire, and was purified on a silica column. The hydroperoxide was prepared as described by O'Brien & Frazer (1966) by direct aerial oxidation of a rotating film of the linoleic acid at room temperature, and was separated from the mixture of oxidation products

by partition between light petroleum and 75% aqueous methanol. The purity of the isolated product, determined by ultraviolet absorption spectrophotometry, iodometry and thin-layer chromatography, was between 90 and 100%. This was stored as an ethanolic solution at −20°C, which remained stable for 4 wk. For injections, a 10% solution of sodium linoleate hydroperoxide, pH 7·4, was prepared by removal of the ethanol in a rotary evaporator followed by the addition of the required amount of sodium hydroxide solution. This was used immediately after preparation.

Animals and treatment. The study was carried out on female rats from the Birmingham outbred Wistar strain reared by specified-pathogen-free foster parents. The rats were distributed by a random-number system between the groups shown in Table 1. The control group, C1, consisted of 50 females which received no additional treatment. Rats of the other groups received sc injections from the age of 6 wk at 5-weekly intervals. Twenty injections of 0·2 ml physiological saline were given to the 20 rats in group C2, each of the 25 rats in group L1 received 10 injections of 0·2 ml 10% sodium linoleate hydroperoxide and each of the 20 rats in group L2 were given 20 injections of 0·16–0·18 ml 10% sodium linoleate hydroperoxide. A diet of rat cake supplied by Spiller's Ltd., London, was given *ad lib.* to all the animals. Water was freely available.

Table 1. *General plan of injection experiments in rats*

Compound administered	Group no.	Total dosage (mg/rat)	No. of rats/group	No. of injections	Age of survivors when killed (wk)
—	C1	—	50	—	110
Physiological saline	C2	36	20	20	100
Linoleate hydroperoxide	L1	200	25	10	110
	L2	353	20	20	100

Rats were kept in cages of not more than five animals, in a barrier-controlled area of the animal house. Animals were examined daily and any abnormalities observed were recorded. Rats that appeared unwell were isolated and those that were moribund, bore palpable tumours or showed a marked decrease in body weight were killed. All surviving rats were killed at the age of 100–110 wk. Autopsies were performed on all animals and histological preparations were made of all tissues that appeared abnormal.

Studies of pituitary-gland changes and related effects. Measurements of the weight of the pituitary gland relative to body weight were made at autopsy on 24 control rats, on ten rats of group L1 and on 14 rats of group L2. In addition, estimates of pituitary-hormone activities in the treated and control rats were made by indirect methods.

A crude estimate of the growth-hormone activity in the animals was made from their body length, their body-weight gain and the proportion of eosinophilic cells in the pars anterior of the pituitary gland (Lee, Evans & Simpson, 1945; Purves, 1966). The body length of all rats was measured at autopsy on the extended animal from the tip of the nose to the junction of the body with the tail. The amount of body weight gained by all the rats during the period of active growth between the ages of 6 and 38 wk was also measured. Histological preparations of the pituitary gland from all animals were stained by Mallory's trichrome technique and also by the periodic acid–Schiff reagent using an Orange G counterstain (Culling, 1957) and the proportion of eosinophilic cells in the pars anterior was estimated.

Adrenocorticotrophic hormone (ACTH) activity was assessed from the weight of the adrenal glands relative to body weight at autopsy and from the levels of 11-hydroxycorticosteroids in the serum and adrenals. The adrenal glands from four control and five treated rats were homogenized in 5 ml 20% ethanol in physiological saline and their content of 11-hydroxycorticosteroids was estimated by the method of Givner & Rochefort (1965) using a Mark I spectrofluorimeter manufactured by the Farrand Optical Co., Inc., New York. The fluorescence was measured at 536 nm after excitation at 470 nm. Serum samples from four control and four treated rats were examined by the same procedure.

Thyrotrophin activity was indirectly assessed from the proportion of cells to colloid vesicles in histological preparations of the thyroid gland (Tala, 1952) and from the serum organic iodine levels. Organic iodine levels in the serum from 12 control and ten treated rats were estimated by the Regional PBI Centre, Staffordshire General Infirmary, Stafford, using an autoanalyser.

Gonadotrophin activity was indirectly assessed from the amounts of oestrogen in 24-hr urine samples and from the weight of the ovaries and of the uterus relative to body weight. At 96 wk, 11 rats from group C2 and 11 rats from group L2 were caged individually in urine-collecting apparatus of the type described by Sharratt (1958). To each of the 24-hr urine samples obtained from these rats was added 1 ml 50% sulphuric acid. The urine samples were hydrolysed at 120°C for 1 hr, steroids were extracted by the method of Brown (1955) and oestrogen levels were measured by a modification of Ittrich's procedure for the Kober reaction (Skramovsky & Haeberle, 1968). The fluorescence at 540 nm after excitation at 516 nm was compared with that of standard solutions of oestriol, using the Farrand spectrofluorimeter. At autopsy, the uterus and ovaries from 14 rats of group C2 and 14 rats of group L2 were freed from the surrounding mesentery and weighed, and the weights were expressed as a proportion of the body weight of each rat.

Statistical methods. Tumour incidence was assessed by the method of Arcos, Argus & Wolf (1968). The significance of the between-group differences in the incidence of tumours for each specific organ was calculated by the chi-squared test where the group numbers were large, and by Fisher's exact treatment of the 2 × 2 tables where small numbers were involved (Fisher, 1954). The significance of the between-group differences in the proportion of tumour-bearing animals was assessed by the same procedure. For evaluating the between-group differences in the age of animals when mammary tumours were detected, White's ranking method and Student's *t* test were used. These comparisons were made only between tumours of similar types. The results of the other tests are expressed as the mean values and the standard error of the mean. The significance of the differences between the means were calculated by Student's *t* test. Fisher and Yates' Statistical Tables were used throughout.

RESULTS

Examination for carcinogenic activity

Table 2 shows the effects of linoleate hydroperoxide injections on the tumour incidence and latent period in female rats. The most notable types of tumours found in this study were chromophobe adenomas of the pituitary and mammary adenomas and carcinomas. Lymphosarcomas and some malignant pituitary tumours were also seen. The incidence and types of tumour in the group of rats given sc injections of physiological saline were similar to those in the control group given no additional treatment and in Table 2 the

Table 2. *Effects of linoleate hydroperoxide injections on tumour incidence and latent periods in female rats*

Site and/or type of tumour	No. of rats affected† in group		
	C1 + C2	L1	L2
Effective no.‡/group....	69	25	17
Mammary gland			
Malignant§	4 (6)	5 (20)*	2 (12)
Benign§	—	1 (4)	4 (24)**
Pituitary gland			
Malignant	4 (6)	1 (4)	0
Benign	2 (3)	0	4 (24)*
Lymphosarcoma	1 (2)	4 (16)*	0
Other organs			
Malignant	8 (12)	2 (8)	2 (12)
Benign	3 (4)	4 (16)	6 (36)
Total no. of rats with benign or malignant tumours...	22 (32)	13 (52)	11 (65)*

†Two or more neoplasms in the same tissue in any one animal are recorded as one tumour and the no. of affected rats expressed as a percentage of the effective no./group are given in parentheses.

‡No. of rats surviving to wk 100, plus tumour-bearing animals that died or were killed earlier.

§The mean ages at which mammary gland tumours were detected were 86, 82 and 75 wk for the malignant tumours in groups C1 + C2, L1 and L2, respectively, and 110 and 90 wk for the benign tumours in groups L1 and L2, respectively.

Values marked with asterisks differ significantly from the control values: $*P < 0.05$; $**P < 0.01$.

results from these two control groups have therefore been combined. The rats of group L1, which had received a total dose of 200 mg sodium linoleate hydroperoxide, showed a significantly higher incidence of malignant mammary tumours and of lymphosarcomas than the controls, and those of group L2, given a total dose of 353 mg linoleate hydroperoxide, had a higher incidence of benign mammary tumours and of benign pituitary tumours. In group L2, the total number of rats bearing tumours was also significantly raised. No tumours occurred at the site of injection of linoleate hydroperoxide and the incidence of tumours other than lymphosarcomas, mammary or pituitary tumours was not significantly greater than that observed among control animals.

With an increase in the total dose of linoleate hydroperoxide from 200 to 353 mg, the incidence of total mammary tumours increased from 24 to 36%, the incidence of total pituitary tumours from 4 to 24% and the total tumour incidence from 52 to 65% of the effective number of animals in the group. The effect was unlike that of a typical carcinogen in that an increase in benign rather than in malignant tumours followed the increase in the total dose of linoleate hydroperoxide.

Malignant tumours of the mammary gland appeared slightly but not significantly earlier among rats given linoleate hydroperoxide injections than among the controls, as shown in Table 2. The average age at which these tumours were detected in control rats was 86 wk, whereas in group L1 the average age was 82 wk and in group L2 it was 75 wk.

Changes in the pituitary gland

Table 3 shows the changes occurring in the pituitary gland of rats after injections of linoleate hydroperoxide. The weight of the pituitary relative to body weight was raised slightly in rats of group L1 and was significantly elevated in rats of group L2. Some increase in growth-hormone activity in these animals was suggested by the increase in body

Table 3. *Effects of linoleate hydroperoxide injections on the pituitary gland of female rats*

Parameter	Values† for group		
	C1 + C2	L1	L2
Weight of pituitary (mg/100 g body weight)	2·5 ± 0·2 (24)	3·0 ± 0·3 (10)	3·8 ± 0·7* (14)
PAS-positive basophils in pars anterior (%)	8·9 ± 1·2 (20)	—	9·4 ± 1·9 (20)
Indices of growth-hormone levels			
Body length (cm)	23·6 ± 0·2 (70)	24·5 ± 0·2* (25)	24·4 ± 0·2* (20)
Body-weight gain from 6 to 38 wk (g)	192 ± 3 (70)	227 ± 10** (25)	216 ± 7** (20)
Eosinophils in pars anterior (%)	13·0 ± 0·7 (20)	—	15·8 ± 0·9* (20)
Indices of ACTH levels			
Adrenal 11-hydroxycorticosteroids (μg/adrenal)	0·41 ± 0·04 (4)	—	0·43 ± 0·04 (5)
Serum 11-hydroxycorticosteroids (μg/100 ml)	25·0 ± 0·7 (4)	—	22·2 ± 0·7 (4)
Weight of adrenals (mg/100 g body weight)	25 ± 2 (10)	—	20 ± 1* (10)
Indices of thyrotrophin levels			
Cells/colloid vesicles ratio in thyroid	68 ± 1 (10)	—	69 ± 3 (10)
Serum organic iodine (μg/100 ml)	1·7 ± 0·2 (12)	—	2·1 ± 0·2 (10)
Indices of gonadotrophin levels			
Urinary oestrogen (μg/24-hr sample)	0·103 ± 0·014 (11)	—	0·109 ± 0·008 (11)
Weight of ovaries (mg/100 g body weight)	44 ± 3 (14)	—	41 ± 3 (14)
Uterus (mg/100 g body weight)	100 ± 10 (14)	—	110 ± 10 (14)
No. of rats with ovarian follicular cysts	4/69 (6%)	5/25 (20%)*	8/20 (40%)**

†Apart from the final entry in each column, values are expressed as the mean ± SEM for the number of determinations indicated in parentheses. The incidence of follicular cysts is expressed as the no. of rats affected/no. examined, with the percentage in parentheses.
Values marked with asterisks differ significantly from the control values: *$P < 0.05$; **$P < 0.01$.

length and body weight that occurred in rats of groups L1 and L2. The increased proportion of eosinophilic cells in the pars anterior which was observed in rats of group L2 was another index of increased growth-hormone or prolactin activity.

There was no significant alteration in the proportion of PAS-positive basophilic cells in the pars anterior and no other evidence to suggest that gonadotrophin activity, thyrotrophin activity or ACTH activity had been altered by the administration of linoleate hydroperoxide to rats. Normal levels for adrenal and serum 11-hydroxycorticosteroids suggested that ACTH activity had remained within normal limits, and the observed decrease in the weight of the adrenal glands relative to body weight could not be ascribed to reduced synthesis of 11-hydroxycorticosteroids. Normal values for the proportion of cells/colloid vesicles in the thyroid gland and for the levels of serum organic iodine suggested that thyrotrophin activity was unaltered by the treatment. Similarly, normal values for the weight of the ovaries and uterus and for the levels of urinary oestrogen indicated that gonadotrophin activity had not been significantly affected by the administration of linoleate hydroperoxide. The observed increase in the proportion of animals with ovarian cysts following linoleate hydroperoxide administration may have been consequent on changes in organs other than the pituitary.

Pituitary-hormone activity in rats with pituitary chromophobe adenomas

Changes in the mammary gland were seen in all the treated rats in which chromophobe adenomas of the pituitary occurred. Mammary fibroadenomas were present in half of these animals and the rest showed secreting fibro-epithelial hyperplasia of the mammary gland. Treated rats found to have these pituitary tumours did not differ significantly from the rest in the group in their body length, relative weight of adrenals or ovaries, urinary oestrogen levels or serum organic iodine levels. The occurrence of pituitary tumours was not related to the occurrence of cystic ovaries.

Pituitary-hormone activity in rats with mammary tumours

In the groups of rats given linoleate hydroperoxide, those bearing mammary tumours did not differ significantly from the rest of the treated rats in their body length, relative weight of adrenals or ovaries, urinary oestrogen levels or serum organic iodine levels. There was no association between the occurrence of mammary tumours and the occurrence of cystic ovaries in these animals.

DISCUSSION

Linoleic acid hydroperoxide is a toxic compound which has been shown to inhibit cell division (Franz & Cole, 1962; Stillwell, Maroney & Wilbur, 1959), to be mutagenic to Neurospora and Aspergillus (Dickey, Cleland & Lotz, 1949; Van Arkel, 1958) and to produce epidermal hyperplasia in the skin of the newt, a reaction suggestive of carcinogenic activity (Glavind & Arffmann, 1970). In a previous communication (Cutler & Schneider, 1973), we reported that the feeding of oxidized linoleic acid to rats resulted in an increase in the incidence of mammary adenocarcinomas and cervical sarcomas in the females and an increase in interstitial-cell tumours of the testis in the males. Administration of a similar diet to mice resulted in an increase in the incidence of benign ovarian tumours and of other benign tumours in the females. Of the numerous oxidation products to be found in the oxidized linoleic acid used in these experiments, linoleic acid hydroperoxide was present in the highest concentration.

In the experiments described in this paper, purified linoleic acid hydroperoxide given by repeated sc injection, has been found to increase the incidence of mammary tumours, pituitary tumours and lymphosarcomas in female rats. No tumours occurred at the site of injection. The incidence of total mammary tumours and total pituitary tumours showed a progressive increase when the total dose of linoleate hydroperoxide was raised from 200 to 353 mg. However, an increase in benign rather than malignant tumours occurred with this increase in the dose of linoleate hydroperoxide.

The observed increase in tumours following administration of linoleate hydroperoxide may have been brought about indirectly through effects on the endocrine glands, since the average weight of the pituitary gland and the proportion of rats with cystic ovaries increased as the total dose of linoleate hydroperoxide was raised. The increase in weight of the pituitary gland was accompanied by a small but statistically significant increase in the body length and body weight of the treated animals, and in the proportion of eosinophilic cells in the pars anterior of the pituitary, an increase suggesting increased mammosomatotrophic activity. There were no signs to indicate any increased ACTH, thyrotrophin or gonadotrophin activity.

Chronic endocrine imbalance in experimental animals, whether brought about by prolonged oestrogen administration, by neonatal gonadectomy, by thyroidectomy, by chronic iodine deficiency or by growth-hormone administration, has been shown to result in tumours of the pituitary gland and other endocrine organs (Furth & Clifton, 1966; Reid, 1954). For example, mammosomatotrophic pituitary tumours and mammary tumours occur after oestrogen administration and thyrotrophic pituitary tumours are seen after thyroidectomy. After gonadectomy in mice of some strains, basophilic pituitary tumours, mammary tumours and oestrogen-secreting adrenocortical tumours occur.

The combination of increased eosinophilic cells in the pars anterior of the pituitary, increased body length, an increased incidence of mammary tumours and an increase of cystic ovaries, which was seen in the group of rats given linoleate hydroperoxide, parallels the changes occurring in some hypothalamic disturbances. For example, lesions in specific areas of the rat's hypothalamus lead to pseudopregnancy, cystic ovaries and concomitant elevation of serum levels of prolactin (Boot, 1970). Cystic ovaries and increased serum levels or prolactin are also seen in rats after administration of drugs that decrease hypothalamic catecholamine levels (Cotchin & Roe, 1967; Wolstenholme & Knight, 1972). These drugs include reserpine, chlopromazine and methyldopa. Compounds that increase the levels of hypothalamic catecholamines, such as L-dopa, iproniazid and pargyline, significantly decrease the levels of serum prolactin in the rat. In several species, prolactin seems to play a part in hormonal mammary carcinogenesis (Boot, 1970), and in the present experiments it may have been involved in the genesis of the mammary tumours occurring after administration of linoleate hydroperoxide.

The majority of the pituitary tumours occurring in rats given linoleate hydroperoxide were chromophobe adenomas, and hormonal changes were confined to secreting fibroepithelial hyperplasia of the mammary gland and mammary tumours. Such changes in the mammary gland occurred in all of the rats with pituitary tumours. Chromophobe adenomas of the pituitary are common in old rats (Kim, Clifton & Furth, 1960) and these show no adrenotrophic, gonadotrophic or thyrotrophic effects, but do show mammosomatotrophic changes, which are particularly marked in animals given transplants of these tumours. These pituitary tumours may be found in association with ovarian abnormalities and spontaneous mammary tumours (Cotchin & Roe, 1967). By use of a tetrachrome stain-

ing technique, it has been shown that the proliferating cells in spontaneous chromophobe adenomas of the pituitary of the rat are those linked with prolactin secretion (Ascheim & Pasteels, 1963), and it has been postulated that spontaneous hypothalamic failure may be the basic cause for release of these cells from their normal physiological inhibition.

Thus, the mammary and pituitary tumours appearing late in the lifespan of female rats after injections of linoleate hydroperoxide were probably due not to direct carcinogenic effects of the metabolites of the administered compound, but to a hypothalamic disturbance leading to mammosomatotrophic effects and increased prolactin secretion.

No sarcomas of the cervix occurred after injections of linoleate hydroperoxide. It is probable that the increased incidence of these tumours that occurred after the feeding of oxidized linoleic acid to rats may have been due to components other than the hydroperoxide present in the oxidation mixture.

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Tumeurs et changements hormonaux produits chez les rats par des injections sous cutanées d'hydropéroxyde d'acide linoléique

Résumé—On donne de l'hydropéroxyde de linoléate par injections se répétées à deux groupes de rats. Ceux du premier groupe reçoivent une dose totale de 200 mg et ceux du second une dose totale de 353 mg. Aucune tumeur ne survient à l'endroit de l'injection chez les rats traités, mais la fréquence des tumeurs mammaires malignes et des lymphosarcomes augmente chez les rats du premier groupe et celle des tumeurs mammaires et pituitaires bénignes augmente dans le second groupe. Ces tumeurs apparaissent tardivement dans le cours de la vie des animaux.

La fréquence totale des tumeurs mammaires et pituitaires augmente en même temps que la dose d'hydropéroxyde de linoléate mais cette augmentation est due plutôt à un plus grand nombre de tumeurs bénignes que de tumeurs malignes. L'administration d'hydropéroxyde de linoléate est aussi suivie d'une hypertrophie de la glande pituitaire avec des augmentations du nombre des cellules éosinophiles de l'hypophyse antérieure, du poids du corps et de sa longeur ainsi que de l'augmentation du nombre de rates porteuses de kystes folliculaires dans leurs ovaires. On suggère que l'augmentation des tumeurs pituitaires et mammaires est une conséquence d'un trouble hypothalomique induit, conduisant à des effets somatotrophiques de la glande mammaire.

Tumore und hormonale Veränderungen hervorgerufen in Ratten durch subkutane Injektion von Linolsäurehydroperoxid

Zusammenfassung—Linolsäurehydroperoxid wurde durch wiederholte sc Injektion an zwei Gruppen von Ratten verabreicht. Die erste Gruppe erhielt eine Gesamtdosis von 200 mg, die zweite eine von 353 mg. In den behandelten Ratten traten keine Tumore an der Injektionsstelle auf; aber das Auftreten von malignen Brusttumoren und von Lymphosarkomen war erhöht bei den Ratten der ersten Gruppe, und das Auftreten von benignen Brusttumoren und benignen Hypophysentumoren war erhöht in der zweiten Gruppe. Diese Tumore traten spät in der Lebenszeit der Tiere auf.

Das Auftreten von beiden Brusttumoren und beiden Hypophysentumoren stieg an, als die Dosis des Linolsäurehydroperoxids erhöht wurde; aber dieser Anstieg war auf eine höhere Zahl von benignen als von malignen Tumoren zurückzuführen. Der Gabe von Linolsäurehydroperoxid folgte auch eine Vergrößerung der Hypophyse mit Erhöhung der Proportion von eosinophilen Zellen im Pars anterior, der Körpergewichtszunahme und Körperlänge und der Proportion von Ratten mit follikulären Zysten in ihren Ovarien. Es wird darauf verwiesen, daß die Hypophysen- und Brusttumore auftraten als Folge einer induzierten Hypothalamusstörung, die zu mammosomatotropischen Effekten führte.

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ORAL TOXICITY OF TEBUTHIURON (1-(5-*tert*-BUTYL-1,3,4-THIADIAZOL-2-YL)-1,3-DIMETHYLUREA) IN EXPERIMENTAL ANIMALS

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Abstract—The toxicity of tebuthiuron (1-(5-*tert*-butyl-1,3,4-thiadiazol-2-yl)-1,3-dimethylurea) has been studied in several animal species. The acute oral LD_{50}s in mice, rats and rabbits were 579, 644 and 286 mg/kg, respectively. In cats, oral doses of 200 mg/kg were not lethal, while 500 mg/kg given orally did not kill dogs, quail, ducks or chickens. The acute TL_{50} in fish was >160 ppm.

A 3-month study in rats fed diets containing 0, 400, 1000 or 2500 ppm tebuthiuron resulted in moderate growth retardation and a reduction in the efficiency of food utilization in the highest dose group. These changes were evident by wk 1 of the study. In the same group, a non-inflammatory diffuse vacuolization of the pancreatic acinar cells was found. No other important evidence of toxicity occurred. A 3-month study in dogs given daily oral doses of 0, 12·5, 25 or 50 mg tebuthiuron/kg resulted in slight anorexia in all the treated dogs, and a slight body-weight loss in dogs of the highest dose group. There were no other treatment-related signs of toxicity. A 1-month study in chickens fed rations containing 0, 400, 1000 or 2500 ppm tebuthiuron produced, in the highest treatment group, a reduction in food intake and suppression of body-weight gain but no other signs of toxicity. A teratology study produced no significant effects in the offspring of rats fed diets containing 0, 600, 1200 or 1800 ppm tebuthiuron.

Skin and eye irritation studies in rabbits and a contact sensitization study in guinea-pigs revealed no remarkable evidence of toxicity.

These studies indicate that tebuthiuron has a low order of toxicity in animals.

INTRODUCTION

Substituted urea compounds are useful herbicides with a low order of toxicity in animals (Hodge, Downs, Panner, Smith, Maynard, Clayton & Rhodes, 1967; Hodge, Downs, Smith, Maynard, Clayton & Pease, 1968; Hodge, Maynard, Downs & Coye, 1958; Zak & Sachsse, 1971). Tebuthiuron (1-(5-*tert*-butyl-1,3,4-thiadiazol-2-yl)-1,3-dimethylurea), a new pre- and post-emergent broad-spectrum herbicide is effective for total vegetation control at application rates of 2–8 lb/acre. This compound is stable when applied to soil, with minimal leaching or lateral movement.

EXPERIMENTAL

Material. Tebuthiuron used in the following studies was of technical grade (purity >97%). This is a colourless solid (m.p. 162–165°C) and is stable to light and non-volatile. Chemical analysis involves chloroform–methanol extraction, reaction with trifluoroacetic anhydride and measurement of the volatile derivative by flame-ionization gas chromatography.

Experimental design and conduct

Acute oral toxicity. Animals used in the acute oral toxicity studies were weanling ICR mice and Wistar-derived rats, young mature New Zealand white rabbits, cats, beagle dogs,

bobwhite quail, mallard ducks and White Rock chickens. Tebuthiuron was administered in single oral logarithmic doses to groups of fasted test animals of both sexes. Goldfish and fathead minnows were exposed to the test compound in aquaria. The animals were carefully observed for several days for signs of toxicity. The LD_{50} values were calculated by the method of Bliss (1938) for mice, rats, and rabbits, and median tolerance limit (TL_{50}) values (aqueous concentrations of the test compound in which 50% of the fish survived a 96-hr exposure) were determined for fish.

Feeding study in rats. Groups of ten male and ten female Wistar-derived rats (28–35 days old and weighing 74–156 g) were maintained on diets containing 0, 400, 1000 or 2500 ppm tebuthiuron for 3 months. The rats were housed in individual wire hanging cages with free access to food (Lilly mill ration) and water. Body weight and food consumption were measured weekly. Prior to autopsy blood was obtained from the orbital sinus of each rat for haematological evaluations and for determination of blood sugar, blood urea nitrogen (BUN) and serum glutamic–pyruvic transaminase (SGPT). At autopsy organs and tissues were carefully examined, liver, heart, kidneys, spleen, thyroids, adrenals and testes and prostate or ovaries and uterus were weighed and organ weight:body weight ratios were calculated. Sections from these organs as well as from the colon, duodenum, ileum, jejunum, lungs, lymph nodes, mammary glands, pancreas, salivary glands, stomach, skeletal muscle, thymus and urinary bladder were prepared and examined for histopathological changes.

Oral study in dogs. Groups of four beagle dogs, two of each sex, were given daily oral doses of 0, 12·5, 25 or 50 mg tebuthiuron/kg (approximately equivalent to dietary levels of 0, 500, 1000 and 2000 ppm) in gelatin capsules for 3 months. Their starting body weights were 7·4–12·2 kg and they ranged in age from 13 to 23 months. The dogs were caged individually and allowed free access to water. Commercial dog food (Wayne Tailwagger®) was offered daily for a 2-hr period. Ophthalmoscopic examinations were performed prior to and at the end of the study. Physical condition was assessed daily and body weights were recorded weekly. Haematological and clinical-chemistry values were determined from venous blood at intervals before and during the test period. The haematological parameters examined were haemoglobin, haematocrit, erythrocyte count, total and differential leucocyte counts, reticulocyte count, clotting time, platelet count, sedimentation rate and prothrombin time. The blood chemistry values were determined using a Technicon SMA 12/60, and included calcium, inorganic phosphorus, glucose, BUN, cholesterol, total protein, albumin, total bilirubin, alkaline phosphatase, lactic dehydrogenase (LDH) and serum glutamic–oxalacetic transaminase (SGOT). Urine was collected at the same intervals for determination of pH, specific gravity, protein, sugar and occult blood.

At autopsy organs and tissues were carefully examined, liver, kidney, heart, spleen, gonads, adrenals and thyroids were weighed and the organ weight:body weight ratios were calculated. Sections from these organs as well as from brain, bone marrow, colon, duodenum, gall bladder, ileum, jejunum, lungs, lymph nodes, mammary glands, pancreas, pituitary, salivary glands, stomach, skeletal muscle, thymus, urinary bladder and prostate or uterus were prepared and examined for histopathological changes.

Feeding study in chickens. Day-old White Cornish chickens were assigned to four groups of ten birds of each sex and fed rations (Lilly mill chick starter) containing 0, 400, 1000 or 2500 ppm tebuthiuron for 1 month. The eyes were examined before and periodically during the test period. Food consumption and body weights were measured regularly and the physical condition of the birds was assessed daily. At the termination of the

study the chickens were killed and body organs and tissues were examined for gross and histopathological changes.

Rat teratology. Groups of 25 adult Wistar-derived female rats weighing 245–454 g were mated and fed diets containing 0, 600, 1200 or 1800 ppm tebuthiuron on gestation days 6–15. Expulsion of the copulatory plug was considered to be gestation day 0. Body weight and food consumption were measured weekly. The dams were killed on gestation day 20.

Examinations were carried out on the ovaries to determine the numbers of corpora lutea, and on the uterus for the numbers and distribution of foetuses and resorptions. Resorptions were categorized as early or late, to indicate when intra-uterine mortality had occurred. Foetal sex, weight and viability were determined. Each foetus was examined for external abnormalities. Approximately one-third of the foetuses in each litter were fixed in Bouin's solution for visceral examination by the sectioning method of Wilson (1965) and the remainder were cleared for skeletal examination (Crary, 1962).

Mean group values were determined for various reproduction parameters. Mean foetal weight for each dietary level was calculated using the number of litters as the number of independent sampling units (Weil, 1970). The reproduction indices calculated were fertility (proportion of mated females pregnant), gestation (proportion of live foetuses), resorption (proportion of implantations that resulted in resorptions) and implantation (the ratio of the number of implantations to the number of corpora lutea).

Eye irritation, dermal toxicity and sensitization. A 71 mg (0·1 ml) quantity of tebuthiuron was instilled into one eye and conjunctival sac of six 2–3-month-old New Zealand albino rabbits. The untreated eye of each rabbit served as the control. The eyes were examined daily for 7 days for signs of irritation.

Under an occlusive rubber dam, 200 mg tebuthiuron/kg was applied to the shaved backs of three male and three female New Zealand albino rabbits, 2–3 months old. The skin of three of the rabbits was superficially abraded before application of the test material. All animals were restrained in stocks during the 24-hr exposure period. Daily examinations for evidence of dermal irritation or toxicity were conducted for 14 days.

Tebuthiuron was tested for sensitization in ten 2–3-month-old female albino guinea-pigs. Each test animal received topical applications of 0·1 ml of an ethanolic solution containing 2% tebuthiuron 3 times/wk for 3 wk. The solution was applied to shaved abraded skin and covered for 6 hr with occlusive rubber sheeting. A second group of ten female guinea-pigs was treated similarly with the ethanolic vehicle only. Ten days after the last of the nine treatments, a challenge application was made, followed by a second challenge 15 days after the first challenge. The test solutions, including the challenges, were applied to the same 4 cm^2 regions of the flank.

RESULTS

Acute oral toxicity

The results of acute oral toxicity studies with tebuthiuron in various species are given in Table 1. The sex of the animal did not influence the toxicity. The major signs of acute toxicity in rodents and rabbits were hypoactivity, anorexia, ataxia and death. Large oral doses of tebuthiuron induced an emetic response in cats and dogs. Chickens, quail and ducks given oral doses of 500 mg/kg showed no signs of toxicity, except for slight hypoactivity in chickens. The only signs of toxicity in fish were hypoactivity and death.

Table 1. *Acute oral toxicity of tebuthiuron*

Species	LD_{50}* (mg/kg)	TL_{50}† (ppm)	LD_0 (mg/kg)
Mouse	579 ± 11		
Rat	644 ± 27		
Rabbit	286 ± 30		
Cat			> 200
Dog			> 500
Quail			> 500
Duck			> 500
Chicken			> 500
Fish		> 160	

*Combined results from several tests expressed as the mean ± SEM.
†Median tolerance $limit_{50}$ of the test compound in the aquaria.

Feeding study in rats

Rats tolerated large daily dietary concentrations of tebuthiuron for 3 months without any clinical signs of toxicity or any mortality. A moderate reduction in rate of body-weight gain (Fig. 1) and a decrease in efficiency of food utilization in males and females in the highest dose group (2500 ppm) was evident from wk 1 of the study. Tebuthiuron had no clinically important effects on any of the haematological and clinical-chemistry values (Table 2). There were a few statistically significant differences in absolute (Table 3) and relative organ weights. However, most of these were in the animals given the highest dietary concentration and were considered to be related to the smaller body weights. There was no evidence of any treatment-related pathological changes in any of the organs weighed.

All rats receiving the diet containing 2500 ppm tebuthiuron showed a diffuse vacuolization of the pancreatic acinar cells. The degree of this change ranged from moderate to severe but the effect was not associated with necrosis nor with the presence of an inflammatory response. One rat given the intermediate dietary level showed very slight pancreatic

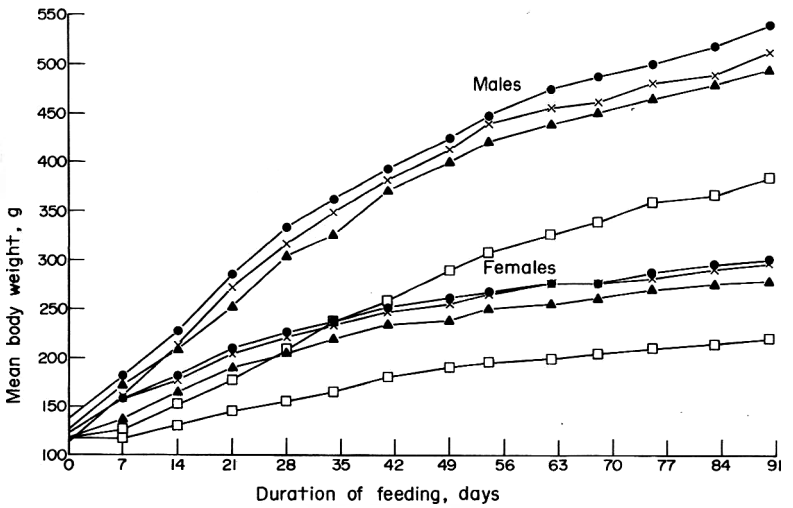
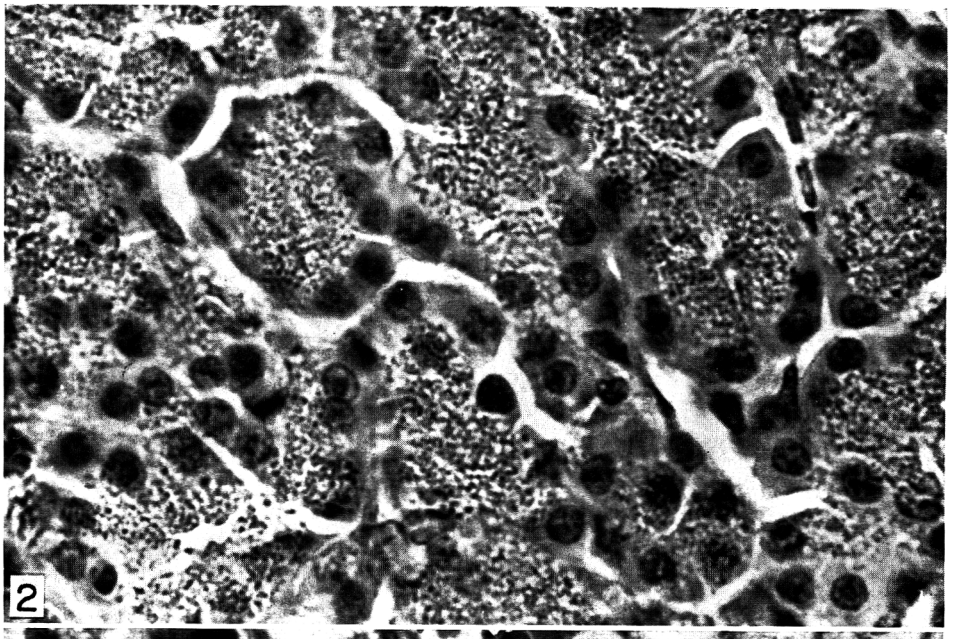


Fig. 1. Growth curves of rats fed diets containing tebuthiuron at levels of 0 (●), 400 (×), 1000 (▲) and 2500 (□) ppm, for 3 months.



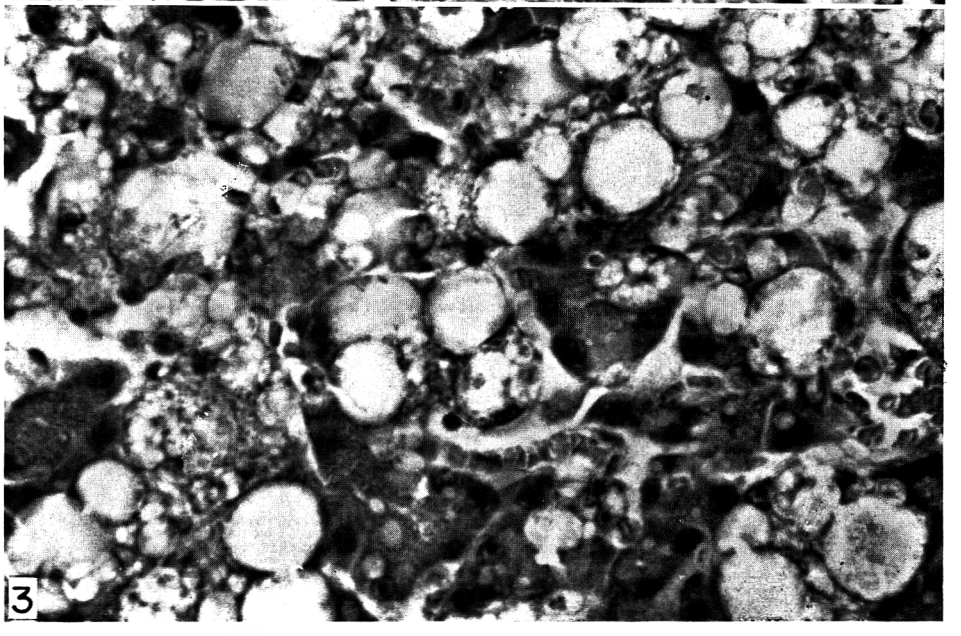


Fig. 2. Section of pancreatic acinar tissue from control rat, showing cytoplasm filled with zymogen granules. Haematoxylin and eosin ×900.

Fig. 3. Section of pancreatic acinar tissue from a rat fed 2500 ppm tebuthiuron for 3 months, showing vacuolated cytoplasm and a minimal number of zymogen granules. Haematoxylin and eosin ×900.

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changes. The vacuoles were variable in size and often contained amorphous material. There was a corresponding decrease in zymogen granules (Figs 2 & 3).

Oral study in dogs

The signs of toxicity were few, being limited to anorexia in all the treated dogs and very slight weight loss in two dogs on the highest dose (Table 4). All dogs survived the test period. There were no adverse treatment-related changes in the haematological findings (Fig. 4), clinical-chemistry values (Fig. 5), urine analyses, organ weights or gross and microscopic examinations of organs and tissues. There was no evidence of pancreatic vacuolization in any of the treated dogs.

Feeding study in chickens

Chickens tolerated the ingestion of 1000 ppm tebuthiuron in the diet for 1 month without any detectable signs of toxicity. The ingestion of food containing 2500 ppm of the test compound resulted in decreases in food consumption and body-weight gain (Table 5). All birds survived the test period, except one male on the lowest dietary level. There were no adverse gross or microscopic changes in organs or tissues.

Rat teratology study

The highest dietary level of tebuthiuron (1800 ppm) caused a slight reduction in maternal food consumption and body-weight gain during the first half of the treatment period. No deaths occurred during the study and there were no clinical signs of toxicity. The reproduction capacity of the animals was unaffected by tebuthiuron. Foetal and uterine parameters and reproduction indices were normal (Table 6). Foetal defects that occurred were independent of treatment (Table 7).

Eye irritation, dermal toxicity and sensitization studies

Tebuthiuron caused no irritation of the cornea or iris of rabbit eyes but there was a slight transient hyperaemia of the conjunctiva. All eyes were normal by the end of the 7-day test period. During the dermal toxicity study, one rabbit died following development of diarrhoea and emaciation. All surviving rabbits gained weight and were without signs

Table 2. *Mean haematological and clinical chemistry values of rats given tebuthiuron in the diet for 3 months*

Tebuthiuron (ppm)	Haematocrit (%)	Haemoglobin (g/100 ml)	RBC (10^6/mm^3)	WBC (10^3/mm^3)	Prothrombin (sec)	BUN (mg/100 ml)	SGPT (IU)	Glucose (mg/100 ml)
				Males				
0	43·4	15·6	8·25	9·38	15·6	12·6	19·2	96·4
400	45·2	16·1	8·45	15·26**	15·9	12·0	18·0	100·0
1000	44·7	16·2	8·30	11·88*	16·4	14·5	18·2	105·2
2500	44·1	16·8**	7·82	10·43	16·1	15·6**	20·2	103·2
				Females				
0	41·5	14·4	7·64	7·59	16·4	13·8	21·8	104·6
400	43·2	15·1	7·89	10·58*	16·4	12·8	20·4	106·0
1000	42·5	15·2	7·70	10·11	15·7	14·3	18·6	113·2
2500	42·8	15·1	6·90**	9·88	15·9	17·2**	18·8	111·8

RBC = Red blood cells WBC = White blood cells BUN = Blood urea nitrogen
SGPT = Serum glutamic–pyruvic transaminase

Values for haematocrit, haemoglobin, RBC and WBC are means for groups of ten animals and those for prothrombin, BUN, SGPT and glucose are means for groups of five animals. Those marked with asterisks differ significantly (Dunnett's t test) from control values: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$.

Table 3. *Body and absolute organ weights of rats given tebuthiuron in the diet for 3 months*

Dietary concn (ppm)	Body weight (g)	Organ weights									
		Liver (g)	Kidney (g)	Heart (g)	Spleen (g)	Thyroid (mg)	Adrenal (mg)	Prostate (g)	Testes (g)	Uterus (g)	Ovaries (mg)
Males											
0	538·1	13·63	3·21	1·53	0·83	23·50	66·30	1·21	3·86		
400	499·9	13·21	3·09	1·57	0·82	22·80	57·10	1·17	3·52		
1000	492·8*	13·65	3·20	1·48	0·78	27·40	64·40	1·31	3·73		
2500	382·6**	12·13	2·73*	1·16**	0·77	21·20	50·50*	1·17	3·74		
Females											
0	295·8	6·82	1·78	1·03	0·55	17·40	66·70			0·49	105·80
400	300·4	7·75	1·78	1·13	0·61	19·30	71·10			0·46	113·70
1000	278·4	7·47	1·66	0·97	0·60	19·70	69·60			0·53	107·90
2500	219·6**	6·65	1·51**	0·80*	0·46	16·00	64·50			0·44	87·60

Values are means for groups of ten animals, and those marked with asterisks differ significantly (Dunnett's *t* test) from control values: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$.

Table 4. *Body weights of dogs given oral doses of tebuthiuron for up to 3 months*

Dose level (mg/kg/day)	Dog no.	Body weight (kg) at day 0*	30	60	90
0	10185	9·3	9·4	9·8	10·2
	10127	12·2	11·9	12·6	12·4
	10192	11·5	11·7	12·4	12·5
	10437	11·1	11·1	11·2	11·6
12·5	10175	9·2	9·4	9·9	10·1
	10145	10·9	10·7	11·3	11·6
	10294	8·8	8·6	9·2	9·4
	10248	11·1	11·1	11·5	11·5
25	10561	9·5	9·5	9·8	9·9
	10146	10·1	10·2	10·6	10·6
	10855	7·4	7·6	7·8	8·3
	10802	10·0	10·0	10·4	10·5
50	10499	8·1	7·0	6·7	7·7
	10808	8·8	8·1	8·4	8·8
	10182	7·5	6·9	7·3	7·1
	10142	9·5	9·5	9·6	9·7

*Initial body weight.

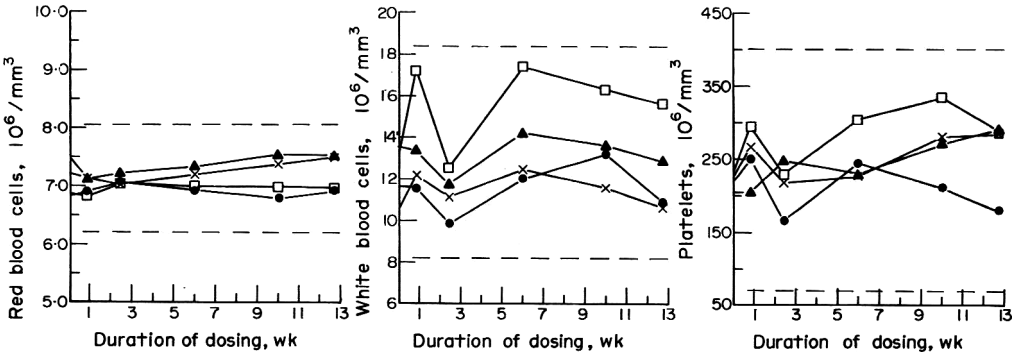

Fig. 4. Selected mean haematological values of dogs given tebuthiuron in oral doses of 0 (●), 12·5 (×), 25·0 (▲) and 50·0 (□) mg/kg for 3 months. Dotted lines denote the limits of normal ranges.

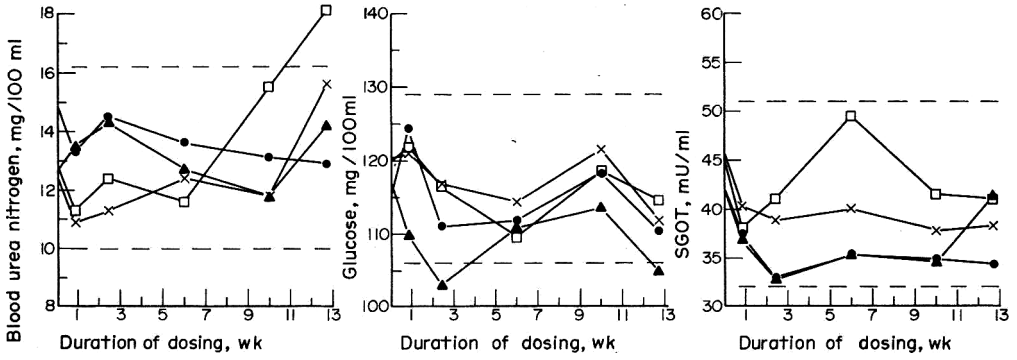

Fig. 5. Selected mean clinical-chemistry values of dogs given tebuthiuron in oral doses of 0 (●), 12·5 (×), 25·0 (▲) and 50·0 (□) mg/kg for 3 months. Although the BUN value is slightly outside the normal range (denoted in each case by dotted lines), it had no clinical significance.

Table 5. *Mean body weights of chickens fed diets containing tebuthiuron for 30 days*

Dietary concn (ppm)	Sex	Mean starting weight (g)	Mean terminal weight (g)
0	M	54·4	658
	F	66·0	581
400	M	55·9	594*
	F	58·8	606
1000	M	53·2	676
	F	54·3	568
2500	M	52·4	454
	F	60·0	510

*Mean for nine surviving chickens. Other values are means for groups of ten chickens.

Table 6. *Reproduction indices of rats fed diets containing tebuthiuron on days 6–15 of gestation*

Dietary concn (ppm)	Fertility index*	Gestation survival index†	Resorption index‡	Implantation index§
0	0·92	1·00	0·09	0·88
600	0·88	1·00	0·08	0·90
1200	0·92	1·00	0·08	0·85
1800	0·84	1·00	0·09	0·90

*No. pregnant/no. mated.
†No. of live foetuses/total foetuses.
‡No. of resorptions/total implantations.
§No. of implantations/no. of corpora lutea.

Table 7. *Summary of structural abnormalities in foetuses from rats fed diets containing tebuthiuron*

Type of abnormality	Incidence of abnormalities* in groups fed dietary levels (ppm) of 0	600	1200	1800	Cumulative control data†
Omphalocele	0/259	1/263	0/300	0/258	4/11,550
Hydronephrosis					
Bilateral	1/92	4/95	8/106	3/95	335/4662
Unilateral	1/92	0/95	2/106	1/95	239/4662
Wavy ribs	1/167	0/168	0/194	0/163	18/6888
Unilateral 14th rib	0/167	0/168	1/194	0/163	6/6888

*No. of affected foetuses/total no. examined.
†Historical data derived from the same strain of rat in this laboratory under conditions comparable with those of the present experiment.

of dermal irritation. Tebuthiuron induced no dermal or systemic responses indicative of contact sensitization in guinea-pigs.

DISCUSSION

The data obtained from these studies indicate that tebuthiuron has a low order of toxicity in animals. The acute oral LD_{50} for mice and rats is about 600 mg/kg. The acute oral LD_0 is >200 mg/kg for cats and >500 mg/kg for dogs, quail, ducks, and chickens. The TL_{50} for fish is >160 ppm.

Repeated oral ingestion of tebuthiuron for 3 months by rats and dogs and for 1 month by chickens resulted in minimal signs of toxicity. There was a decrease in food intake and body-weight gain in the highest dose groups. No important changes occurred in the haematological or clinical-chemistry values or organ weights. There were no pathological changes in dogs or chickens related to the ingestion of tebuthiuron. However, rats fed rations containing 2500 ppm tebuthiuron developed diffuse vacuolization of the pancreatic acinar cells. This pancreatic lesion may have resulted from interference with the production and secretion of digestive enzymes. The moderate reduction in body-weight gains, evident from wk 1 of the study, may be correlated with this pancreatic lesion. Pancreatic lesions resembling those produced by tebuthiuron have been produced in animals by omitting from the diet amino acids, e.g. lysine, and amino acid analogues, e.g. ethionine, β-3-thienylalanine (Herman & Fitzgerald, 1962; Hruban, Swift & Wissler, 1962; Scott, 1966) and by staphylococcal alpha toxins (Schoning, Anderson & Westfall, 1972), caerulein (Tardini, Anversa, Bordi, Bertaccini & Impicciatore, 1971), actinomycin D (Marsh & Fitzgerald, 1971) and puromycin (Longnecker, Shinozuka & Farber, 1968).

Tebuthiuron was not teratogenic in rats. It did not produce any significant or unusual signs of eye or skin irritation or contact skin sensitization.

Hodge *et al.* (1958, 1967 & 1968) and Zak & Sachsse (1971) have reported the animal toxicity of other urea herbicides, namely monuron, diuron, linuron and chlortoluron. These compounds are toxic to plants; but they have a relatively low order of toxicity in animals, manifested by depression of body-weight gain and anaemia. Tebuthiuron produces a depression of body-weight gain and, in rats, a vacuolar pancreatic lesion, but no evidence of anaemia.

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Toxicité orale du tébuthiuron (1-(5-*tert*-butyl-1,3,4-thiadiazol-2-yl)-1,3-diméthylurée) chez les animaux expérimentaux

Résumé—On a étudié la toxicité du tébuthiuron (1-(5-*tert*-butyl-1,3,4-thiadiazol-2-yl)-1,3-diméthylurée) chez plusiers espèces animals. Les DL_{50} orales aiguës chez les souris, les rats et les lapins sont respectivement de 579, 644 et 286 mg/kg. Chez les chats, les doses orales de 200 mg/kg ne sont pas léthales, de même 500 mg/kg donnés oralement ne tuent pas les chiens, les cailles, les canards ou les poulets. La DL_{50} aiguë chez le poisson est supérieure à 160 ppm.

Une étude pendant 3 mois chez les rats dont la nourriture contient 0, 400, 1000 ou 2500 ppm de tébuthiuron montre un retard modéré dans la croissance et une réduction de l'efficacité de l'utilisation digestive chez les groupes qui ont reçu la plus haute dose. Ces changements sont manifestés au bout d'une semaine d'étude. Dans le même groupe on trouve une vacualisation diffuse non inflammatoire des cellules acinaires pancréatiques. Aucune autre manifestation importante de toxicité n'a lieu. Une étude pendant 3 mois chez des chiens auxquels on a donné des doses orales de 0, 12,5, 25 ou 50 mg de tébuthiuron/kg montre une légère anorexie chez tous les chiens traités et une légère perte de poids chez les chiens du groupe recevant les plus hautes doses. Il n'y a aucun autre signe de toxicité dû au traitement. Une étude d'un mois chez les poulets nourris avec des rations contenant 0, 400, 1000 ou 2500 ppm de tébuthiuron montre, dans le groupe dont le traitement est le plus fort, une réduction de la prise de nourriture et une suppression de gain de poids mais aucun autre signe de toxicité. Une étude tératologique ne montre aucun effet significatif dans la descendance des rats nourris avec une alimentation contenant 0, 600, 1200 ou 1800 ppm de tébuthiuron.

L'irritation de la peau et de l'oeil étudiée chez les lapins et l'étude de la sensibilité de contact chez le cobaye ne révèlent aucun signe remarquable de toxicité.

Ces études indiquent que le tébuthiuron a une toxicité faible chez les animaux.

Orale Toxizität von Tebuthiuron (1-(5-*tert*-Butyl-1,3,4-thiadiazol-2-yl)-1,3-dimethylurea) in Versuchstieren

Zusammenfassung—Die Toxizität von Tebuthiuron [1-(5-*tert*-Butyl-1,3,4-thiadiazol-2-yl)-1,3-dimethylurea] wurde in verschiedenen Tierarten studiert. Die akuten oralen LD_{50} in Mäusen, Ratten und Kaninchen waren 579, 644 und 286 mg/kg respektiv. In Katzen waren orale Dosen von 200 mg/kg nicht lethal, während 500 mg/kg, oral gegeben, weder Hunde noch Wachteln, Enten oder Hühner tötete. Die akute TL_{50} in Fischen war >160 ppm.

Eine 3-monatige Studie an Ratten, mit Nahrung gefüttert, die 0, 400, 1000 oder 2500 ppm Tebuthiuron enthielt, resultierte in einer mäßigen Wachstumsverzögerung und einer Verringerung der Wirksamkeit der Nahrungsnutzung in der höchsten Dosisgruppe. Diese Veränderungen waren offenbar in der Woche 1 der Studie. In derselben Gruppe wurde eine nichtinflammatorische diffuse Vakuolisation der Pankreas-Azinarzellen gefunden. Keine anderen wichtigen Beweise von Toxizität traten auf. Eine 3-monatige Studie an Hunden, denen tägliche Dosen von 0, 12,5, 25 oder 50 mg Tebuthiuron/kg gegeben wurden, resultierte in einer leichten Anorexie in allen behandelten Hunden und einem leichten Körpergewichtsverlust bei den Hunden der höchsten Dosisgruppe. Es gab keine anderen Zeichen von Toxizität im Zusammenhang mit der Behandlung. Eine einmonatige Studie an Hühnern, mit Rationen gefüttert, die 0, 400, 1000 oder 2500 ppm Tebuthiuron enthielten, führte in der höchsten Dosisgruppe zu einer Reduktion der Nahrungsaufnahme und Unterdrückung von Körpergewichtszunahme aber zu keinen anderen Zeichen von Toxizität. Eine teratologische Studie bewies keinen signifikanten Effekt auf die Abkömmlinge von Ratten, die mit 0, 600, 1200 oder 1800 ppm Tebuthiuron enthaltender Nahrung gefüttert wurden.

Haut- und Augenreizungsstudien an Kaninchen und eine Kontaktsensitisationsstudie an Meerschweinchen enthüllten keine auffallenden Beweise von Toxizität.

Diese Studien deuten darauf hin, daß Tebuthiuron einen niedrigen Toxizitätsrang in Tieren hat.

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TERATOGENICITY AND DOMINANT LETHAL STUDIES ON HEXACHLOROBENZENE IN RATS

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Abstract—Teratogenicity studies were carried out in rats given single daily oral doses of 0, 10, 20, 40, 60, 80, or 120 mg hexachlorobenzene/kg during days 6–9, 10–13, 6–16 or 6–21 of gestation. The 80 and 120 mg/kg doses caused maternal neurotoxicity and a reduction in foetal weight. In the foetuses, the incidence of uni- and bilateral 14th rib was significantly increased above control values when the fungicide was administered from days 10–13, 6–16 or 6–21 of gestation. This incidence was related to the duration of treatment and the dose. Sternal defects in the foetus were observed following dosing during 6–21 days gestation. Values for live and dead foetuses, resorption sites, foetal weight and visceral and other skeletal anomalies were within control limits. For the dominant lethal test, four groups of 15 male rats were dosed orally with 0, 20, 40 or 60 mg/kg for ten consecutive days. Fourteen sequential mating trials, in each of which a male was caged with two virgin females for 5 days, were conducted. There were no significant differences between the test and control groups with regard to the incidence of pregnancies, corpora lutea, live implants or deciduomas.

INTRODUCTION

Hexachlorobenzene (HCB; C_6Cl_6) has been widely used as a fungicide on cereal grains (barley, oats, wheat and rye). HCB was associated with large-scale human poisoning in Turkey during the years 1955–1959 (Cam & Nigogosyan, 1963). Its distribution has been detected over extensive areas in the Rhine and its estuary (Koeman, Ten Noever de Brauw & de Vos, 1969). The presence of HCB was also confirmed in birds, fish, mussels and animal tissues (Koeman *et al.* 1969) and in human milk and body fat (Acker & Schulte, 1970; Brady & Siyali, 1972; Neuhaus, Brady, Siyali & Wallis, 1973). Morley, Geary & Harben (1973) reported a case of porphyria in a worker occupationally exposed to HCB, which has been detected in the blood of pest-control operators (Siyali & Simson, 1973) and in 97% of 237 random persons examined (Siyali, 1972).

The studies on teratogenicity and dominant lethality reported here are a part of the HCB toxicity screening programme being carried out at the Health Protection Branch, Food Research Laboratories. An earlier report dealt with adverse effects on postnatal survival and the incidence of pregnancy in rats fed diets containing HCB (Somers, Grant & Phillips, 1973).

EXPERIMENTAL

Teratogenicity studies. Nulliparous Wistar rats (200–250 g body weight) were paired overnight with males, and the morning that a sperm-positive vaginal smear was observed was considered to be day 1 of pregnancy. The mated females were randomly assigned to experimental groups. HCB of analytical standard (British Drug Houses, Poole, Dorset)

was administered orally in single daily doses. The doses (in mg/kg with percentage concentrations in parentheses) were 10 (0·2), 20 (0·4), 40 (0·8), 60 (1·2), 80 (0·8) or 120 (1·2). Suspensions were prepared in corn oil (experiments I and II) or in 0·25% aqueous gum tragacanth (experiments III and IV). Controls were given the maximum quantity of vehicle used in the experiment. The dose range and duration of treatment during gestation in the four experiments were: 40–120 mg/kg given on days 6–21 of pregnancy in experiment I, 10–60 mg/kg on days 6–16 of pregnancy in experiment II, 10–80 mg/kg on days 6–9 of pregnancy in experiment III and 10–80 mg/kg on days 10–13 of pregnancy in experiment IV.

Females were weighed on gestation days 1 and 6–15, as well as before and after Caesarean section. Pregnant females were weighed to permit correction of dose with changing weight and to assess maternal toxicity. Females were killed on day 22 of gestation and their viscera including uteri were examined for pathological changes. The foetuses were removed, weighed and examined for viability and external malformations. Early resorptions or deciduomas (Bateman & Epstein, 1971) and foetuses dying late in development were recorded as dead foetuses. Two-thirds of the live foetuses from each litter of experiments I and II and all foetuses except two per litter from experiments III and IV were studied for skeletal anomalies. The pairs of foetuses not included were used in another study not reported here. Extra ribs occurring in rudimentary or well-developed forms (in the cervical or lumbar regions) were designated '14th rib'. Sternal defects were evaluated according to the previously reported criteria (Khera & McKinley, 1972). The remaining foetuses from experiments I and II were fixed in Bouin's fluid and were cut sagittally or transversely into 3–4 mm sections for gross visceral inspection. Three foetuses from each litter of the 80 and 120 mg/kg groups of experiments I and II and their respective control were studied for microscopic changes. For microscopy, mid-sagittal sections from whole foetuses were stained with haematoxylin–phloxine–saffron.

Dominant lethal studies. The dominant lethal test was conducted on male rats randomly distributed into four groups each consisting of 15 males (Khera & Ruddick, 1973). The rats were dosed orally with 0 (0·25% aqueous gum tragacanth), 20, 40 and 60 mg HCB/kg for ten consecutive days after which 14 sequential mating trials were conducted. In each mating trial a treated male was caged with two untreated virgin females for 5 days. The females were killed 9 days after separation from the males, and viable embryos, resorption sites and corpora lutea were counted.

Statistical analysis. Normal distribution was considered as a basis for making intergroup comparisons within or between experiments. Probabilities were computed for values of χ^2, based on degrees of freedom following the methods of Rao (1965). Differences significant at a 5% probability level are reported.

RESULTS

Maternal toxicity associated with a reduction in foetal weight was caused by 80 or 120 mg HCB/kg given from day 6 to 21 of pregnancy in experiment I (Table 1). These effects were not observed with the lower doses in this experiment or with 80 mg HCB/kg or less given for a shorter period in the other experiments. Maternal toxicity was characterized by loss in body weight, hyperaesthesia, tremors and convulsions. The incidence of foetal mortality in all the HCB-treated groups was comparable to that in the control group.

A significant increase in the incidence of 14th rib (uni- and bilateral) in HCB-treated groups compared to that in the relevant control group was observed in experiments I, II

Table 1. *Effects on foetal development in pregnant rats treated with HCB in doses of 10–120 mg/kg for varying periods during gestation*

	Values for experiment																			
	I					II					III					IV				
Dose Parameter (mg/kg)*	0	40	60	80	120	0	10	20	40	60	0	10	20	40	80	0	10	20	40	80
No. of rats pregnant at term	16	12	15	12	13	15	15	16	16	17	7	7	9	8	7	9	10	8	10	9
Mean no. of live foetuses/pregnancy	11·7	12·3	12·0	11·8	12·6	10·4	10·6	11·6	11·5	11·0	13·4	11·7	11·0	13·0	11·7	12·5	12·7	13·8	10·9	11·7
Mean no. of dead and resorbed foetuses/ pregnancy	0·7	1·1	0·9	0·8	0·8	0·6	0·7	0·9	0·7	0·5	0·3	0·7	0·9	0·5	0·9	0·6	1·1	0·3	1·0	1·4
Mean foetal weight ± SD (g)	5·0 ± 0·7	4·7 ± 0·6	4·6 ± 0·7	4·4 ± 0·6	3·9 ± 0·6	5·0 ± 0·8	5·3 ± 0·5	5·0 ± 0·4	4·9 ± 0·4	4·9 ± 0·5	5·2 ± 0·3	5·1 ± 0·5	5·4 ± 0·5	4·6 ± 0·8	5·5 ± 0·2	5·3 ± 0·4	5·2 ± 0·5	5·1 ± 0·8	5·3 ± 0·7	4·9 ± 0·3
Skeletal anomalies:																				
No. found/total foetuses examined	11/125	43/127	55/123	51/84	51/119	5/80	5/79	12/91	19/87	25/96	2/80	8/67	7/89	13/86	8/67	11/92	11/106	16/93	8/90	13/85
Uni- and bilateral 14th rib	6	38	45	47	37	0	4	8	15	25	2	6	1	6	6	2	7	9	8	13
Wavy ribs	4	0	0	0	1	5	0	0	0	0	0	0	2	6	1	8	3	7	0	0
Sternal defects	3	10	18	18	43	0	1	5	5	0	0	2	1	5	0	3	1	0	0	0
Skull, retarded ossification	0	0	2	0	0	0	0	0	0	0	0	0	3	1	1	2	0	2	0	0

*Doses were given daily on days 6–21, 6–16, 6–9 and 10–13 of gestation in experiments I, II, III and IV respectively.

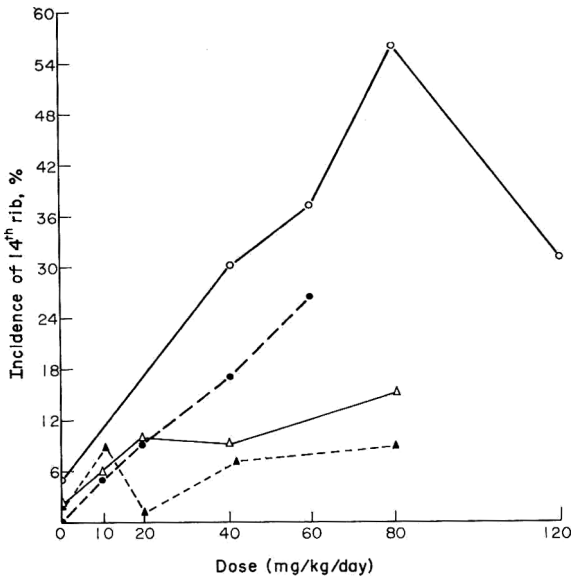

Fig. 1. Incidence of 14th rib (uni- and bilateral combined) related to the dose and length of treatment in experiments I (○), II (●), III (▲) and IV (△).

and IV. The incidence increased as a function of dose and length of treatment (Fig. 1). In experiment III, an apparent increase in frequency of the 14th rib was neither statistically significant nor dose-dependent.

Sternal defects consisting of asymmetrical apposition of sternebrae, hemisternebrae and retarded ossification were significantly more frequent in the test groups than in the control in experiment I and the incidence was dose-related. A low incidence of these defects was

Table 2. *Pregnancies and yield of viable and dead embryos in untreated females mated to HCB-treated male rats*

Mating trial no.	No. of viable embryos/pregnancy				No. of dead embryos/pregnancy				No. of pregnancies/ no. mated (%)			
Dose (mg/kg)*	0	20	40	60	0	20	40	60	0	20	40	60
1	12·5	11·2	13·4	12·3	1·2	0·2	0·5	0·3	21	20	14	25
2	11·5	12·0	11·7	11·9	0·5	0·5	0·6	0·4	46	37	46	38
3	13·1	10·7	12·5	12·6	0·7	0·6	0·4	0·8	39	43	54	46
4	12·9	10·7	10·0	12·3	0·1	0·8	0·4	0·5	50	50	46	56
5	12·6	12·2	11·4	13·2	0·9	0·7	0·6	0·1	50	40	50	52
6	12·1	12·3	11·0	12·8	0·6	0·5	0·4	0·4	50	53	50	57
7	13·5	11·7	12·0	12·8	0·5	0·2	0·8	0·5	50	60	61	46
8	12·3	11·6	12·9	11·1	0·8	1·3	0·3	0·8	64	60	56	46
9	11·3	12·1	12·8	11·1	0·6	0·6	0·1	0·8	57	57	57	46
10	12·0	12·8	9·9	11·6	0·7	0·3	1·0	0·8	79	73	64	71
11	12·8	12·5	12·4	11·9	0·1	0·5	0·4	0·6	79	80	86	64
12	12·4	11·7	12·9	10·8	0·7	0·4	1·0	0·3	68	83	79	64
13	12·7	12·4	12·2	12·5	0·5	0·4	0·4	0·5	86	67	71	68
14	12·8	13·1	13·4	13·1	0·5	0·1	0·7	0·2	79	80	79	71
Mean ...	12·4	12·0	12·0	12·1	0·6	0·5	0·5	0·5	58	57	58	53

*Male rats were dosed daily for ten consecutive days prior to the start of the mating trials.

observed in other experiments but the distribution analysis did not suggest any connexion with the HCB treatment. The incidence of wavy ribs and retarded ossification of skull bones was not related to the treatment.

There were no HCB-related effects in the external morphology. Visceral deformities were not observed and microscopic examinations did not reveal any treatment-related change in the histology of the foetuses.

The data from the dominant lethal assay are summarized in Table 2. Incidences of viable embryos, dead embryos and pregnancies in the test groups were all within the control range for the 14 sequential mating trials.

DISCUSSION

With HCB doses that induced no apparent maternal toxicity the only positive findings were increases in the incidence of 14th ribs and sternal defects. The sternal defects were observed only in experiment I, which was designed primarily to highlight the teratogenic effects of dose levels toxic to the dams, for confirmation during further studies. Since these defects were not reproducible in subsequent trials at doses of up to 80 mg/kg given during organogenesis, their significance in attributing a teratogenic potential to HCB is doubtful.

An extra rib is a frequent observation in teratogenicity studies. Yasuda & Maeda (1972) obtained a dose-related effect in the incidence of lumbar ribs during investigations on a phenoxy acid herbicide and a tetracycline derivative; at higher doses of these compounds malformations suggestive of teratogenic potential were apparent. Kimmel & Wilson (1973) have attempted to evaluate the significance of the 14th rib in a study involving acetazolamide, actinomycin D and sodium salicylate in rats. The 14th rib was classified according to its length as being a rudimentary or an extra rib; the rudimentary rib was found to have little, if any, association with other malformations, whereas an increase in extra rib was generally consistent with other deformities after actinomycin D and sodium salicylate treatment but not after acetazolamide treatment. The authors of both of these studies concluded that the extra rib could be regarded as an indicator of teratogenic potency. It was not possible in the present study to ascribe any predictive value to the 14th rib, since even at doses toxic to the dams, no clearly defined malformations were apparent. If the 14th rib effect were due to HCB treatment alone, a similar incidence at comparable doses in experiments I, II and IV would have resulted. This could be expected since the differentiation of ribs takes place during days 10–13 of gestation in the rat, a period which was included in the dosing schedule of these three experiments. However, the incidence of the 14th rib was related to the length of treatment; it was significantly higher in experiment I, in which the treatment was continued during and after the period of organogenesis, than in experiments II and IV, in which the treatment was restricted to the period of organogenesis. A possible explanation is that stress resulting from the combined effects of maternal asphyxiation during intubation and a low-grade sub-clinical HCB toxicity might be the underlying cause. This view is consistent with that of Green (1962), who considered that an increase in rudimentary or extra ribs suggested that the maternal animal had been stressed sufficiently to express the developmental instability inherent in the species.

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Tératogénicité et études de l'effet léthal dominant de l'hexachlorobenzène chez les rats

Résumé—Des études tératogéniques sont réalisées sur des rats auxquels on a donné une fois par jour des doses orales de 0, 10, 20, 40, 60, 80 ou 120 mg d'hexachlorobenzène/kg pendant les jours 6–9, 10–13, 6–16 or 6–21 de la gestation. Les doses de 80 et 120 mg/kg produisent une neurotoxicité chez les mètres et une réduction du poids des foetus. Chez les foetus, la fréquence d'apparition de la 14^{e} côte uni et bilatérale s'est significativement élevée au-dessus des valeurs témoins quand le fongicide avait été administré pendant les jours 10–13, 6–16 ou 6–21 de la gestation. Cette anomalie est en rapport avec la durée du traitement et avec la dose. On observe un défaut de sternum chez les foetus ayant reçu le traitement du 6^{e} au 21^{e} jour de la gestation. Les nombres de foetus morts et vivants, les sites de résorption, le poids des foetus et des viscères et les autres anomalies du squelette se trouvent dans les limites de la normale. Pour le test léthal dominant, on donne par voie orale à quatre groupes de 15 rats mâles des doses de 0, 20, 40 ou 60 mg/kg pendant 10 jours consécutifs. On a entrepris quatorze essais successifs d'accouplement dans chacun desquels un mâle est mis en cage avec deux femelles vierges pendant 5 jours. Il n'y a aucune différence significative entre les groupes d'essai et les groupes témoin quant à la fréquence des grossesses, des corps jaunes, des implants vivants ou avortés.

Teratogenizität und dominant-letale Studien von Hexachlorobenzol in Ratten

Zusammenfassung—Teratogenische Studien wurden in Ratten durchgeführt, denen tägliche orale Einzeldosen von 0, 10, 20, 40, 60, 80 oder 120 mg Hexachlorobenzol/kg gegeben wurden während der Tage 6–9, 10–13, 6–16 oder 6–21 der Gestation. Die 80 und 120 mg/kg Dosen verursachten Neurotoxizität in den Müttern und eine Reduktion des Fötalgewichtes. In den Föten war das Auftreten einer uni- und bilateralen 14. Rippe beträchtlich erhöht über die Kontrollwerte, wenn das Fungizid von den Tagen 10–13, 6–16 oder 6–21 der Gestation an gegeben wurde. Dieses Vorkommen war abhängig von der Dauer der Behandlung und der Dosis. Sternale Defekte in den Föten wurden nach der Dosierung während der Tage 6–21 der Gestation festgestellt. Werte für lebende und tote Föten, Resorptionsplätze, Fötalgewicht und viszerale und andere skelettäre Ano-

malitäten waren innerhalb der Kontrollgrenzen. Für das dominant-lethal-Test wurden 4 Gruppen von 15 männlichen Ratten während 10 aufeinander folgender Tage täglich mit 0, 20, 40 oder 60 mg/kg dosiert; 14 folgende Paarungsversuche, bei welchen in jedem von diesen ein Männchen mit zwei virginen Weibchen 5 Tage lang in einem Käfig war, wurden ausgeführt. Es gab keine signifikanten Unterschiede zwischen den Test- und den Kontrollgruppen in bezug auf das Auftreten von Trächtigkeit, Corpora lutea, lebenden Implanten oder Deziduomen.

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CITRININ MYCOTOXICOSIS IN BEAGLE DOGS

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Abstract—Three trials were completed in which young beagle dogs were either fed a citrinin-containing culture of *Penicillium citrinum* (Trial I) or were administered pure citrinin either ip (Trial II) or orally (Trial III). Dogs fed the fungal culture and those given 20 or 40 mg pure citrinin/kg body weight developed renal disease. Dogs given 2·5 and 5 mg/kg remained clinically normal and without renal lesions. Clinicopathological evidence of renal damage included a rise in blood urea nitrogen, glucosuria, proteinuria, lowered urinary specific gravity, increased urinary activities of lactic dehydrogenase, glutamic–oxalacetic transaminase and isocitric dehydrogenase and the presence of numerous necrotic renal tubular epithelial cells in the urinary sediment. Emesis and intestinal intussusception occurred in the dogs given 20 or 40 mg/kg ip. Renal lesions consisted of degeneration and necrosis of the tubular epithelium, most prominently in the thick segment of Henle and in the distal convoluted tubules.

INTRODUCTION

Citrinin is a metabolite of fungal species of the genera Penicillium and Aspergillus (Friis, Hasselager & Krogh, 1969; Heatherington & Raistrick, 1931; Jabbar & Rahim, 1962; Pollock, 1947; Timonin & Rowatt, 1944). Soon after its discovery, citrinin was tested as an antibiotic but was rejected because of its marked renal toxicity. In recent years, a citrinin-producing strain of *Penicillium viridicatum* has been implicated as a possible cause of a porcine nephropathy termed "mould nephrosis" in Denmark (Krogh, Hasselager & Friis, 1970).

The acute toxicity of citrinin administered by various routes was studied in rats, mice, guinea-pigs and rabbits (Ambrose & DeEds, 1945 & 1946). The 14-day LD_{50} (sc and ip) of citrinin for rats was 67 mg/kg body weight, while for mice it was 35 mg/kg. When administered sc, the 14-day LD_{50} for guinea-pigs was approximately 37 mg/kg. The iv LD_{50} for rabbits was 19 mg/kg.

When swine were given citrinin daily in oral doses of 20–40 mg/kg, signs of toxicity included growth depression, glucosuria, proteinuria and a rise in concentrations of blood urea nitrogen (BUN). The renal lesions included necrosis and desquamation of renal epithelial cells of the proximal convoluted tubules, dilatation of tubules, thickening of basement membranes and proliferation of cells in the interstitium (Krogh *et al.* 1970).

In dogs, citrinin was found to cause a transient decrease in blood pressure associated with dilatation of blood vessels of the skin, kidneys and extremities. Tachyphylaxis was evident (Ambrose & DeEds, 1946). The nephrotoxicity of citrinin does not appear to have been characterized in the dog. The present report describes the clinical, clinical-pathological and pathological features of citrinin mycotoxicosis in young male beagle dogs.

EXPERIMENTAL

Studies were completed in which dogs were fed a diet containing a fungal culture of *P. citrinum* containing 0·1 mg citrinin/g culture or were given chemically pure citrinin ip or orally in the form of capsules.

Preparation of fungal culture. The fungal culture used in this study was prepared by placing 280 g polished commercial rice and 128 ml water in a 2-litre flask and autoclaving at 12°C for 45 min. An isolate of *P. citrinum* (no. 647), known to produce citrinin, was grown on a potato-dextrose agar plate for sporulation. Flasks of sterilized rice were inoculated with spores of this culture and incubated at 23°C for 2 wk. The cultures were then treated with 125 ml chloroform and left in a hood overnight. After a 5-day drying period at 40·6°C the culture was ground for administration to beagle dogs. Cultures were checked for fungal contamination before drying and after grinding by inoculation on potato-dextrose agar containing 30 ppm chlortetracycline and 220 ppm of a non-ionic detergent. Contamination with other fungi was not found. The ground rice culture did not contain aflatoxin or ochratoxin A, but citrinin was quantitated by extraction and chromatographic procedures (see below). The ground rice culture was mixed with ground commercial dry dog food (Kasco Mealettes®, from Agway, Inc., Syracuse, N.Y.) and the mixture was fed *ad lib.*

Citrinin analysis of rice cultures. Samples of rice (50 g) were extracted five times with chloroform in a Waring blender (3 min). Chloroform extracts were combined, dried with anhydrous sodium sulphate, filtered through glass wool and evaporated to dryness under vacuum. The resulting material dissolved in hot absolute ethanol, filtered through glass wool and allowed to cool to room temperature. The solution was cooled to 0°C for 1 hr. The resulting yellow crystals were collected on pre-weighed filter paper discs and dried for 12 hr at 40°C. The concentration of citrinin was calculated as 0·1 mg/g rice culture. Ultraviolet absorption spectra and R_F values (thin-layer chromatography on Brinkmann pre-coated SIL N-HR plates) were compared with, and found to be identical to, standard citrinin (Saito, Enomoto & Tatsuno, 1971).

Preparation of pure citrinin. P. citrinum NRRL 5907 was grown as a stationary culture on 4 litres of a 4% sucrose and 2% yeast-extract medium in 5 gal carboys for 14 days at 28°C. Culture beers were filtered through a double layer of cheese cloth and adjusted to pH 1·0 with conc. HCl. The resulting precipitate was collected on Whatman no. 4 filter paper using vacuum filtration, dissolved in hot chloroform and filtered through glass wool. The chloroform was removed under vacuum. The resulting solid material was dissolved in hot absolute ethanol in a steam bath and filtered through glass wool. Hot ethanol solutions were allowed to cool to room temperature and then cooled to 0°C for 1 hr. Resulting yellow crystals were collected using vacuum filtration. Citrinin was recrystallized from ethanol three times. The ultraviolet absorption and nuclear magnetic resonance spectra and melting points of this material were identical to those of standard citrinin and published values (Saito *et al.* 1971).

Animals and test procedures

Trial I. Six 8–9-wk-old beagle dogs were purchased for this study from Laboratory Research Enterprises, Kalamazoo, Mich. They were acclimatized in pens in a room with environmental controls for 4 wk. After complete general physical examination the dogs were fed the culture-contaminated diet *ad lib.* Three dogs (group 1) were fed a diet containing 25% of the fungal culture for 16 days. Another three dogs (group 2) were fed a diet containing 50% of the fungal culture for 15 days, but because feed consumption by this group was so low, the dogs were given a diet containing 12·5% of the fungal culture on day 15 for an additional 17 days of feeding.

The general condition of the test dogs was evaluated twice daily. Prior to autopsy, blood was taken by puncture of a jugular vein for haematological and chemical analyses. These included the determination of packed cell volume (PCV) by the microhaematocrit method, total concentrations of plasma protein (TPP) by AO refractometer and blood urea nitrogen (BUN) by paper-chromatographic diagnostic strip assays (Urograph Chromatography Strips, Warner Chilcott, Morris Plains, N. J.). Urine was collected for routine analyses 1 day before autopsy, by manual compression of the urinary bladder through the abdominal wall. The dogs were killed by iv administration of barbiturates and exsanguination. At autopsy, representative portions of most organs were fixed in chilled buffered 10% neutral formalin. Fixed tissues were embedded in paraffin, sectioned at 6 μm and stained with haematoxylin and eosin for histopathological evaluation.

Trial II. Six 8-wk-old male beagles were purchased for this study. They were fed a semi-moist commercial dog food and were kept in metabolism cages in a building with environmental controls. Three dogs (nos 1–3; group 1) were to be administered citrinin in gelatin capsules in a daily dose of 20 mg/kg body weight and three (nos 4–6; group 2) were to receive a daily dose of 40 mg citrinin/kg. Because of the pronounced emetic effect of this treatment the route of administration was changed from day 2. Thereafter, the citrinin was administered in solution (1 N-NaOH adjusted with HCl to a pH of 7·0) by the ip route.

Clinical data collected included body weight, rectal temperature, urine output and water intake. The dogs were examined several times daily and a daily urine analysis included sediment evaluation. Twice during a 1-wk acclimatization period and on test days 1, 3 and 5, blood was collected by puncture of a jugular vein. At the same time, urine was collected and dialysed for enzyme determinations. Haematological parameters studied included, haemoglobin concentration (Hb), total leucocyte count (WBC) and TPP. Concentrations were also determined at the times indicated. Activities of the enzymes glutamic–oxalacetic transaminase (GOT), glutamic–pyruvic transaminase (GPT), isocitric dehydrogenase (ICDH) and lactic dehydrogenase (LDH) were determined in serum and in dialysed urine by kinetic ultraviolet procedures using enzyme test kits (Boehringer Mannheim Inc., New York), twice during the pretrial period and on test days 1, 3 and 5. Six young male beagles served as controls for these studies.

Two of the dogs given 40 mg citrinin/kg daily were found dead on day 3 and were autopsied and four dogs were killed when moribund and autopsied immediately. At autopsy, representative portions of most organs were fixed in buffered 10% neutral formalin. The fixed tissues were processed for paraffin sectioning and stained with haematoxylin and eosin for histopathological examination. Selected sections of liver and kidney were processed in potassium dichromate and stained with Sudan IV for evaluation of neutral lipid content. Sections of kidney were also stained by the trichrome method (Luna, 1968).

Trial III. Two groups each of three dogs were given citrinin for 6 wk, one group in a dose of 2·5 mg/kg body weight and the other in a dose of 5·0 mg/kg. Pure citrinin was triturated with corn sugar and the stock preparation (10% citrinin, w/w) was placed in gelatin capsules. The capsules were prepared twice weekly according to the weight of the test dogs.

The beagles (Laboratory Research Enterprises) were housed and fed as in Trials I and II and were examined twice during a 10-day acclimatization period for collection of baseline data. Blood was obtained by puncture of a jugular vein twice during the pretest period, on test days 1, 3 and 10 and weekly thereafter. Urine was collected for routine analysis and enzyme determinations at the same times. The activities of the enzymes GOT, LDH, and ICDH were determined in serum and dialysed urine by the methods used in Trial II. Concentrations of BUN were monitored with chromatographic test strips at the times given above. The PCV and total leucocyte count were also determined.

The dogs were killed at the end of the 6-wk test period by iv administration of barbiturates and exsanguination. They were autopsied immediately and representative portions of most organs were fixed in buffered 10% neutral formalin. Sections of kidney, ureter, urinary bladder and prostatic urethra from the six dogs were prepared for paraffin sectioning, cut at 5 nm and stained with haematoxylin and eosin. Sections of the kidneys were also stained by the trichrome method.

RESULTS

Trial I

Clinical observations. The dogs of both test groups consumed very little of the test diets. During the first 15 days of the trial the three dogs of group 1 consumed a total of 4 kg of the 25% fungal diet. By day 15 they were very thin and markedly dehydrated, although they collectively consumed several litres of water daily. The dogs in this group were autopsied on day 16 after blood and urine samples had been collected.

In the first 15 days of the trial the three dogs of group 2 consumed a total of 800 g of the 50% culture diet. They were thin but not dehydrated and were bright and alert. On day 15 the concentration of the rice culture in the diet was reduced from 50 to 12·5% and the new diet was offered *ad lib.* for an additional 17 days. During this 17-day feeding period, the dogs of group 2 consumed 12 kg of the 12·5% fungal diet. By day 30, they were somewhat dehydrated and were consuming increasing amounts of water. Their general condition was considered good. They were killed and autopsied on day 32. Rectal temperatures of the six dogs determined immediately before autopsy were between 100 and 102°F.

Clinicopathological observations. On day 15, the three dogs of group 1 had slightly increased concentrations of BUN, the values being 25, 40 and 50 mg/100 ml compared with 12–20 mg/100 ml in six control dogs. The values for PCV, TPP, WBC and differential leucocyte counts of the dogs from both test groups were generally within the normal range. Alterations in urine from the dogs in group 1 consisted of a decrease in specific gravity (as low as 1·004), the presence of glucose in excess of 0·5% and the presence of many necrotic renal epithelial cells in the sediment (Table 1).

Alterations in clinicopathological values for the dogs of group 2 on day 31 (Table 1) were similar to the changes already described for group 1. One of the group 2 dogs had a concentration of BUN in excess of 75 mg/100 ml but the actual level was not established.

Table 1. *Clinicopathological data for dogs fed citrinin-containing cultures of* P. citrinum (*Trial I*)

Parameter	Dog no. 72–...	Values for dogs of: Group 1 (on day 15) 410	411	412	Group 2 (on day 31) 465	466	467	Range for control dogs
Blood								
Packed cell volume (%)		38	44	42	35	33	34	32–38
Total plasma protein (mg/100 ml serum)		7·0	6·6	5·6	6·2	6·5	6·6	5·0–6·4
Blood urea nitrogen (mg/100 ml serum)		40	50	25	75+	45	33	12–20
Leucocyte count (cells/$mm^3 \times 10^3$)		10·0	12·1	9·9	15·0	10·4	9·1	8·0–15·0
Urine*								
Specific gravity		1·006	1·005	1·004	1·030	1·016	1·016	1·020–1·050
Glucose (%)		>0·5	0·3–0·5	0·3–0·5	0·5 +	0·3–0·5	0·5+	None
Protein		Trace	Trace	Nil	Nil	Trace	Trace	None
Sediment		Necrotic renal tubular epithelial cells, too numerous to count			As in group 1, with occasional granular casts			

*Tests for blood, bile, urobilinogen, ketones and pH showed no changes.

Pathological observations. At autopsy, gross alterations were limited to the kidneys, which were pale greyish-tan and slightly swollen. Histopathological changes were also limited to the kidneys and were characterized mainly by necrosis of the renal tubular epithelium and tubular dilatation (Fig. 1). Most of the affected tubules were located deep in the cortex and were principally the thick limbs of Henle's loop and collecting tubules (Fig. 2). The proximal tubules were generally spared. Increased affinity of the cytoplasm for the eosin stain, hydropic change, nuclear pyknosis, karyorrhexis, karyolysis, cytolysis and separation from the tubular basement membrane characterized the changes in the tubular epithelium (Fig. 3). Affected cells were swollen, protruded into the tubular lumen and had pale, finely granular cytoplasm in trichrome-stained sections. Mitosis was observed in some tubular epithelial cells (Fig. 4). Occasionally, groups of dilated necrotic tubules consisted only of basement membranes; other tubules contained variable numbers of necrotic tubular epithelial cells. A few tubules contained homogeneous eosinophilic material but very few granular casts were observed. Tubules with minimal or no histopathological alterations were frequently observed adjacent to necrotic ones. Tubular dilatation was most striking at the cortico-medullary junction but was observed in the cortex and medulla as well. Epithelial cells in the renal pelvis, ureters and urinary bladder were without lesions. Slight hypercellularity of Bowman's capsule was the only glomerular alteration observed. The renal alterations were present in all six dogs, but the changes were more severe and extensive in the kidneys of the dogs of group 1. Other organs examined and considered normal included the brain, spinal cord, sciatic nerve, eye, bone marrow, heart, aorta, trachea, oesophagus, lungs, liver, gall bladder skeletal muscle, tongue, skin, nictitating membrane, parotid salivary gland, pancreas, stomach, duodenum, jejunum, ileum, caecum, colon, rectum, anterior cervical lymph node, popliteal lymph node, mesenteric lymph node, tonsil, thymus, spleen, urinary bladder, ureter, adrenal gland, pituitary gland, thyroid gland, parathyroid gland, gonad, mammary gland, epididymus and prostate gland.

Trial II

Clinical observations. Clinical signs of toxicosis were observed in all six dogs and all were either found dead or were killed in a moribund state by day 5. The initial clinical sign was emesis, all six dogs vomiting within 30 min of receiving the first oral dose of citrinin. The dogs of group 1 received as the first dose 10 mg citrinin/kg and those of group 2 received 20 mg/kg as the capsules were prepared for separate administration of two half-doses each day. The second dose on the first day of dosing (day 0) was halved again in an effort to eliminate the emesis, but all dogs again vomited within 20 min of dosing (dogs of group 1 received 5 mg citrinin/kg and those of group 2 received 10 mg/kg at this dosing). A second effort was made to eliminate emesis by buffering the citrinin with sodium bicarbonate and by further division of the dose. Each capsule of citrinin prepared for administering the daily total in four separate doses contained 5 mg citrinin/kg for group 1 dogs and 10 mg/kg for group 2 dogs and was buffered with 200 mg $NaHCO_3$. All dogs again vomited within 30 min of dosing. Subsequent doses of citrinin were administered twice daily by the ip route.

By day 1, the dogs did not consume all the feed, although the amount offered was not changed from that offered and consumed in the pre-trial period. Anorexia was never complete in any dog, but feed consumption progressively decreased until termination.

The emetic activity of citrinin was not eliminated by ip administration, and absorption of the toxin appeared to be enhanced. The dogs regularly vomited within 3–10 min of

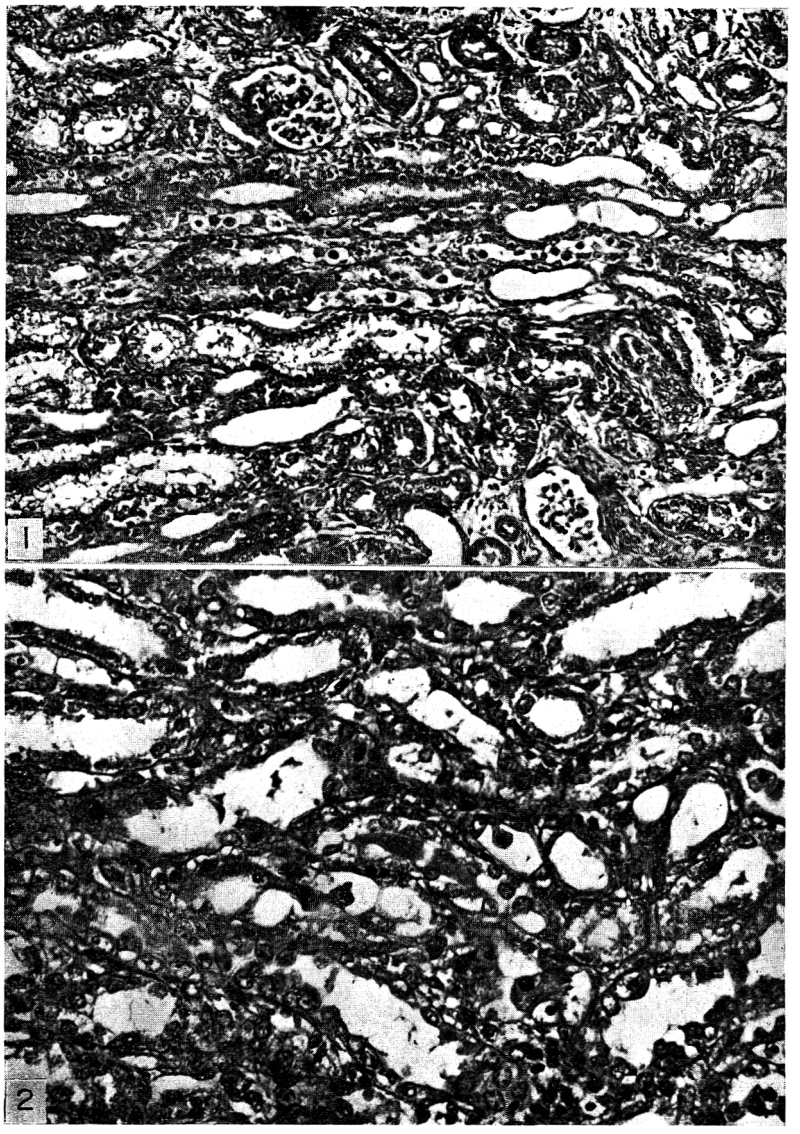

Fig. 1. Section of kidney from dog fed citrinin-containing fungal culture (Trial I), some tubules are slightly dilated and others contain necrotic and sloughed epithelial cells. Degenerative changes are those of cytoplasmic vacuolation of hydropic change. Trichrome × 160.

Fig. 2. Renal tubules from dog fed the fungal culture (Trial I), showing both degenerative and necrotic changes in the epithelial cells, some of which are swollen, protrude into the lumen and have pyknotic nuclei, while others, separated from the basement membrane, are found in the lumina. Trichrome × 400.

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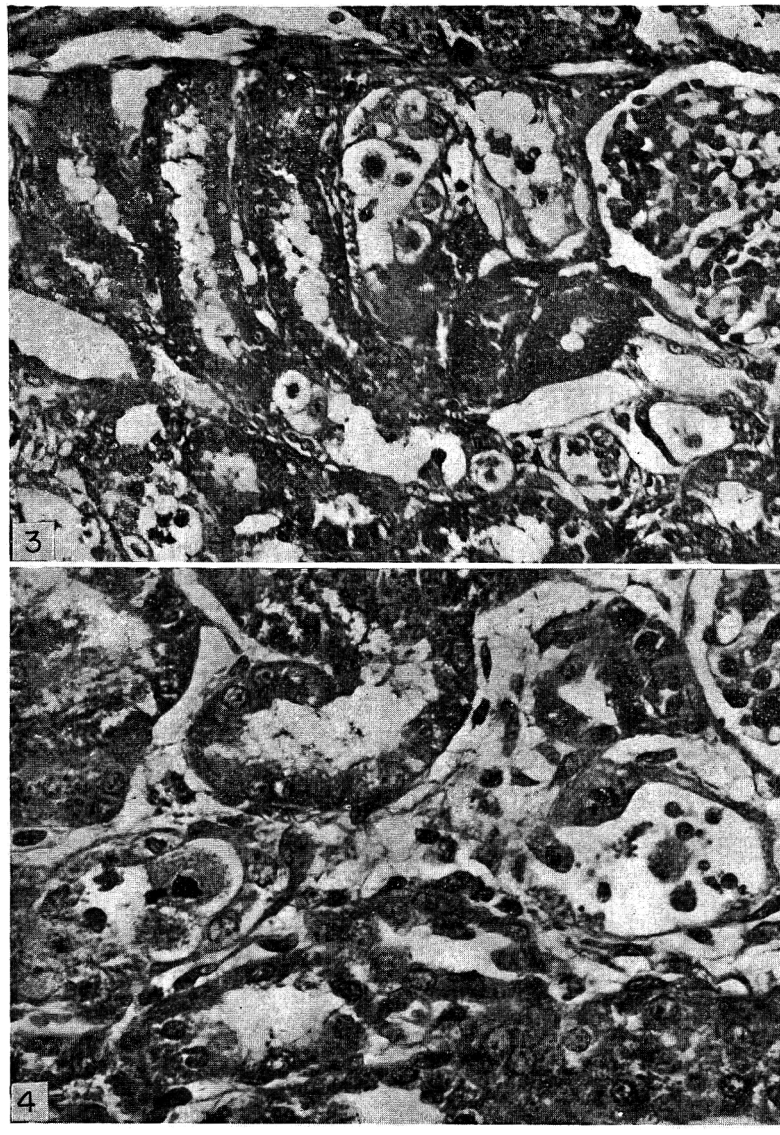
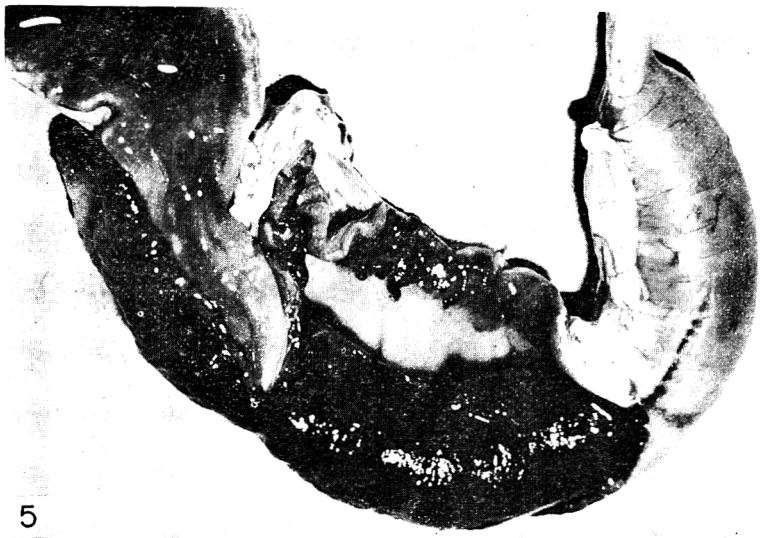


Fig. 3. Kidney section from dog fed the fungal culture (Trial I), showing severely damaged distal tubules located adjacent to apparently normal proximal tubules. The affected tubule adjacent to the glomerulus has an attenuated epithelium and necrotic cells in the lumen. The epithelial cells of the adjacent tubule have vacuolated cytoplasm and pyknotic nuclei. Trichrome × 400.

Fig. 4. Affected renal tubules from dog fed the fungal culture (Trial I). That on left contains a necrotic cell apparently in mitosis, while that on right has a swollen pale cell in mitosis and cellular debris in its lumen. Trichrome × 640.



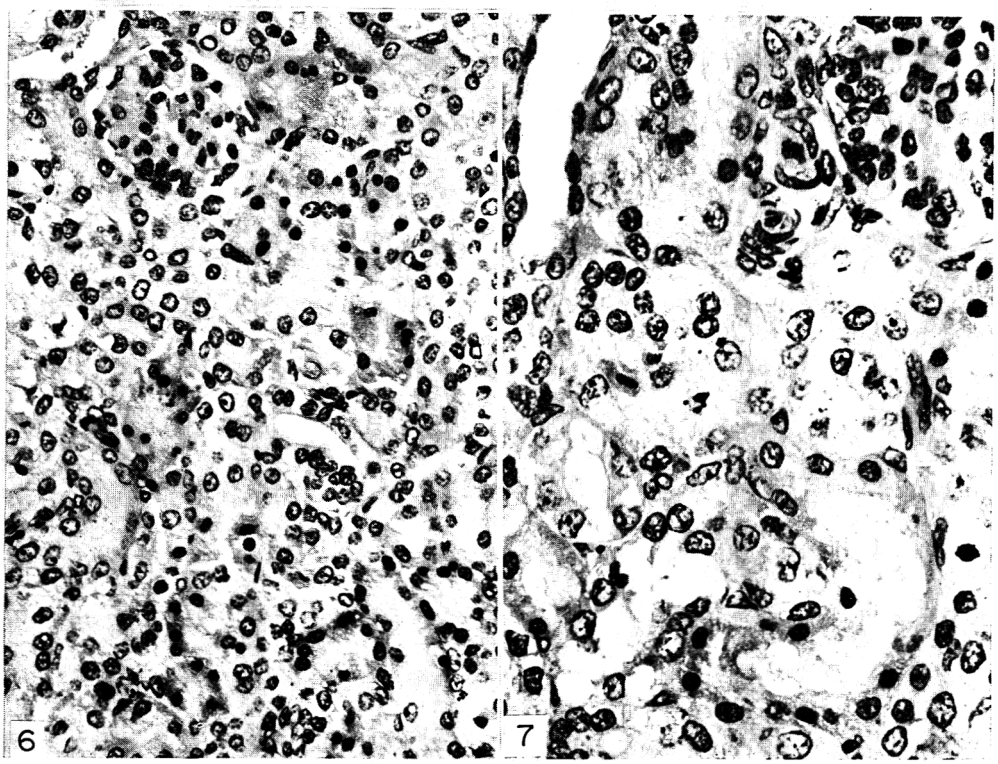


Fig. 5. Open segment of an area of intestinal intussusception from dog given 40 mg citrinin/kg ip daily (Trial II). The lumen contains bloody fluid and blood clcts.

Fig. 6. Necrotic tubules in the kidney of a dog given 40 mg citrinin/kg ip daily (Trial II). Haemotoxylin and eosin × 400.

Fig. 7. Necrotic and degenerative changes in the kidney of a dog given 20 mg citrinin/kg ip daily (Trial II). Haematoxylin and eosin × 640.

ip administration of citrinin. Most dogs defaecated and urinated within minutes of dosing and tenesmus was intense for several minutes after treatment. Another feature of the clinical response in all dogs was a serous nasal discharge, due mostly to increased lachrymation and appearing within minutes of dosing. Lachrymation was recognized by a moist glistening appearance of the eye and a collection of tears at the medial canthus. There was no consistent change in the size of the pupil or the pupillary response to light. Excitement and apparent apprehension followed the dosing with citrinin but within a few minutes the dogs became depressed. After the short (5–10 min) period of retching and vomiting, the dogs tended to lie quietly in a corner of the cage. The clinical signs disappeared within 1 hr of dosing and the dogs resumed normal activity; they were bright and appeared clinically normal. This series of clinical events occurred each time the dogs were dosed.

Polydipsia and polyuria were apparent by day 1 (Table 2). Polydipsia was reflected in more frequent and increased water consumption. The volume of urine was greatly increased in both groups within the first 24–36 hr. By day 2, dehydration was clinically apparent in all dogs. Body weights, not recorded on day 2, were reduced by day 3 in both treatment groups (Table 2). Two dogs (nos 5 & 6) of group 2 were found dead on the morning of day 3. The physical condition of the other dogs deteriorated rapidly and dog 3 was killed *in extremis* on day 3, while dogs 1 and 4 became moribund on day 4 and were killed for autopsy. The last group 1 survivor (dog 2) was killed on day 5.

Table 2. *Mean body weights, urine output and water intake in dogs given daily ip doses of 20 or 40 mg citrinin/kg (Trial II)*

Day of trial	Dose (mg/kg)...	Body weight (kg)		Urine output (ml/24 hr)		Water intake (ml/24 hr)	
		20	40	20	40	20	40
−5		1·7	1·7	66	77	175	177
−4		—	—	36	74	108	160
−3		1·6	1·7	33	61	101	143
−2		—	—	33	79	93	172
−1		1·6	1·8	44	86	132	212
0*		1·6	1·8	51	79	178	168
1		1·6	1·8	58	158	153	287
2		—	—	105	155	113	213
3		1·4	1·5	89	118†	83	200†
4		—	1·6†	110†	200†	150†	450†
5		1·3†	—	115†	—	160†	—

*First day of treatment.

Clinicopathological observations. Physical changes in the urine were not apparent until day 2. The specific gravity of the urine during the pre-trial period ranged from 1·028 to 1·050, but on day 2 the values ranged from 1·012 to 1·026. On day 2 the specific gravity of the urine from dog 4 was 1·007 and on day 3 it was 1·007 for dog 3. Proteinuria was mild and only traces were found. The urine of all dogs of group 2 contained small amounts of glucose by day 1. By the end of day 2, slight glucosuria also occurred in the group 1 dogs and by this time glucosuria was marked in group 2. Also, on day 2 the urinary sediment of all dogs contained masses of necrotic renal tubular epithelial cells. However, hyaline and cellular casts were not present in the urinary sediment of any dog until day 4.

The earliest indication of renal damage was the increase in enzyme activity in the urine of the test dogs (Table 3). In this study, as in others (Szczech, Carlton & Tuite, 1973a; Szczech, Carlton, Tuite & Caldwell, 1974), the activities of the enzymes LDH and GOT

Table 3. *Mean enzyme activities in serum and urine of dogs given daily ip doses of 20 or 40 mg citrinin/kg (Trial II)*

Dose level (mg/kg)	Sample		Enzyme activities (mIU/ml)							
			Glutamic–oxalacetic transaminase		Glutamic–pyruvic transaminase		Isocitric dehydrogenase		actic dehydrogenase	
			Serum	Urine	Serum	Urine	Serum	Urine	Serum	Urine
0	Control		8	4	6	3	3	21	83	25
20	Pre-trial	1	8	2	7	2	3	20	85	24
		2	12	7	11	7	2	13	84	32
	Test day	1	9	66	7	18	3	46	95	415
		3	7	24	8	7	3	25	59	303
		5*	6	58	8	11	5	116	19	449
40	Pre-trial	1	7	11	8	7	3	2	179	26
		2	11	12	9	10	2	9	81	10
	Test day	1	8	26	8	11	3	15	100	100
		3*	8	13	17	5	4	3	86	95

*Data for one surviving dog.

in the urine increased rapidly and to many times the pre-trial values. A marked increase in the activities of these two enzymes in urine was apparent by day 1 in the dogs of both test groups. GPT activity was slightly increased in the urine of dogs of group 1 by day 1 but was not increased in the dogs of group 2. The activity of ICDH in the urine from dogs of group 1 was also slightly increased by day 1 but again this enzyme activity was not increased in the urine of group 2 dogs (Table 3). There were no consistent alterations in the activities of these enzymes in the serum (Table 3).

Increases in values for PCV, Hb and TPP in both test groups by day 3 (Table 4) were considered to be a reflection of the clinically apparent dehydration. There were no consistent alterations in leucocyte counts. Concentrations of BUN were slightly increased in all dogs by day 3 (Table 4).

Pathological observations. A prominent finding at autopsy in five of the six test dogs was intussusception of the terminal ileum (Fig. 5). In most dogs the distal 15–25 cm of the ileum was telescoped for 10–20 cm into the lumen of anterior ileum. The intussusceptions were considered to have preceded death because of the considerable pooling of blood in affected

Table 4. *Mean clinicopathological data for dogs given daily ip doses of 20 or 40 mg citrinin/kg (Trial II)*

Dose level (mg/kg)	Sample		Packed cell volume (%)	Haemoglobin concn (g/100 ml)	Leucocyte count (cells/mm^3 × 10^3)	Total plasma protein (mg/100 ml)	Blood urea nitrogen (mg/100 ml)
0	Control range		32–38	10·5–13·5	8·0–15·0	5·0–6·5	12–20
20	Pre-trial	1	29	9·0	8·9	4·4	15
		2	32	9·8	11·5	4·1	19
	Test day	1	34	9·9	15·0	3·9	13
		3	40	10·6	8·7	4·9	33
		5*	41	11·4	9·4	4·0	55
40	Pre-trial	1	31	9·8	13·2	4·6	23
		2	32	9·6	11·2	4·4	17
	Test day	1	33	9·6	17·1	4·4	17
		3*	42	11·8	17·4	5·0	32

*Data for one surviving dog.

segments; the serosa of the ileum was dark red and the luminal contents consisted of a reddish brown fluid and clots of blood. Other findings at autopsy included marked dehydration in all carcasses and pale kidneys ranging in colour from tan to grey or slate-coloured.

Prominent histopathological alterations in the test dogs included tubular dilatation and necrosis of renal tubular epithelial cells (Fig. 6). Renal tubular dilatation, primarily located in the renal cortex, occurred in two of the three dogs in each group. Necrotic tubular epithelial cells were distributed throughout most portions of the nephron but were uncommon in the proximal convoluted tubules. Necrotic tubular cells had pyknotic nuclei and the cells were often separated from the basement membrane. Occasionally, small groups of necrotic tubular epithelial cells were found, but more often necrosis involved single cells of a tubule. Necrotic cells were primarily observed in the distal convoluted tubules, in the loop of Henle and in collecting ducts. Cloudy swelling, hydropic degeneration and altered cytoplasmic staining were other features of the renal pathology (Fig. 7). The proximal convoluted tubules, with the exception of an occasional necrotic cell, appeared normal in sections stained with haemotoxylin and eosin and in other sections stained with the trichrome method. There was no accumulation of lipid material in the kidney or liver.

Other pathoanatomical alterations were few. Ulcerative oesophagitis occurred in two dogs of group 1 and one of group 2 and was attributed to oesophageal reflux of the acidic gastric contents. Hydropic degeneration as indicated by cytoplasmic vacuolation occurred in the transitional epithelial cells of the urinary bladder and ureters of one dog of group 1 that was killed and in two dogs of group 2 that were found dead. Other organs examined and considered normal included those listed on p. 484 for Trial I.

Trial III

Two dogs given 5 mg citrinin/kg vomited within 30 min of the first dose of citrinin and one of these dogs also vomited after the second dose, but emesis did not occur in any dog at the later dosings. The dogs were bright and alert without any clinical evidence of toxicity, as they ate well and gained weight.

The mean activity of LDH increased in the urine of dogs given 2·5 mg/kg from pre-trial levels of 20 and 24 mIU/ml to 72 and 47 mIU/ml on test days 3 and 10 respectively (Table 5). Otherwise, the activities of enzymes in serum and urine and values for BUN, PCV and total WBC were within the normal range and comparable to control values (Table 6).

At autopsy, there were no gross lesions. The tissues were considered to be histologically normal.

DISCUSSION

The results of these studies establish citrinin as a potent nephrotoxin in the dog, as in rats and swine (Friis *et al.* 1969; Krogh *et al.* 1970). The amount of toxin consumed by the dogs fed the fungal culture induced renal disease and apparently would have resulted in death, but alterations in other organs and tissues were not observed.

Histopathological features of citrinin-induced renal failure in dogs are very similar to those in swine and rats given this mycotoxin. Young beagle dogs are very sensitive to ochratoxin A, which induces a histopathologically distinct nephrosis characterized by considerable cast formation and a total lack of tubular dilatation as well as widespread lymphoid necrosis (Szczech, Carlton & Tuite, 1973b). Our studies indicate that the lesions of

Table 5. *Mean enzyme activities in serum and urine of dogs given daily oral doses of 2·5 or 5·0 mg citrinin/kg (Trial III)*

Dose level (mg/kg)	Sample		Enzyme activities (mIU/ml)					
			Glutamic-oxalacetic transaminase		Lactic dehydrogenase		Isocitric dehydrogenase	
			Serum	Urine	Serum	Urine	Serum	Urine
0	Control		8	4	83	25	3	21
2·5	Pre-trial	1	9	2	79	24	3	21
		2	15	7	70	20	4	22
	Test day	1	5	8	102	22	6	25
		3	8	9	67	72	5	39
		10	12	20	76	47	3	2
		17	12	9	48	23	5	19
		24	13	6	42	22	5	21
		31	11	9	39	24	4	11
		38	10	10	71	29	4	8
5·0	Pre-trial	1	9	5	68	7	3	14
		2	9	4	35	5	3	34
	Test-day	1	11	6	81	5	4	18
		3	11	3	54	7	4	8
		10	14	11	65	30	3	29
		17	9	5	80	10	8	50
		24	17	7	67	18	3	14
		31	7	10	67	22	3	15
		38	9	12	70	35	3	8

the mycotoxic disease induced in dogs by citrinin are limited to the kidneys and that ochratoxicosis in this species is distinguishable in that it induces widespread necrosis of lymphoid tissue.

The renal damage produced by pure citrinin in these studies was similar to that produced by a culture of *P. citrinum* containing citrinin. Most of the necrotic renal tubular epithelial cells were located in the distal convoluted tubules and in the loop of Henle. This serves to differentiate further the renal damage induced in dogs by citrinin from that caused by ochratoxin A, as the latter affected primarily the proximal convoluted tubules

Table 6. *Mean blood urea nitrogen, packed cell volume and leucocyte counts in male beagles given daily oral doses of 2·5 or 5·0 mg citrinin/kg (Trial III)*

Sampling time		Blood urea nitrogen (mg/100 ml)		Packed cell volume (%)		Leucocyte count (cells/mm^3 × 10^3)	
	Dose (mg/kg)...	2·5	5	2·5	5	2·5	5
Pretrial	1	12	14	31	32	27·7	17·3
	2	13	15	31	32	30·0	16·9
Test day	1	16	12	31	33	27·5	20·9
	3	19	16	31	32	25·3	19·5
	10	14	16	30	33	25·9	29·5
	17	15	15	31	32	19·2	12·2
	24	12	15	32	31	25·7	16·4
	31	13	15	31	32	24·5	15·9
	38	12	13	33	33	19·3	14·4

(Szczech *et al.* 1973b). Species differences in response apparently exist, because in guinea-pigs and rabbits citrinin caused necrosis primarily of the epithelial cells of the proximal convoluted tubules (Ambrose & DeEds, 1946). Further points of differentiation include tubular dilatation in dogs given citrinin. Tubular dilatation is not prominent in dogs with ochratoxicosis. The single-cell type of necrosis that occurred in the renal tubules of citrinin-treated test dogs also serves to differentiate citrinin toxicosis from ochratoxicosis in dogs as the latter is characterized by necrosis of groups of renal tubular epithelial cells.

Ambrose & DeEds (1944) commented on hyperaemia of the ears and feet, respiratory stimulation, dyspnoea, prostration, micturition, defaecation and serious rhinorrhoea in rats and rabbits given citrinin. Our dogs exhibited serous rhinorrhoea, micturition and defaecation within minutes of dosing. We observed these signs in all dogs throughout Trial II. The parasympathomimetic effects of citrinin in dogs were striking. Five of the six dogs in Trial II died with intussusceptions, perhaps the result of severe peristaltic activity, and had marked renal damage as well. A minimal and no-effect level of citrinin was established in Trial III as there were no signs of toxicosis in six dogs given daily doses of 2·5 or 5 mg/kg for 6 wk.

The potential for natural citrinin toxicosis in the dog seems negligible because of the emetic effect. In this trial emesis occurred after the dose was divided, after the orally administered citrinin was buffered with $NaHCO_3$ and after the toxin was administered by the ip route. The emesis appears to have a central origin, as there were no histopathological lesions in the stomachs of test dogs and there was no peritonitis or any lesion to explain the emesis that occurred after the toxin was given ip. When dogs received daily doses of citrinin that did not cause emesis (Trial III), there were no clinical signs or histopathological alterations indicative of toxicosis.

The urine analyses and determination of LDH, ICDH and GOT concentrations in urine were useful in detecting and following the nephrotoxic effects of citrinin in this trial, as in our previous studies of ochratoxicosis in beagles (Szczech *et al.* 1973a) and swine (Szczech *et al.* 1974).

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Mycotoxicose de la citrinine chez les chiens bigles

Résumé—Trois essais sont réalisés sur de jeunes chiens bigles; ils sont ou bien nourris avec une culture de *Penicillium citrinum* contenant de la citrinine (Essai I) ou alors il reçoivent de la citrinine pure soit par voie ip (Essai II) soit par voie orale (Essai III). Les chiens qui sont nourris avec la culture fongique et ceux auxquels on a donné 20 à 40 mg de citrinine pure/kg de poids corporel développent une maladie rénale. Les chiens auxquels on a donné 2,5 et 5 mg/kg restent cliniquement normaux et sans lésions rénales. Les signes clinicopathologiques de l'atteinte rénale comprennent une élévation de l'azote uréique sanguin, de la glucosurie et de la protéinurie, un abaissement de la densité de l'urine, une augmentation des activités urinaires de la lactico-déshydrogénase, de la transaminase glutamique-oxaloacétique et de l'isocitricodéshydrogénase et la présence de nombreuses cellules nécrotiques de l'épithélium tubulaire rénal dans le sédiment urinaire. Vomissements et intussusception intestinale ont lieu chez les chiens qui reçoivent 20 ou 40 mg/kg ip les lésions rénales consistent en une dégénérescence et une nécrose de l'épithélium tubulaire, d'une façon plus marquée dans le segment épais de Henle et dans les anses coutournées distales.

Citrinin-Mycotoxicose bei Beaglehunden

Zusammenfassung—Drei Versuche wurden durchgeführt, in welchen junge Hunde der Beaglerasse entweder mit einer zitrininhaltigen Kultur von *Penicillium citrinum* (Versuch 1) gefüttert wurden oder ihnen reines Zitrinin gegeben wurde entweder ip (Versuch 2) oder oral (Versuch 3). Hunde, denen die Pilzkultur verfüttert wurde und denen 20 oder 40 mg reines Zitrinin/kg Körpergewicht gegeben wurde, entwickelten Nierenerkrankungen. Hunde, die 2·5 und 5 mg/kg erhielten, blieben klinisch normal und ohne Nierenläsionen. Klinikopathologische Beweise von Nierenschäden enthielten eine Erhöhung in Blutharnstoff-Stickstoff, Glukosurie, Proteinurie, verringertes spezifisches Gewicht des Harns, erhöhte Aktivität von Milchsäuredehydrogenase im Harn, Glutamat-Oxalazetat-Transaminase und Isozitratdehydrogenase und das Vorkommen von zahlreichen nekrotischen renalen tubularen Epithelzellen im Harnsediment. Emesis und intestinale Intussuszeption traten bei den Hunden auf, denen 20 oder 40 mg/kg ip gegeben wurde. Renale Läsionen bestanden aus Degeneration und Nekrosis des Tubularepithels, am meisten hervorstechend in dem dicken Henle-Segment und in den distalen konvolutierten Tubuli.

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LONG-TERM ADMINISTRATION OF STERIGMATOCYSTIN AND *PENICILLIUM VIRIDICATUM* TO MICE

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Abstract—The long-term toxic effects and carcinogenicity of pure sterigmatocystin (S-p), of sterigmatocystin administered in the form of a rice culture of *Aspergillus versicolor* (S-rc) and of a toxigenic species of *Penicillium viridicatum* were studied by feeding mice on a diet containing 5 ppm sterigmatocystin (S-p or S-rc) and/or a 7·5% content of a rice culture of *P. viridicatum* (P-rc) for periods of 2 wk alternating with 2-wk periods on the control diet, for a total of 54–58 wk. A control group was fed only the purified diet. In mice fed S-p or S-rc alone, the incidence of pulmonary tumours was, respectively, 73 and 49% higher than that in the control group. The incidence of pulmonary tumours in mice fed P-rc was about 16% higher than that in the controls. In the groups fed P-rc with S-p or S-rc, the incidence of pulmonary neoplasms exceeded that in the controls by 54 and 52%, respectively. The tumours were classified as adenomas or adenocarcinomas arising from the bronchial and alveolar epithelium. Adenomatosis was more common in mice fed diets containing sterigmatocystin, the incidence being 27–47% greater than that in controls. The incidence of other neoplasms was not affected by the feeding of the experimental diets. The increased incidence of pulmonary neoplasms in the mice fed S-p, S-rc or P-rc appeared to be directly related to the feeding of these test materials. The possible presence of certain common mycotoxins in the P-rc was eliminated by chemical analysis. It was concluded that P-rc and sterigmatocystin (S-p or S-rc) each had a carcinogenic effect when fed in the diet to mice but no additive effect was found when sterigmatocystin was combined with the P-rc diet.

INTRODUCTION

Neoplasms of the liver in trout fed a commercial diet containing aflatoxin were described in the United States (Ashley & Halver, 1961; Rucker, Yasutake & Wolf, 1961; Wood & Larson, 1961) and in France (Levaditi, Besse, Vibert & Nazimoff, 1960). Sterigmatocystin, closely related chemically to aflatoxin, produced hepatocellular carcinomas in rats (Purchase & van der Watt, 1970) when fed or given by gavage. Dickens & Jones (1965) reported that the sc injection of sterigmatocystin produced sarcoma at injection sites in rats. Purchase & van der Watt (1973) demonstrated that 1 mg sterigmatocystin applied twice weekly to the shaved backs of rats produced a high incidence of papillomas.

Members of the genus Penicillium are commonly found in feedstuffs, where they elaborate toxic metabolites. The three mycotoxins, penicillic acid (Dickens & Jones, 1961 & 1963), penicillin G (Dickens & Jones, 1961, 1963 & 1965) and patulin (Dickens & Jones, 1961), produced sarcomas at sc injection sites in mice and rats. Zwicker, Carlton & Tuite (1973) described a high incidence of pulmonary neoplasms (about 57% higher than in controls) in mice fed a diet containing 7·5% *Penicillium viridicatum* culture, but the toxin and possible carcinogen produced by this fungus is at present unknown.

This study was undertaken to characterize the effects of long-term feeding of pure sterigmatocystin, of sterigmatocystin in the form of a rice culture of *Aspergillus versicolor* and of a toxic rice culture of an Indiana isolate of *P. viridicatum* to male and female white Swiss mice, and to determine the possible additive effect when sterigmatocystin was combined with the *P. viridicatum* diet.

EXPERIMENTAL

Animals. ICR white Swiss mice of both sexes were obtained from Harland Small Animal Industries, Cumberland, Indiana. The mice were started on the experiment as weanlings (approximately 3 wk old, 11–16 g body weight) and were fed test diets containing 5 ppm pure sterigmatocystin (S-p) or 5 ppm sterigmatocystin in the form of a rice culture of *A. versicolor* (S-rc) and/or a rice culture of *P. viridicatum* at a level of 7·5% (P-rc). Details of the treatment regimens and numbers of mice are given in Table 1. The numbers of male and female mice assigned to some of the diets were unequal because of errors in sexing the weanlings.

Table 1. *Experimental groups of mice fed sterigmatocystin and/or a* P. viridicatum *culture intermittently over a test period of 54–58 wk*

Group no.	No. of mice/group		Dietary level of		
			Sterigmatocystin (ppm)		*P. viridicatum* culture (%)
	Male	Female	Culture (S-rc)	Pure toxin (S-p)	(P-rc)
I	19	21	0	0	0
II	42	47	0	0	7·5
III	18	10	0	5	0
IV	16	16	0	5	7·5
V	34	35	5	0	0
VI	33	46	5	0	7·5

Diets. Pure sterigmatocystin was obtained from Makor Chemicals Ltd. (Jerusalem, Israel). The fungal cultures of *P. viridicatum* and *A. versicolor* were prepared as described by Carlton & Tuite (1970) and were added to a purified diet (Mills & Murray, 1960). The concentration of sterigmatocystin in the *A. versicolor* culture was determined by the method of Stack & Rodricks (1971). The experimental diets were fed for periods of 2 wk alternating with 2 wk on the control diet. This feeding schedule was adopted because previous studies had shown that mice would not survive continuous feeding of a 7·5% *P. viridicatum* diet. The groups fed only S-p or S-rc were also pulse-fed, to match the exposure of groups fed S-p or S-rc combined with P-rc.

Conduct of experiment. Mice were housed in groups of five, with sexes separated, in plastics shoebox-type cages with a stainless-steel top and dry sterile bedding. Water and feed were provided *ad lib.* The cages were located in the rooms of an air-conditioned animal house with a room temperature between 21 and 24°C. During the test period of 54–58 wk, mice were weighed weekly for the first month and then at monthly intervals for the remainder of the study. Observations on the general health of the mice were made daily. During the experimental period, some of the mice from several groups either died or became moribund and in either case these were autopsied. Those that died early in the

experiment (i.e. before wk 30) were not included in the computation of the incidences of pulmonary neoplasms.

Post-mortem studies. At termination, mice were anaesthetized and then decapitated for autopsy, and tissues including the brain, eyes, lung, heart, liver, kidney, spleen, stomach and intestines, trachea, testes and bone were fixed in 10% buffered formalin. Portions of these organs and multiple sections of each lung were prepared for paraffin sectioning and the sections were stained with haematoxylin and eosin. The pulmonary changes were evaluated microscopically and placed into three categories—adenomatosis, adenoma and adenocarcinoma—according to criteria presented by Amaral-Mendes (1969).

RESULTS

Clinical signs of toxicity, seen in several mice fed the test diets for 1–2 months, included roughened hair, dehydration and emaciation. Jaundice was seen in some mice and these had hepatic lesions. Anaemia occurred in a number of mice, and most had renal lesions.

The mortality and incidence of pulmonary neoplasms are presented in Tables 2 and 3. The results for both sexes will be discussed together as there was no great difference between male and female mice in the incidence of these neoplasms. The incidence for control mice (4/37) was low and in each case was a single pulmonary nodule that was considered on histopathological examination to be an adenoma.

Table 2. *Mortality and incidence of pulmonary neoplasms in mice fed experimental diets for up to 58 wk*

Group no.	Treatment	Early mortality*		Mice with tumours†		Total incidence of tumours	
		Males	Females	Males	Females	No.	%
I	Control	3/19	0/21	2/16	2/21	4/37	11
II	P-rc	8/42	4/47	10/34	11/43	21/77	27
III	S-p	3/18	0/10	12/15	9/10	21/25	84
IV	S-p + P-rc	3/16	3/16	10/13	7/13	17/26	65
V	S-rc	12/34	2/35	14/22	19/33	33/55	60
VI	S-rc + P-rc	7/33	13/46	16/26	21/33	37/59	63

P-rc = Rice culture of *P. viridicatum* (7·5% in diet)
S-p = Pure sterigmatocystin (5 ppm in diet)
S-rc = Sterigmatocystin as rice culture of *A. versicolor* (5 ppm in diet)
*No. of mice dying before wk 30/original no. in group.
†No. of mice affected/total no. surviving for at least 30 wk.

In mice fed P-rc, the first pulmonary neoplasm was observed in a male mouse after 388 days of feeding and the incidence of these tumours was about 27% (21/77 mice). The pulmonary neoplasms were predominantly adenomas and were multiple in most of these mice. Of the 21 mice with neoplasms two had tumours classified morphologically as adenocarcinoma.

Among mice fed the S-p diet, the first pulmonary neoplasm was seen in a male mouse after 355 days of feeding and the incidence was 84% (21/25), the highest of all groups. In most of these mice the pulmonary neoplasms were multiple. Nine mice had at least one neoplasm considered to be an adenocarcinoma.

Among mice fed S-rc the first pulmonary neoplasm was found in a male mouse after 397 days of feeding. The incidence, the fourth highest, was about 60% (33/55 mice). Pul-

Table 3. *Incidence of pulmonary lesions in mice fed experimental diets for up to 58 wk*

Group no.	Treatment	Incidence of pulmonary lesions*					
		Adenomatosis		Adenoma		Adenocarcinoma	
		Male	Female	Male	Female	Male	Female
I	Control	3/16	1/21	2/16	2/21	0/16	0/21
II	P-rc	5/34	5/43	10/34	11/43	0/34	2/43
III	S-p	10/15	6/10	12/15	9/10	1/15	8/10
IV	S-p + P-rc	5/13	7/13	10/13	7/13	0/13	0/13
V	S-rc	16/22	18/33	14/22	19/33	2/22	1/33
VI	S-rc + P-rc	11/26	25/33	16/26	21/33	1/26	1/33

P-rc = Rice culture of *P. viridicatum* (7·5% in diet)
S-p = Pure sterigmatocystin (5 ppm in diet)
S-rc = Sterigmatocystin as rice culture of *A. versicolor* (5 ppm in diet)
*No. of mice affected/total no. surviving for at least 30 wk.

monary neoplasms were multiple in most of these mice. Three mice with adenoma had, additionally, a neoplasm classified morphologically as adenocarcinoma.

The first pulmonary neoplasm was seen after 405 days of feeding in the mice fed a diet containing S-p and P-rc. This group had an incidence of pulmonary neoplasms of about 65% (17/26), the second highest among the experimental groups. In most of the mice the neoplasms were multiple. .

The incidence of pulmonary neoplasms in mice fed the diet containing S-rc and P-rc was 63% (37/59 mice), the third highest incidence. The first pulmonary neoplasm was seen in a male mouse after 192 days of feeding and in most of these mice the neoplasms were multiple.

Pulmonary adenomatosis (Table 3) was commonly observed microscopically in the lungs of mice of both sexes with pulmonary adenoma. Variations in the incidence of the lesion were observed in test groups and between the sexes. The incidence was generally greater in test females and in the groups that were fed sterigmatocystin. However, some mice in each group had lesions of pulmonary adenomatosis but no neoplasms. Adenomatosis was seen on microscopic examination of the lungs and was characterized by small deep parenchymal or occasionally sub-pleural foci of hypertrophied and hyperplastic cuboidal alveolar epithelial cells (Figs 1 & 2). In these foci the alveoli were filled with cells. There was no apparent host reaction to this change. These foci were variable in size and irregular in shape, and the adjacent pulmonary tissue was not compressed.

The pulmonary adenomas (Figs 3 & 4) were not restricted to any lobe of the lungs and were seen on both dorsal and ventral surfaces. When observed grossly, they appeared as tan-white raised nodules, were firm when palpated, and varied in size from about 2 to 6 mm in diameter. In section, they were occasionally seen to be located near bronchioles, suggesting bronchiolar origin, but most often they appeared to arise in the pulmonary parenchyma without any obvious association with the airways. Mitotic figures were not frequent.

Pulmonary adenocarcinoma (Figs 5 & 6) was observed in some male and female mice fed the experimental diets (Table 3). These tumours were seen grossly as large tan-white, firm, raised masses, which occasionally occupied an entire lobe. In the larger neoplasms a central necrotic area was seen on microscopic examination. In these neoplasms there

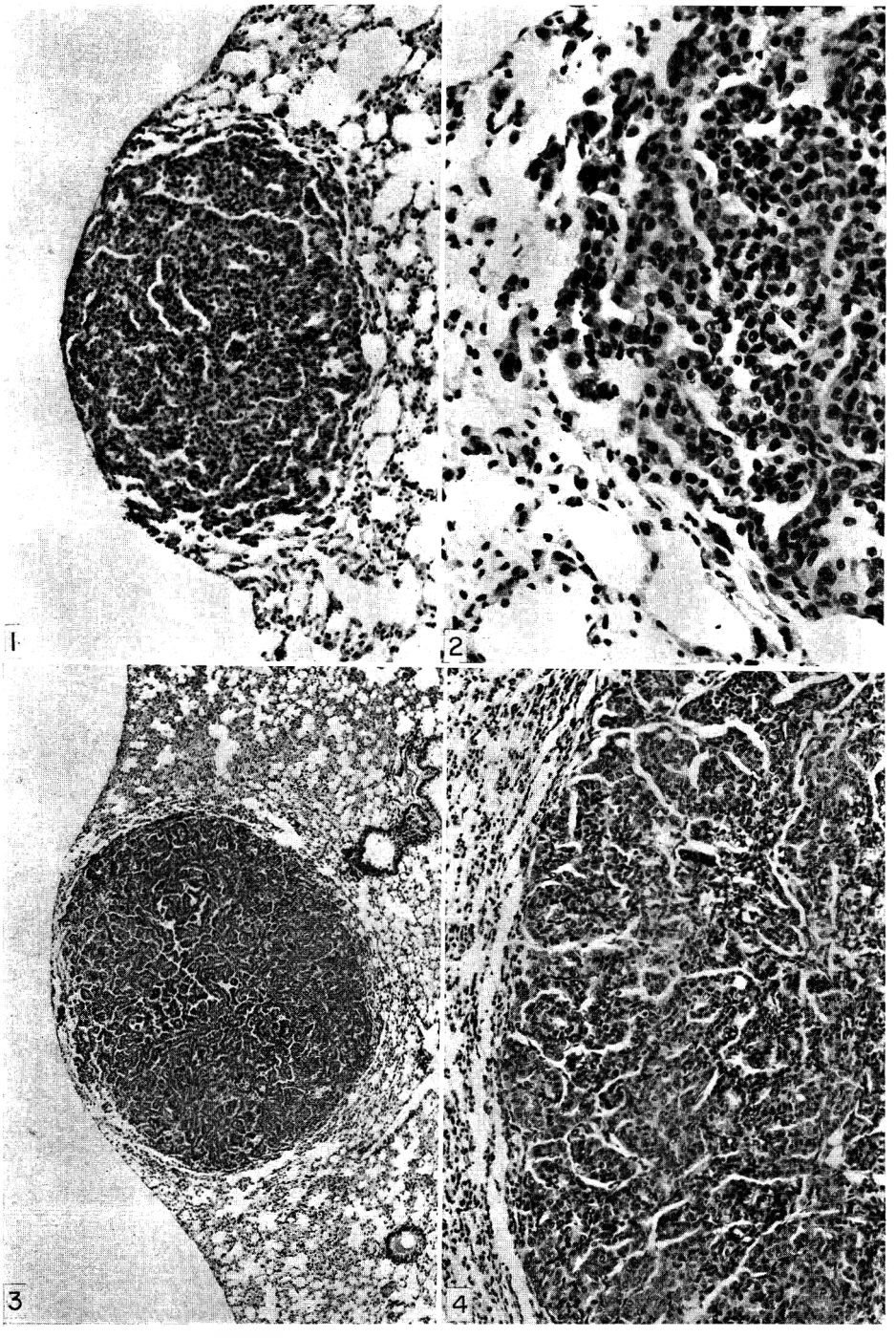

Fig. 1. Small focus of adenomatosis in lung of mouse fed a culture of *P. viridicatum*. Proliferated alveolar epithelium fills and obliterates the pulmonary alveoli. Haematoxylin and eosin ×160.

Fig. 2. Higher magnification of Fig. 1 to illustrate the junction between the lung and the area of adenomatosis. Haematoxylin and eosin ×400.

Fig. 3. Adenoma in the lung of a mouse given sterigmatocystin as a rice culture of *A. versicolor*. A circumscribed, non-escapulated mass compresses the surrounding pulmonary tissue. Haematoxylin and eosin ×64.

Fig. 4. Higher magnification of adenoma in Fig. 3, illustrating the replacement of lung tissue by neoplastic cells arranged essentially in solid masses and cords. Haematoxylin and eosin ×160.

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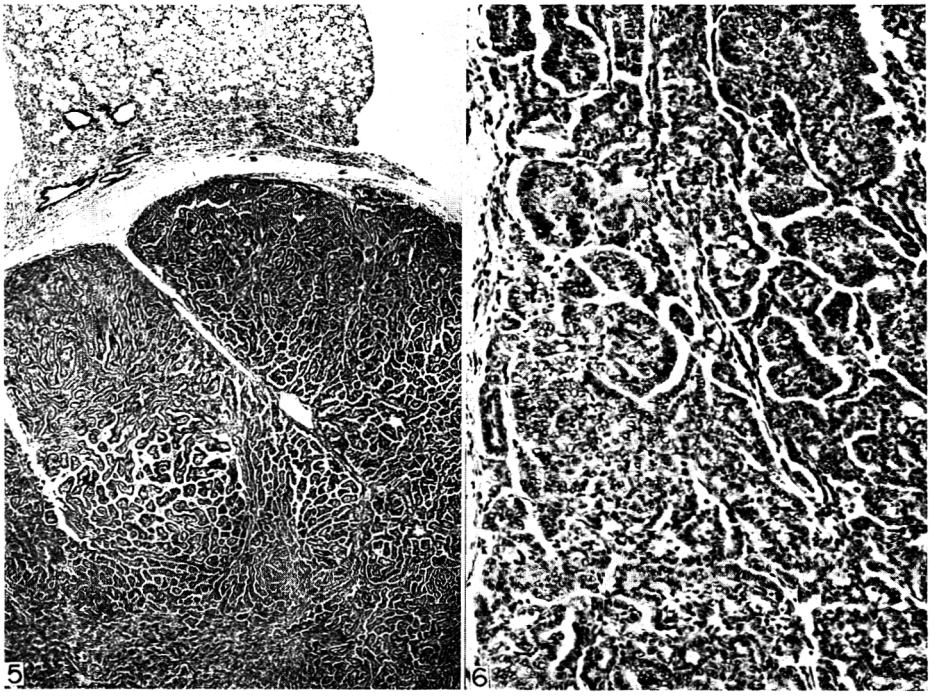

Fig. 5. Large adenocarcinoma in the lung of a mouse fed sterigmatocystin and *P. viridicatum* culture. The highly cellular mass has compressed and extends beyond the pulmonary parenchyma. Haematoxylin and eosin ×40.

Fig. 6. Higher magnification of pulmonary adenocarcinoma in Fig. 5, showing solid masses and cords with mild papillary formations. Haematoxylin and eosin ×160.

was extensive invasion of adjacent pulmonary tissue, due to marked proliferation of large anaplastic epithelial cells with frequent mitotic figures.

The other neoplasm that occurred in the mice used in this study was malignant lymphoma. This tumour occurred in one male mouse and one female mouse of the control group, one female mouse fed S-rc and one male mouse fed P-rc. The incidence of these tumours was not obviously influenced by the feeding of the experimental diets.

DISCUSSION

Neoplasms of the lung were found in all groups, but the incidence was great only in the groups fed the diets containing S-p or S-rc alone or combined with P-rc. There was no evidence of any additive effect between sterigmatocystin and P-rc, since the incidences of pulmonary neoplasms for these groups were lower than for the groups fed diets containing only sterigmatocystin.

The genetic constitution of the strain of mice was found by Heston (1966) to influence the development and incidence of pulmonary neoplasms. Cross-breeding experiments suggested that multiple genetic factors were involved in the development of pulmonary adenoma in mice. It was concluded that genetically susceptible mice can develop a high incidence of pulmonary neoplasms when exposed to a variety of carcinogenic stimuli (Heston, 1966; Kinosita, 1966; Shimkin, 1955). The increased incidence of pulmonary neoplasms ranged from a minimum of 16% for mice fed the P-rc diet to a maximum of 73% for mice fed the S-p diet. The high incidence of pulmonary neoplasms in the mice of the groups fed diets containing P-rc or a form of sterigmatocystin either alone or combined, intermittently during 54–58 wk, appear to be due to sterigmatocystin and a carcinogenic substance produced by the *P. viridicatum* culture acting in a genetically susceptible host.

At least two lines of evidence suggest the presence of a carcinogen in the products of *P. viridicatum* cultures and the carcinogenic activity of sterigmatocystin in mice. First, the incidence of pulmonary adenomas (about 11%) was very low among controls and secondly, other investigators (Percy & Jonas, 1971; Snell & Stewart, 1962; Tannenbaum, 1965) have observed that neoplasms of the lungs are not common during the first year of life in most strains of mice.

The incidence (about 27%) of pulmonary neoplasms in mice fed the P-rc diet in this study was not as great as the incidence of 67% observed by Zwicker *et al.* (1973) in mice fed a similar diet. The reason for the lower incidence of pulmonary adenomas in the mice of this current group is not clear, since experimental procedures for the two studies were similar.

At present no definite evidence is available on the specific carcinogen responsible for the increased incidence of pulmonary neoplasms in the mice fed P-rc, but such common mycotoxins as sterigmatocystin, aflatoxin and ochratoxin A were eliminated by chemical analysis.

The increased incidence of pulmonary neoplasms in the mice of the experimental groups of this study may have been due to the interaction between an oncogenic virus in the pulmonary tissue of the test mice and sterigmatocystin or the unidentified carcinogen in the P-rc. No attempts were made to isolate viruses in this study. Infection of the commercial colony with the viruses of mouse hepatitis, ectromelia, polyoma or lymphocytic choriomeningitis or the pneumotropic and Sendai viruses was ruled out on the basis of serological evidence (Harland Small Animal Industries, Cumberland, Ind., personal communication 1972).

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Administration à long terme de stérigmatocystine et de *Penicillium viridicatum* à des souris

Résumé—On étudie les effets toxiques à long terme et la carcinogénicité de la stérigmatocystine pure (S-p), de la stérigmatocystine administrée sous forme d'une culture sur riz d'*Aspergillus versicolor* (S-rc) et d'une espèce toxigène de *Penicillium viridicatum* en nourrissant des souris avec un régime contenant 5 ppm de Stérigmatocystine (S-p ou S-rc) et ou un régime de 7,5% d'une culture sur riz de *P. viridicatum* (P-rc) pendant des périodes de 2 semaines, alternant avec des périodes de 2 semaines de régime témoin, ceci pendant un total de 54–58 semaines. Un groupe témoin reçoit seulement le régime purifié. Chez les souris nourries de S-p ou de S-rc seul, la fréquence des tumeurs pulmonaires a été respectivement de 73 et 49% plus élevée que chez le groupe témoin. La fréquence des tumeurs pulmonaires chez les souris nourries avec P-rc a été d'environ 16% plus élevée que

celle des témoins. Dans les groupes qui sont nourris de P-rc avec S-p ou S-rc, la fréquence des néoplasmes pulmonaires a dépassé respectivement celle des témoins de 54 et 52%. Les tumeurs sont classés comme adénomes ou comme adénocarcinomes de l'épithélium bronchique et alvéolaire. Les adénomatoses sont plus communes chez les souris recevant les régimes contenant de la stérigmatocystine, la fréquence étant de 27–47% plus grande que chez les témoins. Les fréquences des autres néoplasmes n'est pas affectée par les différents régimes expérimentaux. L'augmentation de la fréquence des néoplasmes pulmonaires chez les souris nourries avec S-p, S-rc ou P-rc apparaît en relation directe avec l'absorption de ces substances d'essai. L'analyse chimique a eliminé la possibilité de la présence de certaines mycotoxines communes chez les P-rc. On en conclut que P-rc et la stérigmatocystine (S-p ou S-rc) ont chacun un effet cancérogène quand ils sont donnés dans le régime des souris mais on n'a trouvé aucun effet additif quand la stérigmatocystine est ajoutée au régime P-rc.

Langzeitverabreichung von Sterigmatocystin *Penicillium viridicatum* an Mäuse

Zusammenfassung—Die langzeittoxischen Effekte und Karzinogenität von reinem Sterigmatocystin (S-p), von Sterigmatocystin gegeben in Form einer Reiskultur von *Aspergillus versicolor* (S-rc) und von einer toxigenen Art von *Penicillium viridicatum* wurden studiert, indem man Mäuse mit einer 5 ppm Sterigmatocystin-(S-p oder S-rc)-haltigen Nahrung fütterte und/oder mit 7·5% Inhalt einer Reiskultur von *Penicillium viridicatum* (P-rc), 2 Wochen lang abwechselnd mit 2 Wochen von Kontrollnahrung während einer Gesamtzeit von 54–58 Wochen. Eine Kontrollgruppe wurde nur mit der reinen Nahrung gefüttert. In Mäusen mit S-p oder S-rc allein gefüttert, war das Vorkommen von Pulmonartumoren 73 bzw. 49% höher als in der Kontrollgruppe. Das Vorkommen von Pulmonartumoren in mit P-rc gefütterten Mäusen war ungefähr 16% höher als in den Kontrollen. In den Gruppen mit P-rc, S-p oder S-rc gefüttert, übertraf das Vorkommen von Pulmonarneoplasmen das in den Kontrollen um 54 bzw. 52%. Die Tumore wurden klassifiziert als Adenome oder Adenokarzinome aus dem Bronchial- und Alveolarepithel entspringend. Adenomatose war häufiger in Mäusen, die mit Sterigmatocystin-haltiger Nahrung gefüttert wurden; das Vorkommen war um 27–47% höher als das in den Kontrollen. Das Vorkommen anderer Neoplasmen war nicht beeinflußt durch die Fütterung mit der experimentellen Nahrung. Das erhöhte Vorkommen von Pulmonarneoplasmen in den mit S-p, S-rc oder P-rc gefütterten Mäusen schien direkt abzuhängen von der Fütterung dieser Testmaterialien. Das mögliche Vorhandensein von gewissen gewöhnlichen Mykotoxinen in P-rc wurde durch chemische Analyse ausgeschlossen. Es wurde geschlossen, daß sowohl P-rc als auch Sterigmatocystin (S-p oder S-rc) einen karzinogenen Effekt haben, wenn sie in der Nahrung an Mäuse verfüttert werden; aber kein additiver Effekt wurde gefunden, als Sterigmatocystin mit P-rc gemeinsam gefüttert wurde.

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ACUTE INTRAPERITONEAL TOXICITY OF OCHRATOXIN A AND B DERIVATIVES IN RAINBOW TROUT (*SALMO GAIRDNERI*)

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Abstract—The acute ip toxicities of five derivatives of ochratoxins A and B, metabolites of *Aspergillus ochraceus*, were determined using 6-month-old Mt. Shasta strain rainbow trout as test animals. Ethyl esters of ochratoxins A and B were lethal to trout when administered in corn oil. The 10-day LD_{50} values of ochratoxin A and B ethyl esters were 3·0 and 13·0 mg/kg body weight, respectively. Ochratoxins A and B, the ip toxicities of which were previously determined in rainbow trout in another study, were again administered in this study under the current experimental conditions. The LD_{50} of ochratoxin A was 5·53 mg/kg body weight (13·72 μmols/kg), while ochratoxin B caused no deaths at a dose of 66·7 mg/kg body weight. The ethyl ester of ochratoxin *a* administered ip in corn oil, and the alanine and leucine analogues of ochratoxin A administered in aqueous bicarbonate, were not lethal to trout at a dose level of 13·72 μmols/kg body weight (the LD_{50} of ochratoxin A). Histopathological damage caused by these toxins in the trout liver and kidney is described.

INTRODUCTION

Toxicity studies on ochratoxins A and B, metabolites of the mould *Aspergillus ochraceus*, have revealed that ochratoxin A is far more toxic than the non-chlorinated analogue, ochratoxin B. The LD_{50} value of ochratoxin was originally reported by van der Merwe, Steyn, Fourie, Scott & Theron (1965b) to be 25 μg/50 g body weight (0·5 mg/kg) in day-old ducklings, and van der Merwe, Steyn & Fourie (1965a) reported that ochratoxin B and C (ochratoxin A ethyl ester) were non-toxic to ducklings at a dose level 1000 times higher than the toxic ochratoxin A level. Further studies by Steyn & Holzapfel (1967a) showed that the LD_{50} values of ochratoxin A and its methyl and ethyl esters were similar, namely 135–170 μg/duckling. Purchase & Nel (1967) reported a similar LD_{50} value of 150 μg/duckling for ochratoxin A. Peckham, Doupnik & Jones (1971) reported a 7-day oral median lethal dose in day-old Babcock B-300 cockerels of 3·3–3·9 mg/kg for ochratoxin A and 54 mg/kg for ochratoxin B. Purchase & Theron (1968) found the oral LD_{50} value of ochratoxin A to be 22 mg/kg for both male and female rats.

Chu & Chang (1971) reported the oral LD_{50} values of ochratoxin A and C to be 166 and 216 mg/day-old chick, respectively. No toxic effect was demonstrated when chicks were fed up to 500 mg ochratoxin *a*, the acid-hydrolysis product of ochratoxin A. Chu, Noh & Chang (1972) found that ochratoxin *a* and *O*-methylochratoxin A ethyl ester were non-toxic to day-old chicks when given orally in doses of 1000 and 500 mg, respectively.

Doster, Sinnhuber & Wales (1972) reported the 10-day ip LD_{50} of ochratoxin A in 6-month-old rainbow trout to be 4·67 mg/kg body weight. Ochratoxin B produced no mortality at levels up to 66·7 mg/kg but produced histologically-detectable abnormalities in

the liver and kidney. Ochratoxins *a* and *b* were non-toxic at levels up to 28·0 and 26·7 mg/kg, respectively.

These results show that the absence of chlorine and/or phenylalanine from the ochratoxin A molecule markedly reduces its toxicity. The present study was conducted in an attempt to determine the effect of other changes in the ochratoxin molecule on ip toxicity in rainbow trout. The compounds studied included ochratoxins A, B and *a* ethyl esters and the leucine and alanine analogues of ochratoxin A.

EXPERIMENTAL

Preparation of ethyl esters of ochratoxins A, B and a. Ochratoxins A and B were prepared and purified as described elsewhere (Doster *et al.* 1972). Ochratoxin *a* was prepared from ochratoxin A by the acid-hydrolysis method of van der Merwe *et al.* (1965a) and was then purified as previously described (Doster *et al.* 1972). Approximately 10 mg of the ethyl esters of ochratoxins A, B and *a* were prepared from pure samples of the free acids by treatment with 16% boron trifluoride in dry ethanol. The resulting esters were purified by column chromatographic procedures similar to those used to purify ochratoxin A and B, except that the columns were eluted with carbon tetrachloride–glacial acetic acid (98 : 2 v/v). The stationary phase in these columns was 1% water-treated silica gel 60 (Brinkman).

Preparation of alanine and leucine analogues of ochratoxin A. Of particular interest was the question of whether replacement of the phenylalanine in ochratoxin A by other amino acids would have any influence on its toxicity. To test this possibility, the alanine and leucine analogues were prepared for us in toxicity studies. This was achieved by hydrolysis of the phenylalanine moiety from ochratoxin A (van der Merwe *et al.* 1965a) followed by replacement of this amino acid with L-alanine or L-leucine methyl esters by amide formation similar to that originally described for the synthesis of ochratoxin A (Steyn & Holzapfel, 1967b), except that the ochratoxin *a* acid chloride was prepared using oxalyl chloride instead of thionyl chloride. The esterifying methyl group was removed by interchange with acetic acid, and the products were purified by preparative thin-layer chromatography on MN-silica gel G-HR (Brinkman) using benzene–glacial acetic acid (9 : 1, v/v) as an eluant. The identity of the analogues was confirmed from mass spectra of their ethyl esters. The ethyl esters of the alanine and leucine analogues of ochratoxin A showed, in accordance with their formulae, molecular ions at m/e 355 and 397, respectively. Yields of the alanine and leucine analogues after purification were 3 and 36%, respectively.

The possibility of racemization in the synthesized analogues was considered. Since the absolute rotation of these analogues is unknown, their optical purity can be reliably estimated by determining the stereochemical integrity of the synthetic procedure. Ochratoxin A was prepared from ochratoxin *a* and L-phenylalanine methyl ester using methods identical to those used for the preparation of the two amino acid analogues. The resulting ochratoxin A (yield 40%) was crystallized from benzene and glacial acetic acid–tetrahydrofuran (3 : 1 v/v). The ultraviolet spectra and chromatographic properties were identical to those of natural ochratoxin A. The product had a specific rotation (sodium D-line) of $-79°$ determined in glacial acetic acid–chloroform (1 : 1, v/v). Roberts & Woollven (1970) reported the specific rotation of (−, ±)ochratoxin A as $+38°$ (the phenylalanine is active, but the isocoumarin is racemic) and that of natural ochratoxin A as $-93°$. Thus, the synthetic ochratoxin A prepared as described in this study was 11% racemized.

Purity of test compound. The purity of the compounds prepared for the toxicity studies was checked by spotting 8–10 mg on a silica gel G-HR chromatoplate and developing the

plate in carbon tetrachloride–glacial acetic acid (9:1, v/v). Examination under long-wave ultraviolet radiation (365 nm) established that only one fluorescent spot was present on the plate. Developed plates were also placed in an iodine chamber for 30 min to allow detection of possible non-fluorescent impurities.

Quantitation. The ochratoxin test compounds were quantitated spectrophotometrically using the molar absorptivity values shown in Table 1. The molar absorptivity value for ochratoxin *a* ethyl ester was determined experimentally. That of ochratoxin A (Doster *et al.* 1972) was used for quantitation of the two ochratoxin A analogues, since insufficient quantities of these compounds were prepared for accurate weighing. The molar absorptivity values, used for the quantitation of ochratoxin A and B ethyl esters, were reported by S. Nesheim (personal communication 1968) and Nesheim (1969), respectively.

Table 1. *Data used in spectrophotometric quantitative analyses of ochratoxin derivatives*

Ochratoxin	Mol wt	λ max (nm)	ε	Solvent*
A, ethyl ester (C)	431	333	6200	I
B, ethyl ester	397	318	6700	$CHCl_3$
a, ethyl ester	284	336	6250	95% ethanol
A, alanine analogue	327	333	5550	II
A, leucine analogue	369	333	5550	II

* Solvent I—benzene–acetonitrile–glacial acetic acid (97:2:1, by vol.); Solvent II—benzene–glacial acetic acid (99:1, v/v).

Quantitatively defined solutions of the toxins were evaporated to dryness *in vacuo*. Final traces of harmful organic solvents such as benzene and carbon tetrachloride were removed from the test compounds by repeated solution in 95% ethanol and evaporation *in vacuo*. The ochratoxin esters were dissolved in corn oil to the following concentrations (in mg/ml): C, 0·600; B ethyl ester, 2·250; *a* ethyl ester, 0·585. The alanine and leucine analogues of ochratoxin A were dissolved in 0·1 N-sodium bicarbonate to concentrations of 0·673 and 0·763 mg/ml, respectively. Subsequent dilutions were made from these stock solutions.

Toxicity experiments. Groups of 6-month-old domesticated rainbow trout of the Mt. Shasta strain, weighing an average of 35 g, were treated ip with ochratoxin test compounds as previously described (Doster *et al.* 1972). Trout were given various dose levels of the test compounds in x μl of the carrier, where x = weight of trout (in g)/0·15. Control trout were similarly dosed with 0·1 N-sodium bicarbonate or corn oil. Mortalities during a 10-day experimental period were recorded, and the LD_{50} was determined by the method of Litchfield & Wilcoxon (1949). Trout surviving after 10 days were killed and immediately autopsied. Gross abnormalities were noted, and the livers and kidneys were excised and preserved in Bouin's fixative. These tissues were embedded in paraffin, sectioned at 4 μm, stained with haematoxylin and eosin and examined by light microscopy.

RESULTS

Mortality

Of the test compounds administered, only the ethyl esters of ochratoxin A and B were toxic. Single ip doses of ochratoxin C (ochratoxin A ethyl ester) equivalent to 2, 3 and 4

mg/kg body weight caused mortalities of 1/10, 5/10 and 9/10, respectively; and doses of ochratoxin B ethyl ester at levels of 10, 12·5 and 15 mg/kg caused mortalities of 1/10, 4/10 and 8/10, respectively. The acute LD_{50} of ochratoxin C was 3·0 mg/kg body weight (6·96 μmols/kg) with 95% confidence limits of 2·37–3·80 mg/kg body weight. The slope function was 1·31 with 95% confidence limits of 0·97–1·77 mg/kg body weight. The acute LD_{50} of ochratoxin B ethyl ester was 13·0 mg/kg (32·75 μmols/kg) with 95% confidence limits of 10·30–16·41 mg/kg body weight. The slope function was 1·21 with 95% confidence limits of 0·89–1·63 mg/kg body weight.

Previous studies conducted in this laboratory (Doster *et al.* 1972) indicated that the ip LD_{50} of ochratoxin A was 4·67 mg/kg body weight in 6-month-old rainbow trout. Ochratoxin B caused no deaths at levels up to 66·7 mg/kg body weight. The ochratoxin A LD_{50} determination was repeated using progeny from the same female trout as those used in testing all other ochratoxin compounds in this study. The LD_{50} value obtained was 5·53 mg/kg body weight (13·72 μmols/kg) with 95% confidence limits of 4·45–6·85 mg/kg body weight. The slope function was 1·28 with 95% confidence limits of 0·94–1·73 mg/kg body weight. Ochratoxin B was again not lethal in ten trout given an ip dose of 66·7 mg/kg body weight, a level 12 times the LD_{50} of A.

The alanine and leucine analogues caused no deaths in ten trout in a dose of 4·49 and 5·06 mg/kg body weight, respectively. Similarly, ochratoxin *a* ethyl ester given at a level of 3·9 mg/kg body weight produced no mortalities in ten trout. The dose levels of these three compounds were equivalent on a molar basis to the ochratoxin A LD_{50} value of 13·72 μmols/kg.

Histological changes

Livers and kidneys from trout dosed with ochratoxin C showed necrotic changes identical to those previously described in trout dosed with ochratoxin A (Doster *et al.* 1972). Livers from trout dosed with ochratoxin B ethyl ester showed definite injury, but this damage was not as severe as that seen in livers from trout dosed with toxic levels of ochratoxin A or C.

In general, these livers had a normal architecture but, in many cases, showed areas of swollen or hypertrophic parenchymal cells. Some livers contained large nuclei scattered around the periphery of the muralia. Some livers also contained an occasional focus of bile-duct proliferation. Kidneys from these trout contained necrotic haematopoietic tissue and swollen or pyknotic tubule nuclei. There was a general loss of tubular patency and casts were present in renal tubules.

Livers from trout dosed with the leucine analogue of ochratoxin A in a dose of 13·72 μmols/kg showed minor signs of injury. This was determined by the presence of occasional foci of bile-duct proliferation as well as of slightly swollen nuclei. Kidneys from these trout showed damage similar to that caused by non-lethal levels of ochratoxin A. The most noticeable abnormality was necrosis of the haematopoietic tissue. Other abnormalities included swollen nuclei in the tubules, loss of tubular patency and cast formation.

Livers from trout dosed with the alanine analogue of ochratoxin A (13·72 μmols/kg), ochratoxin *a* ethyl ester (13·72 μmols/kg), 0·1 N-sodium bicarbonate or corn oil showed no histological abnormalities. Kidneys from the latter three groups of trout were similarly free from histological abnormalities. Kidneys from trout dosed with the alanine analogue of ochratoxin A showed necrotic haematopoietic tissue as well as some swollen nuclei in the tubules. These abnormalities were not nearly as noticeable as those seen in kideneys

from trout dosed with the leucine analogue of ochratoxin A. Furthermore, no casts were evident in the lumen of the tubules, indicating that damage was much less severe in these kidneys.

DISCUSSION

Results of this study and others show that acute toxicity is enhanced when the basic structure of the non-chlorinated isocoumarin moiety of ochratoxin is changed in such a way that its molecular weight is increased to nearly 400 but the anionic nature of the molecule is still maintained. These changes may play a role in altering the distrubution of the molecule in the trout to bring about greater exposure of target sites in various tissues, where the lethal action is apparent.

The influence of molecular weight is borne out by the observation that the relatively non–toxic ochratoxin B molecule (mol wt 369) is made much more toxic when converted to its ethyl ester (mol wt 397). Also, the toxicity of ochratoxin A (mol wt 403) is about half that of its ethyl ester (mol wt 431). It is realized that the use of different vehicles to administer these toxins may have had some influence on their relative toxicities, but this complication was necessary because the esters were not soluble in aqueous bicarbonate and the free acids were only poorly soluble in corn oil. It would be expected that the esters given in corn oil would normally be absorbed more rapidly than the free acids in aqueous bicarbonate (Schou, 1971) and this may have influenced their comparative toxicities.

It is noteworthy that Chu & Chang (1971) did not find the ethyl and methyl esters of ochratoxin A to be more toxic than ochratoxin A to day-old chicks. Also Steyn & Holzapfel (1967a) found ochratoxin B esters to be non-lethal to day-old ducklings. In the report of the latter study, details of the experimental conditions were not provided, making comparisons with the present work impossible. In the investigation conducted by Chu & Chang (1971) chicks were dosed orally with bicarbonate solutions of ochratoxin A and corn oil solutions of ochratoxin A esters.

Since all the toxins were administered orally, it is probable that the ochratoxin esters were hydrolysed to the free acids in the gut of the chicks before absorption took place. In the present study, the ochratoxin esters dosed ip would not be likely to be subjected to conditions which would result in significant ester hydrolysis until after absorption by the liver and/or excretion in the bile.

Perhaps of greater value in exemplifying the effect of molecular weight on possible distribution differences and consequent toxicity are the results obtained for the alanine and leucine analogues of ochratoxin A (mol wt 327 and 369 respectively). Neither of these compounds caused any mortalities in trout when given in a dose that was the molar equivalent of the ochratoxin A LD_{50}. The leucine analogue did, however, induce some histologically-detectable abnormalities in the trout liver and kidney.

Any possible explanation for the difference in toxicity of ochratoxins A and B and their analogues necessitates consideration of the influence of differences in ionic property as well as in molecular size and structure. Chu *et al.* (1972) reasoned that the difference in the apparent dissociation constant of the phenolic hydroxyl group in ochratoxins B and C may cause ochratoxin C to bind more readily than ochratoxin B to important cellular components. It is conceivable that this difference in the ionic properties of ochratoxins A and B could also play a role in altering their distribution, to hasten or hinder their transport to receptor sites within the cells of tissues where their lethal action is apparent. This

hypothetical attempt to relate ochratoxin toxicity to potential differences in biological distribution does not take into account possible differences in ochratoxin transformation to metabolites that are more readily excreted than the parent toxins. Doster & Sinnhuber (1972) noted that ochratoxin B was more readily hydrolysed *in vitro* to its non-toxic isocoumarin moiety than was ochratoxin A. Metabolic propensities may also alter the biological distribution of the ochratoxins and affect their ultimate toxicity.

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Toxicité intrapéritonéale aiguë des dérivés A et B de l'ochratoxine chez la truite arc-en-ciel (*Salmo gairdneri*)

Résumé—On détermine la toxicité ip aiguë de cinq dérivés des ochratoxines A et B, métabolites de l'*Aspergillus ochraceus*, en utilisant comme animal d'épreuve la truite arc-en-ciel de souche Mt. Shasta âgée de 6 mois. Les esters éthyliques des ochratoxines A et B sont mortels pour la truite quand ils sont administrés avec de l'huile de maïs. Les valeur DL_{50}-10 jours pour les esters éthyliques des ochratoxines A et B sont respectivement de 3,0 et 13,0 mg/kg de poids du corps. Les ochratoxines A et B, dont les toxicités ip avaient été déterminées préalablement dans une autre étude, ont été administrées dans le présent travail en utilisant les conditions expérimentales habituelles. La DL_{50} de l'ochratoxine A est de 5,53 mg/kg du poids corporel (13,72 μmoles/kg) tandis que l'ochratoxine B à une dose de 66,7 mg/kg de poids du corps ne produit aucune mort. L'ester éthylique de l'ochratoxine A administré par voie ip dans de l'huile de maïs et les analogues alanine et leucine de l'ochratoxine A administrés dans une solution aqueuse de bicarbonate, ne sont pas mortels chez la truite à une dose de 13,72 μmoles/kg de poids du corps (DL_{50} de l'ochratoxine A). On décrit les dégâts histopathologiques produits par ces toxines dans le foie et le rein de la truite.

Akute intraperitoneale Toxizität von Ochratoxin A und B Abkömmlingen in der Regenbogenforelle (*Salmo gairdneri*)

Zusammenfassung—Die akuten ip Toxizitäten von fünf Abkömmlingen von Ochratoxin A und B, Metaboliten von *Aspergillus ochraceus*, wurden unter Verwendung von 6-Monate alten Mt. Shasta-Stamm Regenbogenforellen als Versuchstiere bestimmt. Äthylester von Ochratoxin A und B waren letal für die Forellen, wenn sie in Maisöl verabreicht wurden. Die 10-Tage LD_{50} Werte von Ochratoxin A und B Äthylester waren 3·0 und 13·0 mg/kg Körpergewicht respektiv. Ochratoxin A und B, deren ip Toxizität früher in der Regenbogenforelle in einer anderen Studie bestimmt worden war, wurden in dieser Studie wieder unter den laufenden experimentellen Bedingungen gegeben. Die LD_{50} von Ochratoxin A war 5·53 mg/kg Körpergewicht (13·72 μmol/kg), während Ochratoxin B keine Todesfälle verursachte bei einer Dosis von 66·7 mg/kg Körpergewicht. Die Äthylester von Ochratoxin A, gegeben ip in Maisöl, und die Alanin- und Leucinanaloge von Ochratoxin A, gegeben in wässrigem Bikarbonat, waren nicht letal für die Forelle bei einem Dosisspiegel von 13·72 μmol/kg Körpergewicht (die LD_{50} von Ochratoxin A). Histopathologischer Schaden verursacht durch diese Toxine in der Forellenleber und -niere wird beschrieben.

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SHORT PAPERS

IMPLANTATION AND EMBRYONIC SURVIVAL IN RATS TREATED WITH AMARANTH DURING GESTATION

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Summary—Samples of amaranth obtained from three manufacturers were administered to rats by gavage or mixed in a semi-synthetic diet, to provide in each case dosage levels of 0, 15, 30, 100 or 200 mg/kg body weight/day, from day 1 to 19 of pregnancy (day 1 being the day sperm were found in vaginal smears). Dams were killed on day 20 of gestation. Prenatal values for corpora lutea, live foetuses, deciduomas (suggestive of early embryonic death or resorption), dead foetuses and foetal weight gave no evidence of any amaranth-related deleterious effect on implantation or embryonic survival.

Introduction

Amaranth, the trisodium salt of 1-(4-sulpho-1-naphthylazo)-2-naphthol-3,6-disulphonic acid has been reported to have adverse effects on embryonic survival in rats following dosing for three generations (Shtenberg & Gavrilenko, 1970) and when given by daily gavage on days 0–19 of pregnancy, day 0 being the day sperm were found in vaginal smears (Collins, McLaughlin & Gray, 1972). Interim results from further multigeneration feeding studies in rats indicated no adverse effect even at high dose-levels, and teratogenicity studies with amaranth given by gavage on days 6–15 of pregnancy produced negative results (*Food Chemical News*, 1973). However, the lingering question as to whether amaranth is embryocidal following daily gavage during the entire period of gestation has led to controversy over its safety for human consumption. These studies were undertaken in an attempt to resolve the controversy.

Experimental

Materials. Three samples of amaranth, one from L. J. Pointing & Son Ltd., Northumberland (lot L5890, certificate 1887; sample A_1), one from Williams (Hounslow) Ltd., Middlesex (lot 9/630M; sample A_2) and one from Kohnstamm & Co., New York (FDA lot Z0287; sample A_3) were tested. The samples were certifiable by Canadian Standard and had a dye content (%) of 91·1 in A_1, 87·0 in A_2 and 92·1 in A_3. Impurities present were NaCl and Na_2SO_4 (3–4%), subsidiary dyes (0·7–2·0%), naphthionic acid (0·1–0·2%) and R acid (0·02–0·2%), the combined ether extracts, the volatile matter and the water-insoluble matter constituting 0·08–0·15, 2·0–4·0 and 0·03–0·05%, respectively.

Animals and diet. Randomly-bred female Wistar rats (175–200 g body weight) obtained from Woodlyn Farms, Guelph, Ontario, were started on a semi-synthetic diet containing, in %: corn starch, 64; casein, 20; Mazola corn oil, 6; alphacel, 5; mineral mix, 4 (Jones & Foster, 1942); vitamin mix AM (Becking & Morrison, 1970). The corn oil was from

Canada Starch Co. Ltd., Montreal, and other ingredients were from General Biochemical Laboratory Park, Ohio.

Experimental procedures

Females were paired overnight with proven sires and those with spermatozoa in the vagina on the following morning (counted day 1 of pregnancy) were randomly distributed among the experimental groups. Each sample of amaranth was administered on days 1–19 of pregnancy in one study by gavage as a single daily dose and, in the other, mixed in the diet.

Gavage study. The doses given by gavage were 0 (distilled water), 15, 30, 100 and 200 mg/kg/day, the four dose levels of amaranth being given in concentrations of 0·33, 0·66, 2 and 4%, respectively. Pregnant rats were weighed daily in order to permit the quantity of solutions administered to be adjusted so that a constant dose/body-weight ratio was maintained during pregnancy.

Diet study. The control group continued to receive the semi-synthetic diet, while the test groups were fed the same diet with amaranth added to provide an intake of 15, 30, 100 or 200 mg/kg body weight/day. The amaranth was thoroughly mixed in the diets and the diets were stored in air-tight containers at 6°C. The animals received diets and water *ad lib.*

Post-mortem studies. All female rats were killed on day 20 of pregnancy and their ovaries were examined for pathological changes and for enumeration of corpora lutea. Early resorptions or deciduoma (Bateman & Epstein, 1971) and foetuses dying late in development were recorded. The foetuses were removed, weighed and examined for viability and external malformations.

Statistical evaluation. The data were handled through t distribution. Population means and standard deviations from test groups were compared with the within-experiment control to compute t values (1-tail test; Dunn, 1964). Differences not significant at $P < 0{\cdot}10$ were considered negative.

Results

There was no evidence of maternal toxicity in any of the experiments. Prenatal values for corpora lutea, live foetuses, deciduomas and foetal weights are summarized in Table 1. No deleterious effect was observed by these criteria. The ratios of corpora lutea to live foetuses plus deciduomas were within control limits.

All embryonic mortality was reflected in the formation of deciduomas except for two foetuses that died late in development—one in the A_1 30 mg/kg group and one in the A_2 control group of the diet study. One exencephalic foetus was observed in the A_1-200 mg/kg group in the gavage test and in the A_2-30 mg/kg and the A_3-100 mg/kg groups of the diet experiment.

Discussion

The present study failed to demonstrate a significant effect on any prenatal parameters that were investigated at doses up to 20 mg amaranth/kg/day given for almost the entire duration of pregnancy. Embryonic survival remained unaffected whether the test compound was given by gavage or mixed in the diet and the incidence of foetal mortality was within control limits in all test groups. Although three exencephalic foetuses were observed in the test groups and none in the control groups, the incidence was consistent with the

Table 1. *Effects on prenatal development of treating pregnant rats with amaranth in daily doses of 15–200 mg/kg/day on days 1–19 of pregnancy*

Test compound	Mode of administration	Dose (mg/kg/day)	No. of pregnant rats/group	Corpora lutea (no./litter)*	Live foetuses (no./litter)*	Deciduomas (% of total implants)	Foetal weight (g)*
A_1	Gavage	0	18	13·9 ± 2·2	11·9 ± 2·8	10	2·1 ± 0·1
		15	17	14·9 ± 1·6	12·5 ± 2·5	11	2·0 ± 0·2
		30	19	14·2 ± 2·7	11·5 ± 2·4	13	2·1 ± 0·1
		100	19	14·1 ± 3·0	11·5 ± 3·0	7	2·1 ± 0·2
		200	17	13·4 ± 2·0	11·4 ± 3·2	7	2·0 ± 0·3
	In diet	0	19	13·3 ± 1·8	11·6 ± 2·3	10	2·1 ± 0·2
		15	19	13·1 ± 2·5	11·6 ± 2·4	5	2·2 ± 0·1
		30	18	13·9 ± 1·8	12·1 ± 1·3	10	2·1 ± 0·2
		100	19	13·7 ± 1·6	11·7 ± 2·0	11	2·2 ± 0·1
		200	18	14·4 ± 2·1	12·8 ± 2·0	7	2·2 ± 0·1
A_2	Gavage	0	18	14·2 ± 1·7	11·5 ± 3·1	8	2·1 ± 0·4
		15	13	13·4 ± 3·2	11·4 ± 3·7	8	2·1 ± 0·6
		30	18	12·8 ± 2·8	10·4 ± 3·9	9	2·0 ± 0·2
		100	16	13·1 ± 1·6	10·9 ± 1·8	8	2·1 ± 0·3
	In diet	0	16	14·4 ± 1·5	11·3 ± 3·1	11	2·1 ± 0·2
		15	18	13·1 ± 2·1	10·3 ± 3·5	8	2·2 ± 0·2
		30	14	13·6 ± 1·2	11·5 ± 2·0	6	2·2 ± 0·2
		100	17	12·9 ± 2·2	11·2 ± 3·1	8	2·1 ± 0·2
A_3	Gavage	0	16	12·9 ± 2·3	11·6 ± 2·3	7	1·9 ± 0·5
		15	18	12·2 ± 2·0	11·3 ± 2·5	7	2·2 ± 0·2
		30	16	13·0 ± 2·3	11·1 ± 3·6	10	2·3 ± 0·3
		100	17	13·4 ± 1·7	11·2 ± 3·3	7	2·2 ± 0·2
		200	16	14·2 ± 2·0	12·8 ± 1·4	7	2·1 ± 0·1
	In diet	0	19	13·2 ± 1·6	11·6 ± 2·1	7	2·1 ± 0·2
		15	18	13·6 ± 2·0	12·4 ± 2·9	5	2·1 ± 0·3
		30	18	12·9 ± 2·1	11·5 ± 3·2	5	2·1 ± 0·6
		100	19	13·6 ± 1·8	12·3 ± 2·0	6	2·1 ± 0·2
		200	18	14·3 ± 1·4	13·3 ± 1·3	6	2·2 ± 0·1

*Values are means ± SD for numbers of litters indicated.

background incidence recorded in Woodlyn Farms' rats over the past several years. The finding did not suggest an adverse effect related to the test compounds.

Our observations that amaranth had no adverse effects on implantation and mean foetal weight were consistent with those of Collins *et al.* (1970) but, contrary to their results, our studies failed to show any amaranth-related embryocidal effect.

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SOME INTER-RELATIONSHIPS BETWEEN VITAMIN C (L-ASCORBIC ACID) AND MERCURY IN THE GUINEA-PIG

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Summary—Young male guinea-pigs were used to study the interrelationships of L-xyloascorbic acid (vitamin C) and orally administered mercuric mercury (8 mg Hg^{2+}/kg body weight). Mercury significantly reduced the concentration of ascorbic acid in the brain, adrenals and spleen of animals receiving maintenance doses of the vitamin. Large doses of ascorbic acid resulted in an increased deposition of mercury in the liver and kidney. Mercury administration depressed the growth rate and altered the weights of the liver and kidney, and these changes were not affected by large intakes of ascorbic acid. The mercury-induced adrenal hypertrophy, however, was prevented by large doses of ascorbic acid.

Introduction

"Shun mercury as poison" was Kramer's advice to scorbutic patients according to George Budd, a London physician, in 1840. Budd himself claimed that in cases of scurvy "....mercury in every form should be religiously avoided [as] we have met with instances in which the scorbutic symptoms seemed to have been much aggravated by mercury, taken before the scurvy made its appearance" (Budd, 1840). Budd was an astute observer with a suitably cautious scientific approach and it is unlikely that he would have emphasized this apparent relationship between scurvy and mercury had he not a strong observational basis for doing so (Hughes, 1973a).

Currently, dietary intakes of vitamin C (L-xyloascorbic acid; ascorbic acid) in Britain may vary from the inadequate to the massive doses recommended by the "megavitamin C therapists" (Brook, 1972; Hughes, 1973b). Any relationship between the tissue levels of ascorbic acid and the uptake and retention of mercury could therefore be of some significance. The observations of Budd (1840) indicated that low tissue levels of ascorbic acid increased a person's susceptibility to mercury poisoning (or, conceivably, that an increased intake of mercury exacerbated the scorbutic condition). As a possible corollary of this one would perhaps expect a state of tissue saturation with ascorbic acid to afford some degree of protection against mercury poisoning.

The experiments described in this note were designed to examine both the effect of mercury on tissue levels of ascorbic acid and the effect of large doses of ascorbic acid on tissue levels of mercury. Guinea-pigs were used because of their total dependence upon dietary ascorbic acid.

Experimental

Animals and diet. Male albino guinea-pigs (initial body weight 325 g) were used for two experiments. They were fed a semi-synthetic scorbutogenic diet, previously described (Williams & Hughes, 1972) and containing no detectable amounts of ascorbic acid. All animals received a weekly supplement of 0·5 ml cod-liver oil.

Experiment 1. Three groups each of eight guinea-pigs were used. Groups A and B received a daily supplement of 5 mg ascorbic acid/kg body weight, an intake sufficient to maintain normal growth rate without raising the tissue ascorbic acid concentrations above 25% saturation. Group C received a daily supplement of 1% ascorbic acid in the drinking-water; this resulted in saturation of the tissues with ascorbic acid (Hughes & Jones, 1970). Animals in groups B and C received a daily dose of 8 mg Hg^{2+}/kg body weight, given as $HgCl_2$ in water. Both the ascorbic acid and the mercury supplements were given in the morning and within 30 min of each other. After 15 days the animals were weighed and killed, and ascorbic acid and mercury were determined in liver, kidney, adrenals, spleen and brain.

Experiment 2. Two groups (A and B), each comprising six guinea-pigs, received an ascorbic acid supplement (5 mg/kg body weight) orally at 09.30 hr daily. Group B also received an oral supplement of 8 mg Hg^{2+}/kg body weight at 17.30 hr daily. This experiment was terminated after 15 days and the tissue levels of ascorbic acid were determined in adrenals, spleen and brain.

Tissue analysis. The animals were killed by stunning followed by decapitation and exsanguination. Organs were quickly dissected out, dried with filter paper and weighed. Ascorbic acid was determined by the 2,6-dichlorophenolindophenol photometric method (Bessey, 1938; Hughes, 1956). Mercury was determined by a cold vapour atomic absorption method (Braun & Husbands, 1971).

Results

The mercury was administered at a level shown, in preliminary experiments, to depress the normal growth rate of guinea-pigs. In the first experiment described in this note, the growth rate of the mercury-treated animals was 50% of that of the group not given mercury (Table 1). This depression of growth rate was independent of the ascorbic acid status of the animal. The relative weights of both kidney and liver were altered by mercury (Table 1), the relative weight of the kidney being significantly increased by mercury treatment while that of the liver was decreased. These changes occurred in both group B and C. However, hypertrophy of the adrenal glands occurred only in group B.

The ascorbic acid levels in the tissues of the mercury-treated group B in experiment 1 were consistently and significantly lower than those in the group (A) not given mercury (Table 2). Similar reductions in tissue levels of ascorbic acid were found when the mercury was administered 8 hr after the ascorbic acid (Table 3, experiment 2). The levels of mercury in the liver, kidney and brain of guinea-pigs given different amounts of ascorbic acid (experiment 1) are shown in Table 4. The higher dietary intake of ascorbic acid (group C) resulted in a marked increase in the amounts of mercury deposited in these tissues.

Discussion

The levels of ascorbic acid found in the tissues of groups not treated with mercury (Tables 2 & 3) were of the order to be expected from the intake level used (Hughes & Jones, 1971), while the pattern of mercury distribution in the tissues studied (Table 4) was similar to that reported for man and for the rat (Hughes, 1957; Emerick & Holm, 1973).

The changes induced by mercury in the relative weights of the kidney and liver, both sensitive criteria in toxicological studies (Feron, de Groot, Spanjers & Til, 1973), were (like the depression in body weight) independent of the ascorbic acid status of the animals. The fact that adrenal gland hypertrophy was limited to the mercury-treated group given only

Table 1. *Body and organ weights of guinea-pigs given different amounts of ascorbic acid and mercury for 15 days (experiment 1)*

Test group	Intake (mg/kg†/day) of		Body weights (g)		Organ weights							
					Liver		Spleen		Adrenals		Kidney	
	Ascorbic acid	Mercury (Hg^{2+})	Initial	Terminal	g	%	g	%	g	%	g	%
A	5	0	323 ± 10·6	464 ± 7·6	22·56 ± 1·19	4·88 ± 0·69	0·844 ± 0·561	0·181 ± 0·013	0·165 ± 0·010	0·036 ± 0·002	3·78 ± 0·11	0·81 ± 0·02
B	5	8	322 ± 11·1	415 ± 13·1**	17·39 ± 0·70**	4·20 ± 0·37*	0·741 ± 0·101	0·179 ± 0·008	0·201 ± 0·013*	0·049 ± 0·003**	4·01 ± 0·20	0·97 ± 0·06*
C	1%‡	8	327 ± 10·0	396 ± 17·1*	15·02 ± 0·75**	3·78 ± 0·37*	0·803 ± 0·203	0·203 ± 0·016	0·153 ± 0·016	0·039 ± 0·003	3·82 ± 0·15	0·97 ± 0·07*

†Of body weight.

‡Administered at a level of 1% in the drinking water.

Values are means for groups of eight guinea-pigs and those marked with asterisks differ significantly (Student's *t* test) from the corresponding value for group A: *$P < 0·05$; **$P < 0·02$.

Table 2. *Ascorbic acid levels in the organs of guinea-pigs given different amounts of ascorbic acid and mercury for 15 days (experiment 1)*

Test group	Intake (mg/kg†/day) of Ascorbic acid	Intake (mg/kg†/day) of Mercury (Hg^{2+})	Ascorbic acid concn (mg/100 g tissue) Brain	Adrenals	Spleen
A	5	0	7·97 ± 0·32	41·5 ± 3·9	11·79 ± 1·02
B	5	8	5·18 ± 0·32**	21·2 ± 3·8**	7·41 ± 1·13*
C	1%‡	8	21·53 ± 1·24	183·1 ± 9·3	59·03 ± 2·98

†Of body weight.
‡Administered at a level of 1% in the drinking water.
Values are means for groups of eight guinea-pigs and those marked with asterisks are significantly lower (Student's *t* test) than the corresponding value for group A: $*P < 0·05$; $**P < 0·01$.

Table 3. *Ascorbic acid levels in guinea-pigs given ascorbic acid and mercury daily at different times for 15 days (experiment 2)*

Test group	Intake (mg/kg†/day) of Ascorbic acid	Intake (mg/kg†/day) of Mercury (Hg^{2+})	No./group	Ascorbic acid concn (mg/100 g wet tissue) Brain	Adrenals	Spleen
A	5	0	5	9·99 ± 0·14	45·12 ± 3·00	14·10 ± 0·68
B	5	8	6	8·68 ± 0·43*	26·34 ± 3·01**	10·05 ± 0·09**

†Of body weight.
‡Ascorbic acid was administered at 09.30 hr each day and mercury at 17.30 hr.
Values are means for the numbers of animals stated and those marked with asterisks differ significantly (Student's *t* test) from the corresponding value for group A: $*P < 0·05$; $**P < 0·01$.

Table 4. *Mercury levels in tissues of guinea-pigs given mercury and different amounts of ascorbic acid for 15 days (experiment 1)*

Test group	Intake (mg/kg†/day) of Ascorbic acid	Intake (mg/kg†/day) of Mercury (Hg^{2+})	Mercury concn (μg Hg^{2+}/g wet tissue) Liver	Kidney	Brain
B	5	8	16·10 ± 1·24 (7)	362·1 ± 41·7 (6)	1·22 ± 0·15 (7)
C	1%‡	8	27·08 ± 4·93*(5)	473·5 ± 29·4*(7)	2·26 ± 0·53 (7)

†Of body weight.
‡Administered at a level of 1% in the drinking water.
Values are means for the numbers of animals indicated in parentheses, and those marked with an asterisk differ significantly (Student's *t* test) from the value for group B: $*P < 0·05$.

5 mg ascorbic acid/kg body weight appeared to suggest that tissue saturation with ascorbic acid 'protected' the adrenal glands from mercury-induced hypertrophy. The increase in adrenal size was presumably a stress-induced change rather than a direct effect of the mercury toxicity; it is generally accepted that high doses of ascorbic acid can prevent the adrenal hypertrophy that normally follows stress situations such as exposure to cold (Dugal & Therien, 1949).

Of the reductions in tissue ascorbic-acid levels associated with mercury treatment (Tables 2 & 3), the depression in brain ascorbic acid is of particular interest as brain ascorbic acid is in general more resistant to change than is the ascorbic acid of the other organs examined (Hughes, Hurley & Jones, 1971). It is therefore quite conceivable that in scor-

butic or near-scorbutic subjects, mercury could still further reduce the already low levels of ascorbic acid and so precipitate or exacerbate the scorbutic symptoms, as described by Budd (1840).

Mercuric cations have a high affinity for thiol groups (Clarkson, 1972) and there is a tendency to ascribe the toxicity of mercury either directly or indirectly to this, although at least one study has indicated that tissue thiol groups are not reduced by administration of mercury to chicks (Miller, Bearse, Russell & Csonka, 1969). Thiol groups are conceivably involved in the abstraction and/or retention of ascorbic acid by the tissues and interference with these mechanisms could account for the mercury-induced depression in tissue ascorbic acid. Thiol groups also have a somewhat more direct role in the 'preservation' of tissue ascorbic acid and in its reduced form (Grimble & Hughes, 1967) and any diminution in this biological reducing capacity of tissue thiols would presumably result in lower levels of tissue ascorbic acid.

Two additional factors must be considered in a study of this type. There is evidence that a depression in growth rate or a reduction in metabolic activity results in a fall in tissue ascorbic acid (Williams & Hughes, 1972). It is therefore difficult in a study of this type unequivocally to distinguish between effects directly attributable to the presence of mercury in the tissues and those resulting from the mercury-induced depression in growth rate.

Again, the lowered tissue levels of ascorbic acid could reflect mercury interference with the absorption of ascorbic acid from the gastro-intestinal tract, although the finding of similar falls in tissue ascorbic acid when the administration of the ascorbic acid and the mercury were temporally separated (Table 4) reduces the credibility of this explanation. A further experiment in which the mercury is introduced orally and the ascorbic acid parenterally would be required to give an unequivocal answer to this.

Somewhat surprisingly, high dietary intakes of ascorbic acid resulted in elevated amounts of mercury being deposited in the tissues (Table 3). There is considerable evidence that ascorbic acid will enhance the absorption of iron (Moore, 1961) but on the other hand, Sahagian, Harding-Below & Perry (1967) reported that ascorbic acid reduced the *in vitro* absorption of cadmium (an element closely related to mercury). Other workers found later that, *in vivo*, large doses of ascorbic acid had no obvious effect on the deposition in the tissues of cadmium administered in the diet (Fox, Fry, Harland, Schertel & Weeks, 1971). If one assumes that the intestinal transport of mercury is related to its affinity for binding sites, and that thiol groups are of importance in this respect, then large doses of ascorbic acid could presumably assist the transport of mercury by maintaining the integrity of thiol groups. It is conceivable, though less likely, that a similar relationship could influence the retention of mercury in the tissues.

The situation carries certain implications if extrapolated to the level of human physiology. Elevated intakes of mercury could conceivably reduce tissue levels of ascorbic acid. Of possibly greater significance is the effect of massive intakes of ascorbic acid on the uptake and retention of mercury. Subjects exposed to higher-than-average concentrations of environmental mercury should perhaps avoid "megavitamin therapy". In this respect, it would be of interest to determine whether, on a population basis, a correlation exists between blood levels of ascorbic acid and of mercury. In more general terms, the possibility emerges that tissue ascorbic acid levels could well be of value as an index to toxicity.

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MONOGRAPHS

Monographs on Fragrance Raw Materials*

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DIETHYLENE GLYCOL MONOETHYL ETHER

Synonym: Ethyl diethylene glycol.
Structure: $CH_2(OH) \cdot CH_2O \cdot [CH_2]_2 \cdot OCH_2 \cdot CH_3$.
Description and physical properties: A colourless hygroscopic liquid with a faint ethereal odour with a musty undertone (Arctander, 1969).
Occurrence: Has apparently not been reported to occur in nature.
Preparation: From ethyl alcohol plus ethylene oxide (Arctander, 1969).
Uses: Use in fragrances in the USA amounts to less than 5000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·05	0·005	0·025	0·5
Maximum	0·3	0·03	0·1	2·0

Analytical data: Gas chromatogram, RIFM no. 72–17; infra-red curve, RIFM no. 72–17.

Status

Browning (1965) has an extensive monograph on diethylene glycol monoethyl ether.

Biological data

Acute toxicity. The acute oral LD_{50}s in rats, mice and guinea-pigs were reported as 5·54, 6·58 and 3·87 g/kg, respectively (Laug, Calvery, Morris & Woodard, 1939). The LD_{50}s in mice, rats and rabbits from iv injection were reported as 3·9, 2·9 and 0·9 ml/kg, respectively, and the sc LD_{50}s in rats and rabbits were reported as 3·4 and 2·0 ml/kg, respectively (Stenger, Aeppli, Lislott, Müller, Peheim & Thomann, 1971). The acute dermal LD_{50} value in rabbits was reported as 10·3 g/kg (Carpenter, 1947).

Short-term toxicity. The no-effect level for diethylene glycol monoethyl ether administered to five rats in the drinking-water for 30 days was 0·49 g/kg, while 0·87 g/kg caused reduction in appetite and 1·77 g/kg caused some organic injury (Smyth & Carpenter, 1948).

In a 4-wk sc study in rats, doses of 100–400 μl/kg/day produced no deaths, although at 200–400 μl/kg/day, dyspnoea, somnolence and mild ataxia appeared, with some growth retardation in females. Doses of 800 μl/kg/day had similar effects and, in addition, growth

*Previous sets of these monographs were published in *Food and Cosmetics Toxicology* (1973, **11**, 95, 477, 855 & 1011; 1974, **12**, 385).

was depressed also in males following some reduction in food intake. Changes in liver, kidney and testes were also seen in rats given 200 μl/kg/day orally (Stenger *et al.* 1971).

Diethylene glycol monoethyl ether containing less than 0·4% ethylene glycol was given for 90 days to rats at dietary levels of 0·5 or 5·0%, to mice at dietary levels of 0·2, 0·6, 1·8 or 5·4% and to pigs in daily oral doses of 167, 500 or 1500 mg/kg (Gaunt, Colley, Grasso, Lansdown & Gangolli, 1968). Three pigs given 1500 mg/kg/day for 14–21 days died with symptoms of uraemia and six out of 20 male mice fed the 5·4% diet died with signs of advanced renal damage. There was reduction of growth in rats and mice fed the highest dietary concentrations. Oxaluria was found in rats and mice at the highest feeding level and a reduction in haemoglobin concentration was seen in all three species at the highest level of administration. The no-effect levels were approximately 250 mg/kg/day in rats, 850–1000 mg/kg/day in mice and 167 mg/kg/day in pigs.

Long-term toxicity. Purified diethylene glycol monoethyl ether fed to rats at approximately 1·0 g/kg/day for 2 yr produced a few oxalate concretions in the kidney of one animal and caused slight liver damage and some interstitial oedema in the testes (Morris, Nelson & Calvery, 1942).

Irritation. Diethylene glycol monoethyl ether is not irritant to the skin of rabbits even upon prolonged and repeated contact (Rowe, 1963). 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 a concentration of 20% in petrolatum and produced no sensitization reactions (Kligman, 1972).

Metabolism. The major part of an administered dose of diethylene glycol monoethyl ether is oxidized in the body or excreted as the glucuronate, administration to rabbits orally or by sc injection being followed by a marked increase in the urinary content of glucuronic acid (Fellows, Luduena & Hanzlik, 1947).

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Laug, E. P., Calvery, H. O., Morris, H. J. & Woodard, G. (1939). The toxicology of some glycols and derivatives. *J. ind. Hyg. Toxicol.* **21,** 173.

Morris, H. J., Nelson, A. A. & Calvery, H. O. (1942). Observations on the chronic toxicities of glycols. *J. Pharmac. exp. Ther.* **74,** 266.

Rowe, V. K. (1963). Derivatives of glycols. In *Industrial Hygiene and Toxicology*, 2nd ed. Edited by F. A. Patty. Vol. II. p. 1561. Interscience Publishers, New York.

Smyth, H. F., Jr. & Carpenter, C. P. (1948). Further experience with the range finding test in the industrial toxicology laboratory. *J. ind. Hyg. Toxicol.* **30,** 63.

Stenger, E.-G., Aeppli, Lislott, Müller, D., Peheim, E. u. Thomann, P. (1971). Zur Toxikologie des Äthylenglykol-Monoäthyläthers. *Arzneimittel-Forsch.* **21,** 880.

DIETHYLENE GLYCOL MONOMETHYL ETHER

Structure: $CH_2(OH)\cdot CH_2O\cdot[CH_2]_2\cdot OCH_3$.

Description and physical properties: A colourless liquid with a faint musty odour (Arctander, 1969).

Occurrence: Has apparently not been reported to occur in nature.

Preparation: A by-product in the manufacture of ethylene glycol monomethyl ether (Arctander, 1969).

Uses: Use in fragances in the USA amounts to about 5000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·05	0·005	0·025	0·5
Maximum	0·3	0·03	0·1	2·0

Analytical data: Gas chromatogram, RIFM no. 72–18; infra-red curve, RIFM no. 72–18.

Status

Browning (1965) has an extensive monograph on diethylene glycol monomethyl ether.

Biological data

Acute toxicity. The acute oral LD_{50} in rats was reported as 6·31 g/kg (Weil, 1972), while with a 50% aqueous solution the LD_{50} values were 9·2 ml/kg for rats and 4·16 ml/kg for guinea-pigs (Smyth, Seaton & Fischer, 1941). The acute dermal LD_{50} in rabbits was reported as 20 ml/kg (Browning, 1965).

Short-term toxicity. The maximum dose having no effect in rats given diethylene glycol monomethyl ether for 30 days in their drinking-water was less than 0·19 g/kg (Smyth & Carpenter, 1948). The highest dosage level survived was 1·83 g/kg.

Irritation. Diethylene glycol monomethyl ether is not appreciably irritating to the skin, but on extensive and prolonged contact, it can be absorbed in toxic and even lethal amounts (Rowe, 1963). 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 a concentration of 20% in petrolatum and produced no sensitization reactions (Kligman, 1972).

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Smyth, H. F., Jr. & Carpenter, C. P. (1948). Further experience with the range finding test in the industrial toxicology laboratory. *J. ind. Hyg. Toxicol.* **30**, 63.

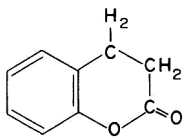
Smyth, H. F., Jr., Seaton, Jane & Fischer, Louise (1941). The single dose toxicity of some glycols and derivatives. *J. ind. Hyg. Toxicol.* **23**, 259.

Weil, C. S. (1972). Statistics vs safety factors and scientific judgment in the evaluation of safety for man. *Toxic appl. Pharmac.* **21**, 454.

DIHYDROCOUMARIN

Synonyms: 3,4-Dihydrocoumarin; hydrocoumarin; 1,2-benzodihydropyrone.

Structure:



Description and physical properties: *Givaudan Index* (1961).
Occurrence: Has apparently not been reported to occur in nature.
Preparation: By reduction of coumarin with a nickel catalyst (Arctander, 1969).
Uses: In public use since the 1930s. Use in fragrances in the USA amounts to less than 10,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·005	0·2
Maximum	0·1	0·01	0·03	2·0

Analytical data: Infra-red curve, RIFM no. 72–19.

Status

Dihydrocoumarin was granted GRAS status by FEMA (1965). The Council of Europe (1970) included dihydrocoumarin in the list of admissible artificial flavouring substances, giving a level of 25 ppm.

Biological data

Acute toxicity. The acute oral LD_{50} value in rats was reported as 1·65 g/kg (1·47–1·83 g/kg) (Moreno, 1972a). The acute dermal LD_{50} value in rabbits was reported as >5 g/kg (Moreno, 1972b).

Subacute and long-term toxicity. In feeding studies, 1000 and 10,000 ppm fed to rats in the diet for 14 wk produced no effects (Hagan, Hansen, Fitzhugh, Jenner, Jones, Taylor, Long, Nelson & Brouwer, 1967). In a 2-yr study, dogs dosed daily with 50 and 150 mg/kg produced no effects (Hagan *et al.* 1967).

Irritation. Dihydrocoumarin applied full strength on intact or abraded rabbit skin for 24 hr under occlusion was moderately irritating (Moreno, 1972b). 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 a concentration of 20% in petrolatum and produced sensitization reactions in all 25 test subjects (Kligman, 1972).

References

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Council of Europe (1970). Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List A(1), Series 1, no. 557, p. 78. Strasbourg.

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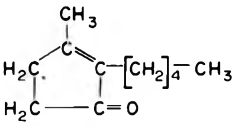
Moreno, O. M. (1972b). Report to RIFM, 1 May.

DIHYDROJASMONE

Synonyms: 2-Pentyl-3-methyl-2-cyclopenten-1-one; 3-methyl-2-(*n*-pentanyl)-2-cyclopentene-1-one.

Structure:



Description and physical properties: A colourless, slightly oily liquid with a floral-like odour (Arctander, 1969).
Occurrence: Has apparently not been reported to occur in nature.
Preparation: Hexyl bromide plus laevulinic ester yields a lactone, which is reacted with polyphosphoric acid or phosphorus pentoxide to produce hydrojasmone (Bedoukian, 1967).
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	0·005	0·0005	0·002	0·04
Maximum	0·03	0·003	0·01	0·4

Analytical data: Gas chromatogram, RIFM no. 72–20; infra-red curve, RIFM no. 72–20.

Status

Dihydrojasmone is not included in the listings of the FDA, FEMA (1965) or the Council of Europe (1970), nor in the *Food Chemicals Codex* (1972).

Biological data

Acute toxicity. The acute oral LD_{50} in rats was reported as 2·5 g/kg (1·79–3·50 g/kg) (Keating, 1972). The acute dermal LD_{50} value in rabbits was reported as 5 g/kg (Keating, 1972).

Irritation. Dihydrojasmone applied full strength on intact or abraded rabbit skin for 24 hr under occlusion was irritating (Keating, 1972). Tested at 4% 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 a concentration of 4% in petrolatum and produced no sensitization reactions (Kligman, 1972).

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Kligman, A. M. (1972). Report to RIFM, 19 July.

DIHYDROMYRCENOL

Synonym: 2,6-Dimethyl-7-octen-2-ol.
Structure: $CH_3 \cdot COH(CH_3) \cdot [CH_2]_3 \cdot C(CH_3):CH \cdot CH_3$.
Description and physical properties: A colourless, somewhat viscous oil (Arctander, 1969).
Occurrence: Has apparently not been reported to occur in nature.
Preparation: By controlled, partial hydrogenation of myrcenol (Arctander, 1969).
Uses: 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	0·005	0·01	0·2
Maximum	0·1	0·01	0·03	0·4

Analytical data: Gas chromatogram, RIFM no. 72–122; infra-red curve, RIFM no. 72–122.

Status

Dihydromyrcenol is not included in the listings of the FDA, FEMA (1965) or the Council of Europe (1970), nor in the *Food Chemicals Codex* (1972).

Biological data

Acute toxicity. The acute oral LD_{50} in rats was reported as 3·6 g/kg (3·0–4·2 g/kg) (Moreno, 1973). The acute dermal LD_{50} value in rabbits exceeded 5 g/kg (Moreno, 1973).

Irritation. Dihydromyrcenol applied full strength on intact or abraded rabbit skin for 24 hr under occlusion was mildly irritating (Moreno, 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 a concentration of 4% in petrolatum and produced no sensitization reactions (Kligman, 1973).

References

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Kligman, A. M. (1966). The identification of contact allergens by human assay. III. The maximization test. A procedure for screening and rating contact sensitizers. *J. invest. Derm.* **47**, 393.

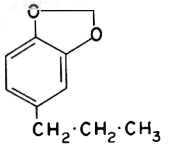
Kligman, A. M. (1973). Report to RIFM, 13 August.

Moreno, O. M. (1973). Report to RIFM, 4 June.

DIHYDROSAFROLE

Synonym: 1,2-Methylenedioxy-4-propylbenzene.

Structure:



Description and physical properties: *Givaudan Index* (1961).
Occurrence: Has apparently not been reported to occur in nature.
Preparation: By catalytic hydrogenation of safrole (Arctander, 1969).
Uses: In public use since the 1950s. Use in fragrances in the USA amounts to less than 2000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·03	0·003	0·005	0·1
Maximum	0·3	0·03	0·05	1·2

Analytical data: Gas chromatogram, RIFM no. 72–123; infra-red curve, RIFM no. 72–123.

Status

The FDA does not permit dihydrosafrole to be used in foods (21 CFR 121.106).

Biological data

Acute toxicity. The acute oral LD_{50} was reported as 2·26 g/kg (1·84–2·78 g/kg) in rats and as 4·30 g/kg in the mouse (Jenner, Hagan, Taylor, Cook & Fitzhugh, 1964). The acute dermal LD_{50} in rabbits was reported as >5 g/kg (Shelanski & Moldovan, 1973). Daily dosage for 4 days with 770 mg dihydrosafrole/kg caused macroscopic liver lesions and one death in six treated rats (Taylor, Jenner & Jones, 1964).

Subacute and long-term toxicity. In a feeding study, 1000 ppm fed to rats in the diet for 2 yr retarded growth in females and caused macroscopic and microscopic liver changes (Hagan, Hansen, Fitzhugh, Jenner, Jones, Taylor, Long, Nelson & Brouwer, 1967). In another feeding study, 250 and 500 mg/kg fed to rats in the diet for 34 and 46 days, respectively, produced no deaths (Hagan, Jenner, Jones, Fitzhugh, Long, Brouwer & Webb, 1965), while 750 mg/kg fed to rats in the diet for 26 days produced three deaths in ten animals.

The tumorigenicity of selected pesticides and industrial compounds was tested by continuous oral administration to both sexes of two hybrid strains of mice, starting at the age of 7 days (Innes, Ulland, Valerio, Petrucelli, Fishbein, Hart, Pallotta, Bates, Falk, Gart, Klein, Mitchell & Peters, 1969). Maximal tolerated doses were given for 18 months. Among the 120 test compounds, seven materials were included as positive controls. Dihydrosafrole which was one of these controls produced tumours of the liver in male mice.

Irritation. Dihydrosafrole applied full strength on intact or abraded rabbit skin was mildly irritating (Shelanski & Moldovan, 1973). Tested at 12% 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 a concentration of 12% in petrolatum and produced no sensitization reactions (Kligman, 1973).

References

Arctander, S. (1969). *Perfume and Flavor Chemicals (Aroma Chemicals)*. Vol. 1, p. 968. S. Arctander, Montclair, New Jersey.

Givaudan Index (1961). *Specifications of Synthetics and Isolates for Perfumery*. 2nd ed., p. 133. Givaudan-Delawanna, Inc., New York.

Hagan, E. C., Hansen, W. H., Fitzhugh, O. G., Jenner, P. M., Jones, W. I., Taylor, Jean M., Long, Eleanor L., Nelson, A. A. & Brouwer, J. B. (1967). Food flavourings and compounds of related structure. II. Subacute and chronic toxicity. *Fd Cosmet. Toxicol.* **5,** 141.

Hagan, E. C., Jenner, P., Jones, W. I., Fitzhugh, O. G., Long, Eleanor L., Brouwer, J. G. & Webb, W. K. (1965). Toxic properties of compounds related to safrole. *Toxic. appl. Pharmac.* **7,** 18.

Innes, J. R. M., Ulland, B. M., Valerio, M. G., Petrucelli, L., Fishbein, L., Hart, E. R., Pallotta, A. J., Bates, R. R., Falk, H. L., Gart, J. J., Klein, M., Mitchell, I. & Peters, J. (1969). Bioassay of pesticides and industrial chemicals for tumorigenicity in mice: A preliminary note. *J. natn. Cancer Inst.* **42,** 1101.

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Kligman, A. M. (1966). The identification of contact allergens by human assay. III. The maximization test. A procedure for screening and rating contact sensitizers. *J. invest. Derm.* **47,** 393.

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DIHYDRO-α-TERPINEOL

Synonyms: *p*-Menthan-8-ol; 1-methyl-4-isopropylcyclohexane-8-ol.
Structure: $CH_3 \cdot C_6H_{10} \cdot C(CH_3)_2 \cdot OH$.
Description and physical properties: A liquid with a more woody pine-like odour than terpineol (Bedoukian, 1967).
Occurrence: Found in American wood turpentine pine oil (Guenther, 1949).
Preparation: By hydrogenation of α-terpineol using a platinum catalyst (Arctander, 1969).
Uses: In public use since the 1950s. Use in fragrances in the USA amounts to less than 1000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·05	0·005	0·03	0·4
Maximum	0·3	0·03	0·1	1·0

Analytical data: Gas chromatogram, RIFM no. 71–45; infra-red curve, RIFM no. 71–45.

Status

Dihydro-α-terpineol is not included in the listings of the FDA, FEMA (1965) or Council of Europe (1970), nor 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, 1973).

Irritation. Dihydro-α-terpineol applied full strength on intact or abraded rabbit skin for 24 hr under occlusion was moderately irritating (Moreno, 1973). Tested at 10% 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 a concentration of 10% in petrolatum and produced no sensitization reactions (Kligman, 1972).

References

Arctander, S. (1969). *Perfume and Flavor Chemicals (Aroma Chemicals)*. Vol. 1, p. 970. S. Arctander, Montclair, New Jersey.
Bedoukian, P. Z. (1967). *Perfumery and Flavoring Synthetics*. 2nd ed., p. 369. Elsevier Publishing Co., New York.
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Kligman, A. M. (1972). Report to RIFM, 13 October.
Moreno, O. M. (1973). Report to RIFM, 5 July.

DIMETHYLBENZYL CARBINOL

Synonyms: α,α-Dimethylphenethyl alcohol; 1,1-dimethyl-2-phenylethanol.
Structure: $C_6H_5 \cdot CH_2 \cdot C(CH_3)_2 \cdot OH$.
Description and physical properties: *Givaudan Index* (1961).
Occurrence: Has apparently not been reported to occur in nature.
Preparation: By Grignard synthesis from benzyl magnesium chloride and acetone (Bedoukian, 1967).
Uses: In public use since the 1950s. Use in fragrances in the USA amounts to less than 2000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·02	0·002	0·01	0·3
Maximum	0·15	0·015	0·1	0·8

Analytical data: Gas chromatogram, RIFM no. 72–130; infra-red curve, RIFM no. 72–130.

Status

Dimethylbenzyl carbinol 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 dimethylbenzyl carbinol in the list of admissible artificial flavouring substances, at a level of 5 ppm. The *Food Chemicals Codex* (1972) has a monograph on dimethylbenzyl carbinol.

Biological data

Acute toxicity. The acute oral LD_{50} in rats was reported as 1·35 g/kg (1·02–1·68 g/kg) (Moreno, 1973). The acute dermal LD_{50} in rabbits was reported as >5 g/kg (Moreno, 1973).

Subacute toxicity. In feeding studies in rats, neither 10,000 ppm fed in the diet for 16 wk nor 1000 ppm fed in the diet for 28 wk had any effects (Hagan. Hansen, Fitzhugh, Jenner, Jones, Taylor, Long, Nelson & Brouwer, 1967).

Irritation. Dimethylbenzyl carbinol applied full strength on intact or abraded rabbit skin was not irritating (Moreno, 1973). Tested at 8% 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 a concentration of 8% in petrolatum and produced no sensitization reactions (Kligman, 1973).

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DIMETHYLBENZYL CARBINYL ACETATE

Synonyms: α,α-Dimethylphenethyl acetate; benzyldimethyl carbinyl acetate.
Structure: $C_6H_5 \cdot CH_2 \cdot C(CH_3)_2 \cdot O_2C \cdot CH_3$.
Description and physical properties: EOA Spec. no. 186.
Occurrence: Has apparently not been reported to occur in nature.
Preparation: By acetylation of dimethylbenzyl carbinol (Bedoukian, 1967).
Uses: In public use since the 1930s. Use in fragrances in the USA amounts to about 50,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·03	0·003	0·02	0·3
Maximum	0·2	0·02	0·10	0·40

Analytical data: Gas chromatogram, RIFM no. 70–35; infra-red curve, RIFM no. 70–35.

Status

Dimethylbenzyl carbinyl 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 dimethylbenzyl carbinyl acetate in the list of temporarily admissible artificial flavouring substances. The *Food Chemicals Codex* (1972) has a monograph on dimethylbenzyl carbinyl acetate.

Biological data

Acute toxicity. The acute oral LD_{50} in rats was reported as 3·30 g/kg (2·55–4·05 g/kg) (Moreno, 1971). The acute dermal LD_{50} in rabbits was reported as >3 g/kg (Moreno, 1971).

Irritation. Dimethylbenzyl carbinyl acetate applied full strength on intact or abraded rabbit skin for 24 hr under occlusion was moderately irritating (Moreno, 1971). Tested at 4% in petrolatum, it produced 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 volunteers. The material was tested at a concentration of 4% in petrolatum and produced no sensitization reactions (Kligman, 1971).

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Kligman, A. M. (1971). Report to RIFM, 2 April.
Moreno, O. M. (1971). Report to RIFM, 24 March.

3,7-DIMETHYL-1-OCTANOL

Synonym: Tetrahydrogeraniol.
Structure: $CH_3 \cdot CH(CH_3) \cdot [CH_2]_3 \cdot CH(CH_3) \cdot [CH_2]_2 \cdot OH$.
Description and physical properties: *Food Chemicals Codex* (1972).
Occurrence: Has apparently not been reported to occur in nature.
Preparation: By reduction of geraniol or by reduction of citronellol, citronellal or citral (Arctander, 1969).
Uses: In public use since the 1930s. 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	0·02	0·2
Maximum	0·2	0·02	0·1	0·8

Analytical data: Gas chromatogram, RIFM no. 72–129; infra-red curve, RIFM no. 72–129.

Status

3,7-Dimethyl-1-octanol 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) listed 3,7-dimethyl-1-octanol (tetrahydrogeraniol), giving an ADI of 5 mg/kg. The *Food Chemicals Codex* (1972) has a monograph on 3,7-dimethyl-1-octanol.

Biological data

Acute toxicity. The acute oral LD_{50} in rats was reported as >5 g/kg (Shelanski & Moldovan, 1973a). The acute dermal LD_{50} in the rabbit was reported as 2·4 g/kg (1·7–3·4 g/kg) (Shelanski & Moldovan, 1973a).

Irritation. 3,7-Dimethyl-1-octanol applied full strength on intact or abraded rabbit skin produced irritation (Shelanski & Moldovan, 1973b). Tested at 8% 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 8% concentration in petrolatum and produced no sensitization reactions (Kligman, 1973).

References

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Kligman, A. M. (1973). Report to RIFM, 12 February.
Shelanski, M. V. & Moldovan, M. (1973a). Report to RIFM, 30 January.
Shelanski, M. V. & Moldovan, M. (1973b). Report to RIFM, 16 February.

DIMETHYLPHENYLETHYL CARBINOL

Synonyms: α,α-Dimethyl-γ-phenylpropyl alcohol; 1,1-dimethyl-3-phenylpropanol.
Structure: $C_6H_5 \cdot [CH_2]_2 \cdot C(CH_3)_2 \cdot OH$.
Description and physical properties: EOA Spec. no. 277.
Occurrence: Has apparently not been reported to occur in nature.
Preparation: By reaction of phenylethyl magnesium chloride with acetone (Bedoukian, 1967).
Uses: In public use since the 1950s. Use in fragrances in the USA amounts to less than 7000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·02	0·002	0·01	0·3
Maximum	0·1	0·01	0·03	0·4

Analytical data: Gas chromatogram, RIFM no. 72–131; infra-red curve, RIFM no. 72–131.

Status

Dimethylphenylethyl carbinol is not included in the listings of the FDA, FEMA (1965) or the Council of Europe (1970), nor in the *Food Chemicals Codex* (1972).

Biological data

Acute toxicity. The acute oral LD_{50} in rats was reported as 2·2 ml/kg (1·8–2·7 ml/kg) (Shelanski, 1973). The acute dermal LD_{50} in rabbits was reported as 3·5 ml/kg (2·6–4·3 ml/kg) (Shelanski, 1973).

Irritation. Dimethylphenylethyl carbinol applied full strength on intact or abraded rabbit skin produced a moderate to severe irritation (Shelanski, 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 a concentration of 4% in petrolatum and produced no sensitization reactions (Kligman, 1973).

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Kligman, A. M. (1973). Report to RIFM, 25 May.
Shelanski, M. V. (1973). Report to RIFM, 16 February and 20 February.

Fd Cosmet. Toxicol. Vol. 12, pp. 539–543. Pergamon Press 1974. Printed in Great Britain

Review Section

REVIEWS OF RECENT PUBLICATIONS

List of Additives Evaluated for their Safety-in-Use in Food. First series. Joint FAO/WHO Food Standards Programme, Codex Alimentarius Commission, CAC/FAL 1–1973. Food and Agriculture Organization of the United Nations, Rome, 1973. pp. 88. £0·40. [Obtainable in UK through HMSO.]

With the growing number of reports issuing from the Joint FAO/WHO Expert Committee on Food Additives, it has become an increasingly time-consuming process to track down the evaluation of a particular additive. The present publication, which lists all additives evaluated by the Committee, with their acceptable daily intakes and with reference to the appropriate reports in which the toxicological evaluations and specifications can be found, is therefore very welcome. It is also good to see that the many additives evaluated at the 17th Meeting, the report of which is still unpublished, have been included.

The additives have been classified into four lists, according to the conclusions reached by the Joint FAO/WHO Expert Committee with regard to their acceptability for use in food. List A(1) includes additives that have been allocated an unconditional or conditional acceptable daily intake or that have not been limited toxicologically, while list A(2) includes those that have been accepted for use on a provisional basis. List C(1) contains substances regarded as unsafe for use in food, whereas additives which have been restricted for toxicological reasons are relegated to list C(2); at present only cyclamates and hydrogen peroxide appear on this last list. Each list is further subdivided according to additive function.

The reason for this particular format would appear to be that the publication is directed primarily at those concerned with regulating the use of food additives, but since it will obviously be of use to a much wider audience as an index to existing FAO/WHO reports, it is perhaps a pity that the information was not presented in the form of a single alphabetical list. Fortunately, just such a list was recently published elsewhere (*CRC Critical Reviews of Toxicology* 1973, **3**, 75), but it did not include additives considered at the 17th Meeting.

The publication under review also contains a list of additives approved by the Codex Alimentarius Commission, on the basis of the Joint FAO/WHO Expert Committee evaluations, for use in specific foods, with details of the foods and the maximum level (if any) in each case. A list of the Recommended International Codex Standards is also given. The functions of the Codex Alimentarius Commission, the Codex Committee on Food Additives and the Joint FAO/WHO Expert Committee on Food Additives are discussed in the short introduction, which also defines terms used in the document and includes a statement of the general principles for the use of food additives, adopted by the Ninth Session of the Codex Alimentarius Commission.

Safe Use of Pesticides. Twentieth Report of the WHO Expert Committee on Insecticides. *Tech. Rep. Ser. Wld Hlth Org.* 1973, **513**. pp. 54. £0·40.

The WHO Expert Committee on Insecticides has, in this report, widened its scope to cover the safe use of pesticides not only in vector control, as before, but also in agricultural applications. In all cases a balanced view has been taken, with consideration of the toxicological data and a careful weighing of the risks against the ensuing benefits.

A good example is provided by DDT, which is still essential in some areas for malaria eradication. Despite the fact that one of two recent studies by the International Agency for Research on Cancer has revealed liver tumours in male mice fed only 0·3 mg DDT/kg/day, a daily intake similar to the highest human exposure known at present, no tumours or other adverse effects have been detected in a small group of heavily exposed men who have been followed for 20 years. Long-term studies on two larger groups have now been organized, and it is hoped that more will soon be initiated. The Committee urges that comprehensive metabolic studies in a variety of animal species, including man, and carcinogenicity tests now in progress in species other than mice, should be completed before any more drastic restrictions on its use are imposed.

The WHO programme for the evaluation and testing of new insecticides has been in operation for 12 years, during which more than 1600 compounds have been evaluated. In recent years growing attention has been paid to biodegradability, and there has been a continuous decline in the number of new compounds submitted. The high rejection rate is illustrated by the fact that only three compounds are in operational use for malaria eradication and control, while a few others are regarded as suitable for controlling the vectors of other diseases. The further trials on indoor application of propoxur and fenitrothion recommended in the Committee's Sixteenth Report (*Cited in F.C.T.* 1968, **6**, 754) have been completed with evidence of only slight and transient side-effects, and these compounds have now joined malathion as suitable alternatives for DDT, when use of the latter is not appropriate. Nine other compounds—the carbamates bux, landrin, mobam and *o*-cyclopentylphenyl methylcarbamate, and the organophosphorus compounds dicapthon, phenthoate, phoxim, chlorphoxim and jodfenphos—have been examined in village-scale field trials, allowing recommendations for larger-scale trials to be made in most cases. Larvicides, for use against mosquitoes and blackfly, and insecticides for human louse control have also been evaluated, with particular reference in the latter category to mobam, abate, carbaryl and propoxur.

Studies of products for aircraft disinsection have yielded no evidence of toxic hazard for the synergized pyrethrins or pyrethroids or for dichlorvos, which acts as an alkylating agent *in vitro* but apparently not *in vivo*, presumably because it is readily demethylated. The technique of ultra-low-volume application of insecticides is also discussed, as are alternative methods of insect control such as the use of chemosterilants.

Of the molluscicides, which still provide the most effective way of controlling schistosomiasis, the most appropriate for aquatic use are niclosamide and frescon (*N*-tritylmorpholine), both with a fairly low toxicity to mammals. More toxicity data are required on yurimin for soil application, while organotin compounds are commanding increasing interest for use in slow-release devices.

Following the widespread development of resistance to anticoagulant rodenticides among rats and mice, materials of high acute toxicity have again come into prominence. Use of red squill, norbormide and zinc phosphide with "ordinary care" is endorsed by the

Committee, while maximum precautions are considered necessary in the handling of sodium fluoroacetate, fluoroacetamide and strychnine. Arsenic trioxide, phosphorus, thallium sulphate, 1-naphthylthiourea and gophacide are regarded as too dangerous for use.

The health hazards relating to transportation, storage and use of agricultural pesticides are discussed in the report. Recommendations include the generation of more specific data on accidental poisoning, the issuance of insecticide data sheets, the development of a classification scheme for pesticides according to the hazard they present, the promotion of medical and other services to deal with poisoning, the provision of emergency aid in poisoning outbreaks, and further education and training in pesticide toxicology. Progress in diagnosis and treatment of pesticide exposure is reviewed, and a summary of the treatment of poisoning is given in an annex reprinted from the Sixteenth Report, with minor amendments and with the addition of a section on organochlorine compounds.

Annual Report 1972 of HM Chief Inspector of Factories. Department of Employment. HMSO, London, 1973. pp. xvii + 133. £1.

The Work of the Inspectorate during 1972 reflected the increasing public concern with matters of environmental and industrial health and particularly with the problem of lead. A considerable proportion of the resources of the Industrial Hygiene Unit during the year was deployed in surveys and investigations of lead hazards in a wide variety of industries. These studies merited a whole chapter in the report. Greatly increased facilities for the estimation of blood levels of lead have become available in the laboratory of the Employment Medical Advisory Service, and new techniques using only very small blood samples have been developed. Analysis of the incidence of cases of lead poisoning in preceding years showed that three sections of the lead trade—lead smelting and refining, manufacture of electrical accumulators and ship breaking—stood out as needing special attention, and events brought the first category into prominence. In the case of one refinery in the Isle of Dogs, the children of workers proved to have abnormally high lead levels acquired from their father's contaminated clothing. This incident led to a general move within the industry to improve standards of cleanliness.

Severe problems of hygiene control presented by the lead/zinc smelter at Avonmouth caused the closing of the plant for part of the year and the setting up of a committee of enquiry. The various measures recommended by this committee had been largely implemented by the end of 1972. After the plant reopened in April 1972, both environmental and biological monitoring gave encouraging results and from then until the end of the year there were no cases of reportable lead poisoning. A working group with representation from all sides of the lead smelting and refining and electrical accumulator sections of the industry was set up as a result of this incident and formulated a Code of Practice (now published), which it is hoped will be observed on a voluntary basis. A further working party is now considering the effect of particle size on lead absorption, the need for more detailed knowledge about levels of lead absorption, and possible long-term effects.

The Inspectorate has been involved in many discussions and meetings aimed at formulating practical methods of handling and using highly hazardous substances, including weak carcinogens. Visits to firms using methylene-bis-*o*-chloroaniline as a hardener for urethane rubbers continued, and advice was given where necessary on improving handling

precautions, but no further evidence relating to the possible carcinogenicity of this material was acquired during the year. Chloromethyl ethers, on the other hand, are now regarded as presenting such a carcinogenic risk that no detectable exposure should be permitted, and it is intended to develop a mass spectrographic method for determining extremely low concentrations; a TLV of 0·001 ppm has been suggested. Investigations into the basic hazards associated with organic isocyanates have led in some cases to greater control of exposure, in others to the substitution of methylene diisocyanate for the more volatile toluene diisocyanate, and a technical data note on the subject has been prepared. Other problems dealt with by the Inspectorate have arisen from the generation of sulphur dioxide from fuel oil, of nitrous fumes from natural gas burned in oxygen, and of cobalt from the grinding of the tungsten carbide tips of tools.

The report observes that the asbestos industry generally has made great strides in complying with the Asbestos Regulations, 1969, but 14 successful prosecutions were still recorded during the year. A new programme involving extensive atmospheric sampling by asbestos workers should in time lead to a better knowledge of hygiene standards. The construction industry has received increasing attention, and the importance of analysing old lagging before demolition and of using specialist sub-contractors to remove asbestos has been stressed. The cotton-dust problem has led to a programme of dust sampling in 34 mills, which should permit a comparison of the different methods used to determine the TLV of 0·2 mg/m^3 recently recommended by the American Conference of Governmental Industrial Hygienists and the hygiene standard of 0·5 mg/m^3 approved by the Joint Standing Committee on Health and Welfare in the Cotton Industry. Elsewhere in the report, it is interesting to learn that work has commenced in the Council of Europe on the compilation of a European list of acceptable levels for contaminants in working areas.

The endeavours of the Inspectorate to safeguard and improve the working environment are always praiseworthy, and this report makes interesting and informative reading.

Annual Report of the Scientific Adviser 1972. Greater London Council Scientific Branch, Director-General's Department. Greater London Council, 1974. pp. 136. £4·00.

The confusion caused by the lack of international harmonization in food-additive regulations is well illustrated in this report, which deals not only with the activities of the Branch in the food field but also with such wide-ranging subjects as water pollution, refuse disposal, building materials, environmental studies, fire prevention and the supply of school meals. Non-permitted colourings were found in 44 of the samples of foods analysed, and 18 of these contained Brilliant Blue FCF, which is permitted in the United States but not in Britain. Patent Blue V, permitted in the EEC but as yet only proposed for addition to the UK list, was identified in a further sample, while another contained the EEC-listed colouring, Ponceau 6R. Other colourings identified had either been banned from use in the UK (Ponceau MX) or had never been permitted in the UK or many other countries (Xylenol Red B, Rhodamine B and Orange II). In 12 samples of blackcurrant-containing foods, the presence of a non-permitted red colouring was associated with the use of Red 10B and was ascribed to decomposition of this somewhat unstable compound. In addition, 51 samples transgressed against the Preservatives in Food Regulations, largely through the use of benzoic acid, sulphur dioxide or sorbic acid in foodstuffs for which such use

is not permitted or at levels higher than are allowed in the regulations. Despite the ban on cyclamate since January 1970, two samples still contained this artificial sweetener.

An unusual problem was posed by the "catty" odour of some pork luncheon meat, which was eventually attributed not to urine, as originally suspected, but to a reaction of sulphur-containing substances naturally present in the meat with minute traces of residual solvents derived from the lacquering of the can.

A number of investigations involved metallic contamination, and it is reassuring to find that only one of 20 baby foods examined exceeded the 0·5 ppm limit for lead; moreover, this sample was taken early in the year, before the permitted level had been reduced from 2·0 ppm. Nine of 2500 other samples exceeded the maximum limits prescribed in the Lead in Food Regulations, the highest lead level, found in a sample of turmeric, being 108 ppm. Excessive levels of copper and zinc were also discovered in a few cases, but the mean level of 0·15 ppm mercury in white fish was well below the US guideline level of 0·5 ppm. Five toys bore paint containing more than the prescribed 0·5% lead, and in one case the yellow paint on a multicoloured toy xylophone was found to contain 15% lead. More seriously, several instances of lead poisoning resulted from the use of an eye cosmetic subsequently identified as pure lead sulphide, and a further case was attributed to the mouth-piece of a flute made of soft lead alloy with a 77% lead content.

The Scientific Branch has also been concerned with atmospheric pollution, and it is encouraging to see that the sulphur dioxide concentration in London air still seems to be declining, although smoke levels have stayed virtually constant since 1968. The sulphur dioxide situation may be reversed, however, if several planned power stations are built in the Greater London area. Oxford Street shoppers will be pleased to learn that pavement widening and traffic restrictions have resulted in a drastic reduction in carbon monoxide levels. The relation between atmospheric and blood levels of lead has also been under consideration. One of the major difficulties in this area of study is the great variation in analytical results obtained in different laboratories, a fact brought to the fore at an international conference held in the Netherlands during 1972.

This report always makes interesting reading, from which all those concerned with conforming to present regulations should benefit.

Fd Cosmet. Toxicol. Vol. 12, pp. 545–549. Pergamon Press 1974. Printed in Great Britain

BOOK REVIEWS

Chemical Mutagens. Principles and Methods for Their Detection. Vols 1 and 2. Edited by A. Hollaender. Plenum Press, New York, 1971. pp. cxxx + 610. £8.20 per volume.

Concern about mutagens in our environment has been increasing during the last few years. There seems to be a trend of opinion among informed observers that human mutations are being observed much more frequently than anticipated. Furthermore, laboratory investigations are continually adding to the list of compounds with allegedly mutagenic properties. The test organisms employed to detect such activity cover a wide range from phage to mammals, while the experimental methods used and the type of mutation thought relevant are numerous. A lot can be said in favour of most of the procedures used, if they are considered as a means of acquiring basic knowledge on the genetics of the organism concerned, and it is true that most workers do not profess to claim any more for their methods than this limited objective.

Unfortunately, however, the data thus acquired tend to attract the attention of scientists who, with much broader interests, seek in the results of such investigations significant indications of mutagenic potency which the original workers never intended. The gap between these two approaches is extremely difficult to define and even more difficult to bridge, so it was with some relief that we studied the two volumes on chemical mutagens named above.

The first volume may be described as laying the foundation for the conduct and interpretation of the tests. It deals with molecular mechanisms of mutation, the central role played by DNA, and a number of test systems involving mainly viruses and bacteria. The second volume deals primarily with descriptions of methods, although some guidance is also given on the interpretation of results. Among the more important tests mentioned are the dominant lethal test, the host-mediated assay and direct cytogenetic observation, all performed on mammals. Tests designed to detect the induction of recessive mutations and chromosome aberrations in cells cultured *in vitro* are also described.

On the whole these two volumes provide an authoritative introduction to a complicated and difficult subject. They will be useful to toxicologists and other scientists interested in public health who, reluctantly or otherwise, have to grasp the thorny problem of identifying mutagens in our environment. All such readers will be glad to know that the standard of production is high, the chapters are written in a readable style and the diagrams and references are both apt and numerous.

Residue Reviews. Residues of Pesticides and Other Contaminants in the Total Environment. Vol. 46. Edited by F. A. Gunther. Springer-Verlag, Berlin, 1973. pp. ix + 250. DM70.20.

This volume of Residue Reviews is devoted to the problem of residues of organophosphorus esters in meat. An introductory chapter stating the aims of the authors (K. Kaemmerer and S. Buntenkötter) and their approach to the subject, is followed by 45 monographs on individual organophosphorus esters.

The two major sources of the organophosphates that may contaminate the edible tissues of livestock are veterinary application and secondary exposure from the ingestion of plant fodder treated with herbicides or insecticides during growth or storage. The former offers the highest risk of residues in the meat since high doses may be given for therapeutic purposes, but the significance of the residues depends on the time between treatment and slaughter. Exposure from secondary sources involves smaller quantities but tends to be more continuous.

Some authorities have considered that, in spite of their comparatively high acute toxicity, these esters do not represent a serious residue problem as they are rapidly decomposed and cleared. The animal itself acts as a screen, developing visible symptoms in response to unacceptable levels of a highly toxic material and generally affecting some degree of detoxication and clearance, although it is important to remember that in some cases metabolites may be more toxic than the parent compound, not less. The authors point out the danger of accumulation from secondary sources in some cases, however, and the risk of high tissue concentrations arising from veterinary use in individual animals shortly before slaughter. Perhaps the most salient point they make, but one which is unfortunately not covered by the brief of this book, concerns the lack of consideration that has been given to the possible combined effects of residues of several different chemicals occurring together in the same meat product.

Details of the innate toxicity, metabolism, stability and type of use of any compound are among the data needed for an assessment of any possible hazard to man. The well-referenced monographs give this information concisely, together with available residue data and published tolerances. Perhaps the only factor detracting from the quality of the book is the standard of translation from the original German, as the resulting text is not particularly easy to read. However, this does not alter the fact that this is a useful survey of the whole question of organophosphorus residues occurring in meat intended for human consumption.

Health Effects of Environmental Pollutants. By G. L. Waldbott. The C. V. Mosby Company, St. Louis, 1973. pp x + 316. £3.40.

This book differs from many in the field in reviewing the problem of environmental pollution in terms of its effect on human health. It is clear that the interests of the author lie mainly in the sphere of atmospheric pollution and inhalation toxicity, but this is not in itself a serious limitation since respiratory disease, much of which is caused by some sort of pollution, either environmental or voluntary (tobacco smoking, for example), is a major cause of death, especially in this country. Moreover the effects of atmospheric pollution act more directly on the human population than some other forms of environmental contamination.

In the case of chronic diseases caused by pollutants, especially when the victims are free from clinical signs and symptoms until many years after exposure, it is often difficult to correlate the effects with the cause, and the author has included data from animal experiments to help fill the gaps in existing knowledge, particularly with regard to the biological mechanisms of the toxicity. One facet of the book which should prove particularly interesting to the layman is the story of how particular sources or types of pollution came to be discovered and the descriptions of some of the environmental conditions that lead to their occurrence.

Dealing with pollution in its many forms, the author attempts to cover in a relatively short book what is now a vast subject. He does in fact manage to deal with most of the important aspects, presenting his data in a form which is readily comprehensible to the layman as well as the clinician. An aid to this is the short glossary of technical terms included at the end of the book. Also included are an author index and a comprehensive subject index.

Information Resources in the Environmental Sciences. Edited by G. S. Bonn. University of Illinois Graduate School of Library Science, Champaign-Urbana, Ill., 1973. pp. 238. $6.00.

The title of this slim, well-produced volume is perhaps a little misleading. One might expect an arid listing, source by source, of holdings in the environmental sciences, but instead this is a call to arms for all environmentalist librarians. No longer should they merely collect, catalogue, file and collate: the time has come for them to infiltrate, activate, prise open the secrets of government and industry alike, fight censorship and apprise scientists and the public of things they could never find out on their own.

This thread runs through this collection of 15 papers originally presented at the eighteenth 3-day Institute held in 1972 by the University of Illinois Graduate School of Library Science at Allerton Park, itself currently at the centre of an environmental controversy. The literature of environmental science is particularly diffuse, partly because the subject is still young enough not to have established set borders and partly because it overlaps with so many other sciences. Chapters on the complexities of US government agencies and on information sources in the private sector are helpful for those seeking to tap such wells, as are details of information centres and services and of library publications and directories, some of which may be an eye-opener for the parochial British reader.

The problems of keeping pace with the rapidly expanding numbers of journals and other publications in this field, in terms of time and cost, are valid points in favour of the alert and in-touch librarian who can assess the needs of his own library and liaise successfully with others. One author estimates that the number of books relevant to the environment published in 2000 AD will be double the present annual output of 13,500—a problem of almost epidemic proportions. Other contributors deal with university requirements, regional environmental libraries and information centres, public and school libraries and the stresses placed on libraries concerned with environment by the varied requirements of library users.

The question of 'activist' librarians is a more uneasy proposition. Librarians are seen classically as being passive and neutral, but it is the contention of M. Schneiderman, author of the first paper in this book, that librarians, while not sponsoring any particular ideas on the data they hold, should sponsor the full use of these data. Neutrality automatically favours the well-informed, who readily take full advantage of library facilities, but the active provision of digested material, abstracts, bibliographies and other aids can assist the less well-informed. It is a laudable concept that library resources should be used as fully as possible, as exemplified in the review of available sources in environmental science given in this book, but there is a danger that at some point the cart may overtake the horse—the librarian may become a research analyst with a point of view.

Although this volume does not have much to offer specifically to the toxicologist, it will be of interest to any information scientist who wishes to look into environmental docu-

mentation, particularly in the USA. It is well referenced, with useful appendices of relevant information centres and services.

Laboratory Primate Handbook, By R. A. Whitney, Jr., D. J. Johnson and W. C. Cole. Academic Press, New York, 1973. pp. xi + 169. £4.40.

This manageable little handbook sets out "to provide knowledge essential for maintaining nonhuman primates in a laboratory environment". It seeks to achieve this aim in ten short chapters, each of which is split into small sections with the subject matter clearly defined by bold headings.

The first chapter deals with the classification of living primates, identifying many of the common members of monkey families from both the new and old worlds. Primate procurement and production, followed by housing and care, are the subjects of chapters 2 and 3. The latter includes a useful table giving recommended cage sizes.

The important topics of personnel protection, quarantine and diagnostic procedures are given only a few pages, but the essential information is there, as is the case with the chapter on restraint and special techniques. Under this heading some suitable anaesthetics are discussed. The sources of twelve vitamins and their role in disease resistance, are set out in another chapter, which also deals with other nutrient requirements and provides references to the work of a number of other authors.

Localized infectious diseases and parasites are discussed under the headings of the particular organ systems commonly involved. Consideration is also given to generalized infectious diseases, special attention being paid to tuberculosis and viral diseases. Some 20 pages of the book are taken up with physiological data, normal haematological values being given for 18 species and blood chemistry data for ten. Some reproduction data on 25 species are also included.

The fact that this handbook is American in origin makes the last quarter of it, devoted to Federal policies, regulations and standards and to US primate research centres less helpful to the British reader than the rest of the book. The final contributions—a list of suggested reading matter and the bibliography—are of much more general value and, as a whole, the handbook is readable and well illustrated. The ease with which reference can be made to any particular topic may be jointly attributed to the size of the book, the clear identification of subject matter in the text and the provision of a good subject index.

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Information Section

ARTICLES OF GENERAL INTEREST

QUESTIONS OF HALOETHER CARCINOGENICITY

The highly reactive α-haloethers have achieved wide usage in industry, in the preparation of ion-exchange resins, in the treatment of textiles, as intermediates in organic syntheses and as solvents for polymerization reactions. Since these alkylating agents offer a potential occupational hazard, both in terms of skin contact and inhalation exposure, several workers have recognized the need to test them for possible carcinogenicity and to determine whether any specific structural feature can be correlated with tumorigenic activity. In at least one case, that of chloromethyl methyl esther (CMME), used in the manufacture of ion-exchange resins, carcinogenicity studies have also been prompted by reports of lung cancer in workers routinely contacting either this material or bis-chloromethyl ether (BCME), one of the by-products of CMME manufacture.

Studies in rats and mice have strongly suggested that BCME is a carcinogen in these species, and there is a growing volume of data to incriminate CMME as well. Thus, an enhanced incidence of adenomas occurred in the lungs of newborn mice following a single sc injection of the maximum tolerated dose of BCME (*Cited in F.C.T.* 1970, **8,** 109) and an increased frequency of lung tumours was recorded in mice following repeated vapour inhalation of 1 ppm BCME for up to 130 days over a 6-month period (Leong *et al. Archs envir. Hlth* 1971, **22,** 663), but in both cases the evidence was inconclusive for CMME. Laskin *et al.* (*ibid* 1971, **23,** 135) exposed rats 101 times to 0·1 ppm BCME for 6 hours/day on 5 days/week, and all died within 659 days of the initial exposure. A high incidence of nasal tumours and squamous carcinomas of the lung was observed (nine tumour-bearing animals were found among the 19 on which gross and histological examination had been completed), but unfortunately the incidence of tumours in the control animals was not disclosed in the preliminary report cited here.

Van Duuren *et al.* (*J. natn. Cancer Inst.* 1969, **43,** 481; *ibid* 1972, **48,** 1431) reported CMME to be an initiating agent in two-stage carcinogenesis in mouse skin, and on sc injection the compound induced a 33% incidence of sarcomas at the injection site in mice. Similar injection in rats, however, resulted in only a 5% incidence of injection-site sarcoma. The studies reported by Van Duuren *et al.* (1969, *loc. cit.*) confirmed the potent reactivity of BCME, but this and the later study indicated a lack of significant carcinogenic activity on the part of certain other related compounds, including octachlorodi-*n*-propyl ether, α,α-dichloromethyl methyl ether and bis-(β-chloroethyl) ether. Bis-(α-chloroethyl) ether produced a 13% incidence of sarcomas at the injection site (Van Duuren *et al.* 1972, *loc. cit.*).

The latter paper also reports on the relation between the biological activity of CMME, BCME and related compounds and their chemical reactivity, as reflected in their rates of hydrolysis. Although both compounds were rapidly hydrolysed, the breakdown products, formaldehyde, methanol and hydrochloric acid, are not known carcinogens. In a more rigorous test for a possible relationship between hydrolysis rate and carcinogenicity,

Nichols & Merritt (*ibid* 1973, **50,** 1373) showed the solvolytic rate ratio of CMME:BCME to be 1:5000 in methanol. With a more highly ionizing medium, 25% aqueous dimethylformamide, the rate differential would be of the order of 10^6, which would be expected to make CMME a more potent carcinogen than BCME. However, since the reverse is true, solvolytic reactivity cannot be a major factor in the carcinogenic potency of compounds in this group.

The first hint that BCME might be carcinogenic to man came in 1965, following the early death from lung cancer of two men who had worked for a US manufacturer of ion-exchange resins, using a process involving BCME (*Lancet* 1973, **ii,** 365). Further cases of lung cancer among workers in a factory using CMME have since been identified by the National Institute for Occupational Safety and Health (*Occup. Hlth Safety Lett.* 1972, **2,** 6; cited from Nelson, *New Engl. J. Med.* 1973, **288,** 1123). More recently, Figueroa *et al.* (*ibid* 1973, **288,** 1096) reported a roughly eightfold enhancement of lung-cancer incidence among men exposed to CMME at another chemical plant. Histological studies were carried out in 13 of these subjects, all of whom were aged between 33 and 55 years, and 12 were found to have oat-cell carcinomas. Smoking habits were taken into account, but the results strongly suggested an occupational hazard.

These findings are undeniably disturbing, but the information so far available is far from complete. The potent carcinogenicity of BCME is generally accepted, but this compound is no longer used *per se* in industry. More questionable is the case of CMME, since this is usually contaminated with BCME, possibly at levels as high as 6–8% (Nelson, *loc. cit.*). The extent to which its contaminant may be responsible for the reported effects of CMME has not been established with any certainty and seems an obvious target for research. Another question demanding further study is whether the reported reversible reaction between gaseous formaldehyde and hydrochloric acid in moist air to form BCME (Nelson, *loc. cit.*) could be of practical significance under laboratory and industrial conditions. Finally the need for carefully designed and monitored epidemiological studies is clear. CMME is produced in the UK, several other EEC countries, Russia, East Germany and Japan, as well as in the USA. Perhaps the two UK plants in which CMME has been produced for over 15 years, and in which stringent precautions are taken for the protection of the workers, could be used as a focus for such studies.

[J.J.-P. Drake—BIBRA]

CARDIAC RESPONSES TO FLUOROCARBON PROPELLANTS

The controversy over the safety of fluorocarbon propellants continues. One of the workers responsible for the studies which initially gave rise to concern (*Cited in F.C.T.* 1971, **9,** 728) has recently reviewed his group's experimental work on this subject in the light of more recent developments (Harris, *Archs intern. Med.* 1973, **131,** 162). Several authors have disagreed with the original claim that fluorocarbons sensitize the mouse to partial asphyxia, producing abnormal heart rhythms. Egle *et al.* (*J. Am. med. Ass.* 1972, **222,** 786) tried to duplicate this finding. They included a study of the effects of partial asphyxia in pentobarbitone-anaesthetized mice both after exposure to nitrogen and after exposure to fluorocarbon. The fluorocarbon mixture (28% trichloromonofluoromethane, F11, and 72% dichlorodifluoromethane, F12) was administered either with or without a

bronchodilator, isoproterenol being used in some studies and the longer-acting albuterol in others. No significant difference was found between any of the groups of mice; all the animals eventually developed irreversible bradycardia and some degree of AV block. These authors suggest that the cardiac effects observed in partial asphyxia after haloalkane exposure may have been due merely to anoxia, which would have been aggravated by prior inhalation of either nitrogen or fluorocarbon.

This conclusion finds support in some of the earlier studies (*Cited in F.C.T.* 1971, **9,** 728). However, it does not explain why the challenge of partial asphyxia can still elicit a response 15 minutes after exposure to fluorocarbon as the fluorocarbon would rapidly leave the lungs via the blood and exhaled air.

An earlier study of the production of cardiac sensitization to a challenge dose of adrenaline by fluorocarbons (*ibid* 1971, **9,** 751) has now been extended to demonstrate in dogs the effects of exercise-induced endogenous adrenaline release on propellant-induced cardiac arrhythmias (Mullin *et al. Am. ind. Hyg. Ass. J.* 1972, **33,** 389). Eight beagle dogs wearing electrocardiogram (ECG) electrodes were trained to run in an enclosed treadmill (speed 3000 fpm). The animals were then exposed to F12 (5, 7·5 or 10% v/v), 1,2-dichlorotetrafluoroethane (F114; 2·5, 5·0 or 10%) or F11 (0·5, 0·75 or 1·0%), while on the treadmill for 16 minutes. The ECG was recorded continuously during the exposure period and during 2- and 3-minute control periods before and after exposure, respectively. Body movement interfered with the trace, preventing the recording of mild changes in heart rate. In many cases the dogs became anaesthetized and were unable to run properly for the full test exposure, and this may have prevented the development of a cardiac effect in some cases. Marked responses (multiple ventricular beats) were produced within 1·5 and 3 minutes by 5 and 10% F114 and by 10% F12, but these responses were all in the same dog. Because of this and the reduced exposure time of many dogs, these results are difficult to interpret. Even so, there is a wide margin between the high dose of fluorocarbon required to sensitize the beagle heart to endogenous adrenaline and the dose effective with injected adrenaline, and the authors interpret this as an indication that there should be no danger from the use of these compounds under the intended conditions.

The cardiac response to aerosol inhalation has also been examined in terms of acid-base relationships (Flowers & Horan, *Chest* 1973, **63,** 74). Dogs were exposed to a mixture of F11 and F12 (65 and 35% respectively in 28 cases and 35 and 65% in two), under pentobarbitone anaesthesia and artificial ventilation. Blood samples were obtained from a cannula in the right femoral artery, and used for determinations of oxygen tension (pO_2), carbon dioxide tension (pCO_2), pH, oxygen saturation, base excess and bicarbonate at the beginning and then at 5-minute intervals (or more frequently) during the experiment. An intracardiac catheter was used to monitor heart function electrographically, and the animals were exposed to the fluorocarbon mixture until the first sign of cardiac abnormality appeared, when samples for blood-gas determinations were again taken and the animal was put back on controlled ventilation.

Thirteen of the dogs developed various disturbances of rhythm, including sinus bradycardia and junctional or ventricular escape rhythms, leading to death in electrical asystole (in the majority) or ventricular fibrillation. The remaining 13 (four having been eliminated by laboratory accidents) developed sinus slowing, but no other signs of disturbance except, in a few cases, ventricular tachycardia or premature beats. Analysis of blood samples showed that survivors had a greater base-line base deficit than the non-survivors. The pH was slightly above 7·3 in the ultimate survivors and slightly below 7·4 in the non-survivors.

This value dropped somewhat in survivors during the test period and rose in non-survivors. There were no significant changes in bicarbonate or in oxygen tension or saturation at any point.

The authors suggest several theories to explain in part why these cardiac effects may be pH-dependent, as the existing evidence concerning the relationship between blood-gas parameters and heart rhythm is conflicting. They consider it unlikely that death in their animals was due to catecholamine sensitivity, because the dogs generally died in asystole, rather than developing ectopic tachycardia and ventricular fibrillation. While these data leave many questions unanswered, they do suggest that the acid-base response of a subject to a fluorocarbon may be one of the factors determining the likelihood of severe toxic reaction.

Another approach to the question of the toxic effect of fluorocarbons is demonstrated in a further study by Kilen & Harris (*J. Pharmac. exp. Ther.* 1972, **183,** 245), concerned with investigating the inotropic effect of propellants by recording electronically the contractility of an *in vitro* preparation of the left ventricular papillary muscle of the rat. Exposure to various concentrations of F12 (1·06–11·35 mg/100 ml organ-bath fluid) was studied at normal and reduced levels of oxygenation, and compared with the effect of reduced oxygenation alone. At a level of 11·35 mg/100 ml, F12 reversibly reduced the amount and rate of development of isometric force in the isolated cardiac muscle. This negative inotropic effect was enhanced by hypoxia, and was dose-related, and the findings thus indicated a direct effect of the fluorocarbon on the ventricular muscle, at least under *in vitro* conditions. The question of possible species variation in the cardiotoxic effects of propellants arises, and thus it is noteworthy that a dose-related depression in isometrically developed force and the rate of its development has also been demonstrated in preparations of human papillary muscle (Harris *et al. Circulation* 1971, **44,** suppl. II, 119).

However, Azar *et al.* (*Am. ind. Hyg. Ass. J.* 1972, **33,** 207) have investigated the acute toxicity of F12 in two young male volunteers exposed to concentrations of 1000 or 10,000 ppm for 2·5-hour periods. A complete physical and clinical examination and a series of psychomotor tests were carried out at the start of the study and the ECG continued to be monitored during exposure periods. Two exposures of each subject to each concentration were then carried out on four separate days over a 2-week period, intermediate exposures to air under test conditions serving as the control. F12 could not be detected in the atmosphere at the concentrations used, and the subjects did not know when they were being exposed to the compound. On a clinical level the subjects tolerated the exposures quite well, and no ECG abnormalities were observed. Analysis of expired air showed that F12 was rapidly eliminated from the lungs, and breath samples were back to control levels 24 hours after exposure. A slight decrease in psychomotor performance was found after exposure to the higher concentration of F12, but this was not a lasting effect. Azar *et al.* (*loc. cit.*) suggest, on the basis of this study and previous work, that man should be able to tolerate single, brief exposures (less than 2·5 hours) to 10,000 ppm F12 without permanently affecting his health.

The evidence on inhalation toxicity has been supplemented by a recently published report of a series of subacute and long-term toxicity studies on six fluorocarbons (Smith & Case, *Toxic appl. Pharmac.* 1973, **26,** 438). Mice were exposed to a mixture of F12, F11, F114 and trichlorotrifluoroethane (F113), to provide a daily dose of 970 mg/kg for 5 days/week for 23 months, rats were exposed to an F12/F114/ethanol mixture at a dose rate of 164 mg/kg/day on 7 days/week for 93 days, and beagle puppies inhaled a dichloromono-

fluoromethane (F21)/monochlorodifluoromethane (F22) mixture in a daily dose of 1714 mg/kg on 5 days/week for 2 weeks. Adult dogs fitted with face masks breathed on 7 days/week a mixture of either F114, F12 and ethanol (700 mg/kg/day) for 90 days or F11, F114, F12 and Span 85 (560 mg/kg/day for 90 days or 2240 mg/kg/day for 1 year). Body weights, haematology, blood chemistry and various other parameters were monitored in some or all species and histological studies were carried out at autopsy, but in no cases were there any pathological findings, beyond sedation and ataxia in the puppies and occasional drowsiness in the adult dogs during exposure. No changes in ECG traces were detected in the dog studies. These authors estimate that a 50-kg patient using correctly a medical aerosol containing fluorocarbon propellant would be exposed to 11 mg/kg/day, a far lower dose than the levels to which these animals were exposed.

Although the question of fluorocarbon cardiotoxicity is still not fully solved, it would seem from some of these papers that both from an industrial and medical point of view, fluorocarbons are unlikely to constitute a hazard if they are used according to instructions and if specified restrictions on exposure are observed. The Threshold Limit Value for fluorocarbons in factory atmospheres is 1000 ppm for example. However, abusers of fluorocarbons, like the abusers of many other products, are still at risk.

[F. A. Charlesworth—BIBRA]

EFFECTS OF CARBON TETRACHLORIDE ON LIPID METABOLISM

Introduction

The quest for a common biochemical explanation for the diverse hepatotoxic responses to carbon tetrachloride (CCl_4) still continues, despite innumerable theories which, for three decades, have provoked much speculation. The currently-favoured idea, which provides the theme of this article, assigns an aetiological role to the peroxidation of liver microsomal lipids by this solvent. In a previous review (*Cited in F.C.T.* 1970, **8,** 87) evidence was reported suggesting that CCl_4 *per se* was not the direct toxic agent. It was postulated that the C-Cl bond split to form a free radical, which attacked methylene-group hydrogens of the polyenoic fatty acids constituting the structural lipids of the membrane of the endoplasmic reticulum.

Free radicals and protein denaturation in lipid peroxidation

As a sequel to the work mentioned above, Rao & Recknagel (*Expl mol. Path.* 1969, **10,** 219) reported an experiment in which they examined the time-scale of incorporation of ^{14}C label from CCl_4 into microsomal lipids. On the basis of the lipid peroxidation theory, rapid incorporation of the $CCl_3^{\cdot}$ free radical into the fatty acid chain was to be expected, and this was confirmed by feeding $^{14}CCl_4$ to rats by stomach tube. Lipids of the liver-microsomal fraction were rapidly labelled, the process being complete within 5 minutes. After this time there was a slight decrease in the degree of labelling, which then remained at a level of 80% of the maximum for the remainder of the experiment. Incorporation of label into mitochondrial lipid or into microsomal proteins was much less effective than in the case of the microsomal lipids. In view of this preferential reactivity, the authors concluded that C–Cl bond cleavage and microsomal lipid peroxidation were key steps in CCl_4 hepatotoxicity.

In an examination of the time-course of changes induced in the fatty acid pattern of microsomal phospholipids by CCl_4 poisoning, Comporti *et al.* (*Ital. J. Biochem.* 1971, **20,** 156) found the only consistent alteration to be a decrease in the arachidonic acid content of the microsomal phospholipids, 4 and 12 hours after poisoning. No effective variation in the level of this acid was detected in liver triglycerides, however, 12 hours after poisoning. These data support the idea of a selective free-radical attack on the polyenoic fatty acid content of microsomal membranes, the stereochemical organization of lipid-protein complexes possibly resulting in exposure of only a limited number of unsaturated lipid molecules. CCl_4-induced lipid peroxidation can be usefully monitored *in vitro*, since such fatty acid decomposition yields malonaldehyde, which can be detected by its reaction with thiobarbituric acid. This reaction was exploited by Slater & Sawyer (*FEBS Lett.* 1970, **11,** 132) to demonstrate that CCl_4-induced lipid peroxidation in a rat-liver microsomal system was directly proportional to the square root of the CCl_4 concentration in the incubation mixture. This suggested that the induction of peroxidation was not merely a function of the lipophilic action of CCl_4 on the membranes of the endoplasmic reticulum and supported the free-radical initiation theory of lipid decomposition.

The process of lipid peroxidation may perhaps contribute to the liver damage associated with CCl_4 through denaturation of proteins and other macromolecules. Since measurements of enzyme activity offer a particularly sensitive means for detecting such changes, this possibility was examined by means of a study of the lipolytic activity of the liver in CCl_4-poisoned rats and *in vitro* experiments designed to probe the mechanism of the inhibition of hepatic lipase by CCl_4 metabolites (Ugazio & Torrielli, *Life Sci.* Part I, 1969, **8,** 197). A sharp decrease in hepatic lipolytic activity was demonstrated within 4 hours of intragastric treatment with 0·25 ml CCl_4, and was also apparent in rat-liver homogenates after *in vitro* treatment with CCl_4.

Turning to CCl_4 metabolites, these authors showed that the free-radical scavenger, propyl gallate, protected lipase activity from *in vitro* inhibition by CCl_4. Such a finding lends support to the idea that the effective CCl_4 metabolites are free radicals. Free-radical involvement may also be inferred from a reported correlation between the bond-dissociation energies of several halomethanes and their ability *in vitro* to stimulate malonaldehyde production, already identified as an index of lipid peroxidation (Slater & Sawyer, *loc. cit.*).

A more controversial issue would seem to be whether CCl_4 inhibits lipolytic activity solely by means of free-radical attack or through the additional involvement of lipid peroxidation products. Some support for the latter possibility was gleaned from *in vitro* experiments in which rat-liver lipolytic activity was impaired by treatment of the homogenate with lipoperoxides obtained by ultraviolet-irradiation of unsaturated fatty acids (Ugazio & Torrielli, *loc. cit.*). This lipolytic inhibition was also interpreted as being a possible contributory factor in the onset of CCl_4-induced fatty liver.

One set of liver enzymes profoundly affected by CCl_4 is the group of mixed-function oxidases associated with the microsomal fraction. Since the system is also very sensitive to lipoperoxidation, it was considered worthwhile to search for a possible link between this reaction and the CCl_4-induced depression of drug metabolism (Glende, *Biochem. Pharmac.* 1972, **21,** 2131). In liver-microsome preparations, peroxidative decomposition of lipids was paralleled by a reduced biotransformation of foreign compounds, as measured by the activity of aminopyrine demethylase. An aetiological connexion between these parameters was also suggested by the observation that addition of up to 4 μl CCl_4/ml to micro-

somal preparations protected from lipid peroxidation by ethylenediaminetetraacetic acid did not affect residual enzyme activity.

Searching for the biochemical site of inhibition of drug metabolism by CCl_4, Glende (*loc. cit.*) monitored changes in the activity of NADPH-cytochrome *c* reductase, and found that this actually increased as CCl_4-induced peroxidative damage to the membrane progressed. This enzyme is representative of the flavoprotein stage of the electron-transport chain responsible for demethylation of aminopyrine, and it seems therefore that the lipoperoxidation effect is remote from the flavoprotein stage of the electron-transport system. To complement these studies, the earlier work of Slater & Sawyer (*loc. cit.*) appears to exclude cytochrome *P*-450 as a site for CCl_4 attack. It was shown that when inhibitors were added to microsomal suspensions from rat liver, at concentrations sufficient to decrease the rate of drug metabolism at the *P*-450 site, they actually enhanced the stimulatory action of CCl_4.

Catecholamine hypothesis

It has been suggested (*Cited in F.C.T.* 1970, **8,** 92) that CCl_4-hepatotoxicity is evoked by a direct action on the central nervous system, the resulting discharge of sympathetic nerves being accompanied by a release of catecholamine. In one recent study in which [^{3}H]adrenaline was injected iv into rats pretreated with CCl_4, the rate of disappearance of radioactivity from the serum was similar to that in controls not treated with CCl_4 (Shafrir & Khassis, *Israel J. med. Sci.* 1969, **5,** 975). Differences were observed, however, in the tissue distribution of radioactivity, in that less was taken up by the liver and more was bound to other tissues, notably adipose tissue, in the CCl_4-treated rats than in the controls. Diminished activity of two enzymes responsible for metabolizing catecholamines in the liver (catechol-*O*-methyl transferase and monoamine oxidase) suggested that these amines may play a part in the increased release of free fatty acids (FFA) from peripheral adipose tissue associated with CCl_4 administration.

Several authorities (*Cited in F.C.T.* 1970, **8,** 92) have expressed reservations about the soundness of this theory, however, and far more direct evidence will be required before it achieves the status currently held by the lipid peroxidation hypothesis.

Processes involved in the development of fatty liver

Some doubts were cast on the scope of the lipid peroxidation hypothesis by Scheig & Klatskin (*Life Sci.* Part I, 1969, **8,** 855), who failed to obtain a quantitative relation between CCl_4-induced peroxidative decomposition and fatty-liver production (triglyceride accumulation) in rats. Production of malonaldehyde in the untreated 9000 **g** supernatant (containing cell sap and microsomes) of liver homogenate from untreated rats was slightly higher in fasted than in fed animals, and was four times greater in 400 g than in 200 g unfasted rats. *In vitro*, addition of CCl_4 to the liver preparations derived from 400 g unfasted animals enhanced malonaldehyde production, but failed to do so in preparations either from fasted rats of this weight or in fasted or fed rats weighing 200 g. Invariably, however, such animals do show large increases in hepatic triglyceride levels after CCl_4 dosage. These observations are not in keeping with a purely lipoperoxidative aetiology for CCl_4-induced fatty infiltration. We have previously indicated (*Cited in F.C.T.* 1970, **8,** 89) that the development of fatty liver in response to CCl_4 probably results primarily from an impaired secretion of hepatic triglyceride into the plasma. On this basis, we might also expect the solvent to affect the concomitant secretion of triglyceride into the bile. Indeed, following ip

injection of CCl_4 at a rate of 0·3 mg/kg, significant decreases have been reported in the blood and biliary triglyceride levels of cannulated rats, with concomitant increases in hepatic triglyceride content (Juggi, *Biochem. Med.* 1972, **6,** 111). The author discussed these results in terms of a direct toxic effect of CCl_4 on "the hepatic triglyceride secretory mechanisms", but such a basic conception of the intricate biochemical changes leading to such secretion may be somewhat misleading. In fact, triglyceride egress appears to rely on a dual mechanism, whereby the availability of the apoprotein moiety of the secreted lipoprotein complex is as important as that of the triglyceride moiety itself (*Cited in F.C.T.* 1970, **8,** 89).

In recent years it has been considered unlikely that a breakdown in the hepatic metabolism of phospholipids is a major factor in the accumulation of triglyceride caused by CCl_4. However, some correlation between these two events is still conceivable, since hepatic triglyceride and glycerophosphatides are synthesized from a common precursor, 1,2-diacylglycerol. To investigate this possibility, a group of Japanese workers (Sugano *et al. Biochem. Pharmac.* 1970, **19,** 2325) examined the changes in fatty acid composition of these glycerides in rat liver after CCl_4 administration. CCl_4 decreased the concentration of total phospholipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in the liver. There was at the same time a change in the pattern of the phospholipid components, the percentage of PC being decreased and that of PE increased. The only significant change in the distribution of the various molecular species of PC and PE in CCl_4-treated animals compared with that of controls treated with liquid paraffin was a large increase in the monoenoic fraction. Changes induced by CCl_4 were more prominent in female rats, and also in the livers of animals killed 20 hours rather than 6 hours after the CCl_4 dose.

The observed effects of CCl_4 on phospholipid composition could be explained in terms of a reduction in the synthesis of PC and PE from the diglycerides as a result of the degeneration of the endoplasmic reticular membrane, where most phospholipid synthesis occurs. However, such alterations are also explicable in terms of a block in conversion of PE to PC. Significant changes in liver triglycerides were associated with increased palmitic and decreased linoleic acid levels in CCl_4-treated rats. In the case of PC and PE, however, decreases were observed in arachidonic, docosahexaenoic and palmitic acid levels, and increases in oleic, stearic and linoleic acids. Present evidence was thus deemed inadequate to correlate the changes in hepatic phospholipids with the mechanism of fatty infiltration.

Another possible mechanism of fatty-liver production involves the induction by various agents of FFA release from adipose tissue, since the uptake of FFA by the liver is in direct proportion to their availability. We have already touched upon this in connexion with the hypothesis of catecholamine release and the work of Shafrir & Khassis (*loc. cit.*), who found that following intragastric administration of CCl_4 to rats, a fall in serum triglyceride levels slightly preceded an enhanced rate of FFA release. The peak value for such release, at about 24 hours, coincided with the maximal accumulation of triglyceride in the liver.

The influence of increased FFA mobilization on eventual fatty infiltration in the liver was assessed by administration of glucose and insulin (known to have a suppressing effect on FFA release from adipose tissue) to CCl_4-treated and control animals. The CCl_4-treated group alone showed a marked decrease in liver triglyceride content after gastric intubation with 3 g glucose, although the level was still higher than that in control animals. Glucose supplementation of CCl_4-treated rats also resulted in a notable lowering of serum FFA content and a suppression of excessive adipose-tissue FFA release, compared with that in animals not receiving the carbohydrate. These findings suggested a correlation

between the reduced triglyceride accumulation in the liver and the suppression of excessive FFA mobilization.

To trace the pattern of fat release, adipose tissue triglycerides were labelled *in vivo* by incorporation of [1–^{14}C]palmitate. CCl_4 administration was found at least to double the proportion of label transported to the liver within 24 hours and concomitantly muscle labelling was enhanced. Glucose supplementation markedly reduced the flow of label into both of these tissues. These parallel changes in muscle radioactivity support the view that the corresponding hepatic triglyceride changes may be attributable, at least in part, to alterations in the FFA supply.

Conclusions

Much of the information reviewed here appears to support the concept that free radical liberation from CCl_4 triggers the rapid peroxidation of microsomal lipids in the liver, thus explaining the early functional and structural damage to the endoplasmic reticular membrane observed in CCl_4 intoxication. Whether these early changes are the sole aetiological basis for the numerous hepatotoxic responses of CCl_4 is still unclear. Certainly in the case of triglyceride accumulation, no integrated concept of the mechanism involved can be formulated at present.

[J.J.-P. Drake—BIBRA]

THE COMPLEXITIES OF PYRROLIZIDINE ALKALOID TOXICITY

Introduction

Composites of the genus Senecio and of a few closely related genera have come under suspicion as factors contributing to the liver disease and primary liver tumours common in industrially developing countries where natural products are relied upon for medicinal and cooking purposes (*Cited in F.C.T.* 1973, **11,** 343). The alkaloids incriminated in these toxic effects include retrorsine, monocrotaline, fulvine, lasiocarpine, heliotrine and senkirkine. All of these are esters of 1-hydroxymethyl-1,2-dehydropyrrolizidine and are probably metabolized by liver microsomal-enzyme systems to hepatotoxic pyrroles (*ibid* 1971, **9,** 895; *ibid* 1972, **10,** 873).

Distribution and uses

According to Rose (*S. Afr. med. J.* 1972, **46,** 1039) there are more than 300 Senecio species widely distributed over Southern Africa, a number of them being used as food and medicine by people of the Transkei and Ciskei. In addition, pyrrolizidine alkaloids are present in some of the Boraginaceae (Heliotropium, Amsinckia, Echium, Cynoglossum and Trichodesma) and Leguminosae (Cortalaria species). Most of the alkaloid content occurs in the aerial portions before flowering and in the roots at the end of the growing season. Alkaloids are predominantly present as *N*-oxides, especially during the flowering period of the plant. Of 35 species of Senecio collected in the Transkei and the Eastern Cape, 15 are locally used as spinach, seven as snuff, one for smoking and others for various inhalations. Some species are also used extensively as purgatives, administered by mouth and by enema. *S. coronatus* has a special use over the entire area studied: a cold infusion of the chopped roots in water is given by mouth and also as an enema for the ritual purging

of infants at weaning. A decoction of the roots is also drunk for tuberculosis, haemoptysis and rectal prolapse.

Toxicity studies

In domestic animals the most common signs of acute Senecio poisoning are staggering gait, diarrhoea and nervous incoordination, followed by coma and death within a few days (Rose, *loc. cit.*). In man, diarrhoea, liver enlargement, ascites and death, after intervals ranging from 14 days to 2 years, have been reported. Post-mortem examinations have revealed haemorrhages in most organs, with centrilobular liver necrosis in the acute stage, followed by enlargement of hepatocytes in the mid-zone and peripheral regions of the lobules. In man, liver cirrhosis with venous occlusion and bile-duct proliferation are found at a relatively late stage (Rose, *loc. cit.*).

Schoental & Cavanagh (*J. natn. Cancer Inst.* 1972, **49,** 665) described two spinal cord tumours and one brain tumour in rats exposed to one of a variety of pyrrolizidine alkaloids. A spinal cord tumour, associated with paraplegia and a distended urinary bladder occurred in a rat given an sc injection of 600 mg retronecine/kg neonatally and in another rat from the litter of a female given 5% *Heliotropium ramosissimum* in the diet on days 0–15 of gestation and days 10–31 after parturition, the offspring being fed the test diet directly for 10 days when 6 month old. A cerebral astrocytoma, or possibly several, occurred following the ip injection of a weanling rat with 300 mg hydroxysenkirkine/kg. As tumours of the central nervous system were virtually unknown in untreated rats of this particular colony, it may be concluded that some tumours affecting the central nervous system may be attributed to exposure to natural pyrrolizidine alkaloids during the perinatal stage of life.

Lipomatous kidney tumours are also rare in this type of rat, but Schoental *et al.* (*ibid* 1971, **47,** 1037) found six such tumours among small groups of rats treated neonatally, as weanlings or *in utero* with retrorsine (in three oral doses of 30, 30 and 8 mg/kg over 10 months), with crude alkaloids from *H. supinum* (in a single intragastric dose of 400 mg/kg) or with seeds of *Amsinckia intermedia* (at a dietary level of 25% during pregnancy). The compounds involved here all contained activated double bonds, which would be expected readily to undergo epoxidation.

The effects of prolonged administration of retrorsine to vervet monkeys have been described by Van der Watt *et al.* (*J. Path.* 1972, **107,** 279). Intragastric doses of 20 mg/kg were given once weekly for 30 weeks and thereafter every 14 days until hepatic coma developed. After the first 25–30 doses, vomiting usually occurred 6–12 hours after treatment and was followed during the subsequent period up to 72 hours by diarrhoea and melaena. After 30 weeks, these toxic reactions were not consistently observed. The earliest date for the appearance of jaundice was 23 weeks from the start of treatment. Progressive weight loss occurring 2–3 weeks before death was followed by dullness, incoordination and, finally, irreversible hepatic coma. Monkeys dying between 20 and 27 weeks after the start of the experiment showed lung oedema, while those dying later had a variable degree of oedema of the abdominal and thoracic lymph nodes, ascites, hydrothorax and hydropericardium. Hepatic fibrosis was apparent in animals dying at 50 weeks or later. Vaso-occlusive liver lesions started with subendothelial oedema and luminal fibrin deposition in small central veins, and led to venous occlusion and later recanalization. Marked iron storage disease resembling Banta siderosis, occlusion of kidney nephrons, and atrophy of the germinal epithelium of the testes also occurred.

Pulmonary and myocardial changes rather than liver lesions were the principal findings in a study of stumptail monkeys (*Macaca arctoides*) given monocrotaline in an sc dose of 30 mg/kg when 1 month old (Chesney & Allen, *Cardiovasc. Res.* 1973, **7**, 508). Survival time of the treated monkeys ranged from 199 to 325 days (mean 241 days) and death was preceded by marked pulmonary hypertension and vasoconstriction. Enlargement of individual endothelial cells and hypertrophy of the medial musculature led to partial or complete obliteration of pulmonary vessels, and ultrastructural studies demonstrated numerous fibrin and platelet thrombi in the capillaries and arterioles. In some cases, oedema was prominent in the endothelial lining of arterioles and muscular arteries. Myocardial hypertrophy and a marked dilatation of the right ventricle also occurred in the treated monkeys.

In view of the probable role of pyrrole derivatives in the hepatotoxic action of the pyrrolizidine alkaloids, Pleština & Stoner (*J. Path.* 1972, **106**, 235) carried out some experiments actually using monocrotaline pyrrole. Rats injected iv with 5–10 mg/kg became ill after 2–3 weeks and half of them died by the end of month 2. Those injected with 30 mg/kg showed respiratory embarrassment after about 15 hours, and by 48 hours convulsions and death, associated with pleural effusions, generally ensued. The relative lung weight in such animals was significantly greater than that in controls at 6 hours, as maximal at 9 hours and thereafter decreased and gave way to pleural effusion. Protein concentration in the pleural fluid did not exceed that in normal lung lymph and was much lower than that observed after treatment with α-naphthylthiourea (ANTU). Carbon labelling located the pyrrole-induced lesions in the capillaries, whereas ANTU damages venules. 5-Hydroxytryptamine has been suggested as an effective intermediate in the pulmonary activity of monocrotaline, but this too affects the venules rather than the capillaries. Moreover, the reaction of the rat to monocrotaline pyrrole was not modified by pretreatment with *p*-chlorophenylalanine, which would be expected to inhibit 5-hydroxytryptamine synthesis.

Metabolism

Continuing earlier studies on the metabolic conversion of many pyrrolizidine alkaloids to pyrroles by the microsomal enzymes of the rat liver, Mattocks (*Chemico-Biol. Interactions* 1972, **5**, 227) compared the total hepatic pyrrole levels at different times after administration of various alkaloids (ip or by intubation) and attempted to relate the findings to the acute toxicity of the alkaloids. While the individual toxicities of the alkaloids differed widely, the hepatic levels of pyrrolic metabolites a few hours after administration of the LD_{50} were closely comparable. This suggested that the pyrroles were the cause of the toxic effect, although the possibility of other toxic metabolites could not be ruled out. Sex differences in sensitivity were apparent, retrorsine and monocrotaline being more toxic to males than to females. Both liver pyrrole concentration and toxicity were reduced by pretreatment with SKF 525A. On the other hand, phenobarbitone pretreatment increased the rate of conversion to pyrrolic metabolites, increasing acute toxicity when normal metabolism was low, as with monocrotaline, with retrorsine (in female rats) and with heliotrine. When metabolism was normally rapid, as with lasiocarpine, phenobarbitone tended to reduce the rats' susceptibility, possibly by accelerating further conversion to a secondary metabolite.

These findings support those of an earlier study (*Cited in F.C.T.* 1973, **11**, 161), which showed also that the sensitivity of rats to lasiocarpine and heliotrine given repeatedly in small doses increased with age. Mattocks & White (*Chemico-Biol. Interactions* 1973, **6**, 297)

found that liver-microsomal preparations from newborn rats showed a very low capacity for converting retrorsine to pyrrolic metabolites, but that within 5 days of birth this capacity increased markedly, only to decrease again after 30 days in the livers of females. Similarly, when retrorsine was given to rats within 1 hour of birth, pyrrolic metabolites were at a low level 2 hours later, whereas in animals treated when 1–17 days old, the pyrrole levels after 2 hours were much higher. After 30 days the production of metabolites in females was only about 30% of that in males. The susceptibility of rats of different ages to the hepatotoxic effects of this pyrrolizidine alkaloid reflected the capacity of their livers to produce and retain pyrroles.

The overall toxicity of monocrotaline to rats has also been shown by Allen *et al.* (*Toxic. appl. Pharmac.* 1972, **23,** 470) to depend on the level of hepatic microsomal activity. Prior treatment with phenobarbitone was reflected in poorer weight gain, earlier and more severe lung lesions, and earlier death from a dose of 60 mg monocrotaline/kg. Treatment with the enzyme inhibitor chloramphenicol prevented death after this dose, preserved a normal weight gain, and ameliorated the acute lesions, though it did not prevent the appearance of chronic liver lesions. That there is a considerable species difference in the interaction of enzyme inducers and retrorsine toxicity appears from a paper by White *et al.* (*Chemico-Biol. Interactions* 1973, **6,** 207). LD_{50} values for retrorsine varied from 65 mg/kg in mice to 279 mg/kg in the quail and over 800 mg/kg in the guinea-pig. Pretreatment with phenobarbitone decreased the susceptibility of mice but increased that of guinea-pigs. In more susceptible animal species it was found that liver levels of pyrrole 2 hours after an ip dose of retrorsine were generally higher than those in resistant species.

Allen & Chesney (*Expl mol. Path.* 1972, **17,** 220) reported a difference in organ sensitivity in stumptail monkeys of different ages exposed to monocrotaline. They attributed this to differences in microsomal-enzyme levels and the resulting differences in liver concentrations of metabolites. Monkeys were given four sc injections of monocrotaline (comprising doses of 30, 60, 60 and 60 mg/kg) at 2-month intervals, either from the age of 4 weeks or 15 months. Towards the end of the course, those in the younger age-group showed an increase in haemoglobin, haematocrit, red cell mass and arterial pCO_2 and a decrease in arterial pO_2 and pH, together with an increase in right-heart and pulmonary-artery pressures. As in the study mentioned earlier (p. 561), bilateral myocardial hypertrophy with dilatation of the right ventricle was seen *post mortem*. The lungs were firm, with thickened arterial walls and narrowed lumina. The myocardium of the right ventricle was oedematous, the endocardium was fibrosed and the arteries were disrupted. The most prominent findings in the older monkeys, however, were ascites, distended abdominal veins and a rise in portal venous pressure. Post-mortem findings in these animals included small, firm, granular livers, with venous occlusion extending from the centrilobular to the large hepatic veins and associated with dilated blood-filled sinuses.

Protection

Previous observations have suggested that a low-lipotrope diet will protect male rats against the acute effects of monocrotaline, presumably by reducing liver microsomal-enzyme activity and thus pyrrole production (*Cited in F.C.T.* 1972, **10,** 861). A possible method of protecting sheep likely to graze on Senecio and other toxic species has been indicated by Lanigan (*Aust. J. agric. Res.* 1972, **23,** 1085), who had previously demonstrated that the suppression of methane-forming bacteria in rumen fluid *in vitro* markedly increased the rate of metabolism of heliotrine (*idem, ibid* 1971, **22,** 123). When sheep were

given an intraruminal dose of chloral hydrate (10 mmols), bromoform (0·5 mmols) or iodoform (0·5 mmols) to inhibit methane production, followed 18 hours later by 2 g *Heliotropium europaeum* alkaloids (32% heliotrine, 36% lasiocarpine, 18% europine, 8% heleurine and 6% supinine), the normal time for disappearance of the alkaloids from the rumen (7–8 hours) was shortened to 2–3 hours. In further experiments, marked differences in the rate of disappearance of heliotrine and of lasiocarpine became apparent, and there were indications that the induction of lasiocarpone metabolism was possibly stimulated by the simultaneous breakdown of some other pyrrolizidine alkaloid. There appears, however, to be no direct evidence as yet that these effects on the rate of metabolism would necessarily afford protection for the target organs of the pyrrolizidine metabolites.

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HEXACHLOROPHENE

Introduction

Hexachlorophene (HCP) has been a widely used bacteriostatic agent for almost 25 years and is noted for its effectiveness against the gram-positive organisms that usually colonize normal skin. It is used by the cosmetics industry in the manufacture of talcum powders, deodorants and shampoos at concentrations between 0·1 and 0·5%, and in medicinal products for topical application at higher levels of 0·5–3%. Until recently, this phenol derivative was generally regarded as innocuous at the concentrations to which man is normally exposed, except for isolated cases of allergic dermatitis or photosensitivity arising from its contact with hypersensitive human skin. Early in 1971, however, the US FDA published a study showing that signs of central nervous system disorder could be evoked in rats fed 25 mg HCP/kg/day and demonstrating that these effects were associated with a spongy degeneration of the white matter of the brain (*Cited in F.C.T.* 1972, **10,** 275). Readers will be familiar with succeeding events leading up to the American restrictions on the use of HCP, and with the ensuing flurry of activity on behalf of other regulatory authorites. The aim of this article is to present and evaluate the more recent studies aimed at clarifying the benefit-risk balance associated with current usage of this material as a cleansing agent. The cosmetic use of HCP, generally involving the more transient use of HCP on restricted areas of the body, is not considered in detail here.

HCP in infant skin care

Official anxiety concerning the safety of HCP used in the antiseptic skin care of newborn infants was increased by reports of pathological lesions that developed in rhesus monkeys washed daily in a 3% solution of HCP/FDA announcement, 8 December 1971). At about the same time, it was reported that HCP was absorbed into the blood stream of babies bathed daily with a 3% solution for several days (*Cited in F.C.T.* 1972, **10,** 114). The range of HCP levels in the umbilical-cord blood was 0·003–0·182 ppm, while the blood levels at the time the infants were discharged from hospital ranged from 0·01 to 0·65 ppm. Similar results have been observed in babies exposed to HCP in powder form. Thus, after 15 healthy neonates had been treated in hospital for 8 days with a dusting powder containing 0·33% HCP, blood levels of the phenol derivative were in the range 0·04–0·5 ppm (Alder *et al. Lancet* 1972, **ii,** 384). These upper levels are more than half those known to

be associated with histological changes in the white matter of the rat central nervous system (see below).

Among the first epidemiological surveys relating to the use of 3% HCP in hospitals was that carried out in Australia at Geelong Hospital, Victoria (Plueckhahn, *Med. J. Aust.* 1973, **1,** 93). A preliminary report on this study was mentioned earlier (*Cited in F.C.T.* 1973, **11,** 341). No evidence of neurotoxicity was observed in any of the 26,114 infants given this type of antiseptic skin care while in the hospital. The mean HCP concentration in the blood of these infants was approximately 0·05 ppm by the third or fourth washing, and the maximum concentration recorded was less than 20% of the minimum level found to be neurotoxic to rats or newborn monkeys. Reviewing the existing animal data, Plueckhahn (*loc. cit.*) put the critical blood level of HCP at which spongy changes occur in the white matter of the central nervous system of rats and monkeys at between 0·5 and 1·0 ppm. A retrospective review of 34 neonatal and 75 postnatal deaths occurring in the Australian infants failed to reveal any macroscopic changes or histological evidence of spongy change in the white matter of the cerebrum or cerebellum.

On a smaller scale, Abbott *et al.* (*Aust. paediat. J.* 1972, **8,** 246) determined the blood levels of HCP in 2·5–4-kg infants treated with pHisoHex (containing 3% HCP) and reported concentrations of 0·068–0·612 ppm after three applications of the antiseptic. When the HCP treatment was modified to provide for the dry-washing of the infants with pHisoHex on the day of delivery only, the maximum blood level attained was still of the order of 0·5 ppm. The relatively high blood levels of HCP recorded in this survey, compared with those observed at Geelong, cannot be attributed merely to contamination by HCP on the skin, since great care was taken to avoid this complication during blood sampling. It is difficult to make comparisons, however, because of the differing regimes of HCP application at the two hospitals. At Geelong, for example, babies had 2 ml of pHisoHex applied with cotton wool over their entire body (*Cited in F.C.T.* 1973, **11,** 341). This was then washed into a lather with water and removed with a fresh supply of cotton wool. A further 2 ml pHisoHex was then applied and allowed to persist on the skin surface. In the study by Abbott *et al.* (*loc. cit.*), 7·5 ml pHisoHex was applied over the total body surface; this was washed off and the process was repeated. After the final washing no visible pHisoHex remained on the skin. The blood concentrations of HCP in ten full-term infants undergoing daily body-bathing with a 3% solution for between 13 and 82 days in an Intensive Care Unit were reported by M. D. Cunningham at a meeting of the American Academy of Pediatrics held in October 1972 (cited from Plueckhahn, *loc. cit.*). The overall mean HCP level was 0·5 ppm, and subsequent screening revealed no neurological abnormalities and a satisfactory rate of development.

There is thus no current evidence clearly against the hospital use of 3% HCP emulsions on full-term babies weighing over, say, 1·5 kg. The value of HCP in the management of nursing epidemics of staphyloccocal infections is undisputed (Plueckhahn, *loc. cit.*) and until an effective alternative (such as chlorhexidine) has been thoroughly tested, the risk associated with its continued use is likely to be exceeded by that which could accompany its withdrawal. Hospital staff should nevertheless be aware of the relatively small margin of safety existing between present levels of infant exposure and the threshold toxicity level for this bactericide, and every care should be taken to avoid excessive use.

It seems that stronger evidence is available to support the view that the bactericide should not be applied to premature babies. Thus Powell *et al.* (*J. Pediat.* 1973, **82,** 976) described the occurrence of spongiform changes in the brains of infants who had died in

the perinatal period. In the case of babies weighing less than 1·4 kg, no cases of spongiform myelination were observed in those subjected to less than four HCP exposures and six of the seven infants affected in this way had been washed nine times in 3% HCP. In a larger study (Shuman *et al. J. Am. med. Ass.* 1974, in press; cited from Powell *et al. loc. cit.*), vacuolation was found in the long myelinated tracts of 21 infants, 18 of whom had had multiple exposures to 3% HCP and most of whom had been born prematurely. Although they showed no abnormal neurological signs, five infants of low birth-weight, who were bathed daily for 21–56 days with a 3% HCP solution diluted about eightfold with water, had blood-HCP levels of 0·21–1·1 ppm (Kopelman, *J. Pediat.* 1973, **82,** 972).

Some 5 years back, the dermal absorption of HCP during total-body bathing of burned babies was implicated as a factor in the development of burns encephalopathy (Larson, *Hospitals* 1968, **42,** 63). Lockhart (*Pediatrics, Springfield* 1972, **50,** 229) also draws attention to a more recent case reported to the FDA, involving a 10-yr-old boy with 25% body burns who was treated with frequent applications of an antiseptic preparation containing 3% HCP and died in coma within 2 weeks. Some 2 days after the last application, the blood level of HCP was recorded as 2·2 ppm. In a similar case, Mullick (*ibid* 1973, **51,** 395) described signs of neurotoxicity in two children treated with 3% HCP baths for burns. Both children died and in them, and in two infants treated similarly for congenital ichthyosis, severe vacuolization of the white matter of the cerebrum and cerebellum was evident *post mortem*. Such findings are clearly sufficient to contraindicate the use of this antiseptic on the skin of burned babies.

Topical application to adults

Reports of the acute toxic effects of the misuse of HCP products are now plentiful, but there appears to have been no documentation of toxic effects in individuals using HCP products as recommended by the manufacturers. It is thus of interest to compare the blood concentrations of HCP in surgical personnel, routinely using HCP formulations for the cleaning of hands and forearms, with those levels known to be accompanied by toxic signs in animals. Butcher *et al.* (*Archs Surg.* 1973, **107,** 70) recorded blood concentrations of HCP in individuals who scrubbed with 3% HCP detergent, and reported a mean value (0·2 ppm) some seven times as high as that of randomly chosen patients. On the other hand, the blood level (0·06 ppm HCP) among users of a soap containing 0·25% HCP was only twice the patient baseline. Follow-up blood samples taken 2–3 weeks after exposure to the HCP product was terminated indicated a rapid return to the initial background level of HCP contamination. These findings show that HCP levels are higher in users of HCP detergent than in those using the HCP soap and the variation in blood levels was greater in the former group, although the reason for this is not clear.

A similar baseline blood level (0·02 ppm) was reported by Ulsamer *et al.* (*Fd Cosmet. Toxicol.* 1973, **11,** 625), who carried out controlled studies on the use of HCP products for whole-body or hand washing and for mouth washing. Blood levels found ranged from 0·38 ppm in the blood of an individual who used a 3% HCP cleanser for daily total-body washing over a prolonged period to less than 0·005 ppm in some of the test subjects who used 1 or 3% HCP cleansers for hand-washing only. Blood levels resulting from prolonged use of a soap containing 0·75% HCP ranged from 0·02 to 0·14 ppm, similar to those (0·02–0·12 ppm) following daily use of a mouthwash containing 0·5% HCP for 3 weeks.

Oral toxicity in experimental animals

Reports of neurotoxicity arising from topical application of HCP inevitably focused attention on the possibility of food contamination following the use of this chemical as a foliage fungicide and bactericide in agriculture. It was thus deemed important to determine a threshold level of oral intake for the central nervous system effects in experimental animals.

In a group of female rats fed 100 ppm HCP in the diet for 98 days, 80% developed focal areas of spongy degeneration in the white matter of the brain, although there were no signs of the leg weakness which was apparent in rats fed 500 ppm HCP in the diet (Gaines *et al. Toxic. appl. Pharmac.* 1973, **25,** 332). A no-effect level of 20 ppm HCP in the diet was demonstrated in this study, compared with a value of 50 ppm indicated by another 16-weeks feeding study in rats (Nakaue *et al. ibid* 1973, **24,** 239) and with some unpublished results from Biotest Laboratories cited by Lockhart (*loc. cit.*) which established a no-effect level of 65 ppm HCP in the diet (leading to a blood level of 0·4–0·8 ppm) with respect to the development of brain lesions.

Lockhart (*loc. cit.*) also refers to some unpublished work by G. Kennedy (in 1971) on the reversibility of HCP-induced brain lesions. Rats given doses of 40 mg HCP/kg orally for 6 weeks developed hind-limb paralysis and lethargy and showed "moderate" focal vacuolization of the brain and spinal cord. After 6 weeks on the HCP diet, animals were sacrificed after various intervening periods and pathological examinations were conducted over an 84-day period. Vacuolization decreased steadily during this time but had not disappeared completely at the end of the observation period. Clearer evidence for the reversibility of neuropathic responses to HCP treatment has been documented by de Jesus & Pleasure (*Archs Neurol.* 1973, **29,** 180). Rats fed diets containing 25–35 mg HCP/kg showed little, if any, neurological impairment, but animals on diets containing 50–70 mg/kg showed hind-limb weakness after 1–2 weeks, and this effect became severe within 1 month. The motor-nerve conduction velocity (MNCV) of sciatic nerves was decreased in rats on both levels of treatment, and occasional demyelination was evident in some internodes. One week after discontinuation of HCP feeding, neuromuscular dysfunction had cleared and the MNCV had returned to normal.

Histological abnormalities of the type observed in rats appear to be absent from dogs fed relatively high levels of HCP, at least on an acute basis. Thus, Scott *et al.* (*J. Am. vet. med. Ass.* 1973, **162,** 947) reported a general lack of gross and histopathological changes in two 12-kg dogs each given a single dose of 450 mg HCP in gelatin capsules. The animals died within 24 hours of treatment after developing diarrhoea, vomiting, hypersalivation, tachypnoea, depression and hypocalcaemia.

Caldwell *et al.* (*Biochem. Pharmac.* 1972, **21,** 2425) found HCP to be a very potent uncoupler of oxidative phosphorylation. Following oral dosing of rats with 20 or 100 mg HCP/kg, liver mitochondria from these animals exhibited a decrease in oxidative phosphorylation but no modification in the activities of electron-transport enzymes when compared to controls. The maximal effect occurred about 12–15 hours after dosing and complete recovery occurred in about 36 hours. These authors also noted a substantial increase in adenosine triphosphatase activity in liver mitochondria from rats given the lower dose, but increasing the HCP dose to 100 mg/kg caused no further increase in this enzyme activity.

An elimination study discussed by Kimbrough (*Pediatrics, Springfield* 1973, **51,** 391) demonstrated a fairly rapid excretion rate from rats that had been fed 25 mg/kg/day for

55 days, the half-life in kidney, liver and blood being about 10 hours and that in brain about 19 hours. Other unpublished studies cited by Plueckhahn (*loc. cit.*) have indicated that the excretion of HCP occurs rapidly within 24 hours of absorption and is virtually complete within 1 week. The metabolism of HCP involves glucuronide conjugation, the glucuronide being excreted in the bile (Gluck, *Pediatrics, Springfield* 1973, **51,** 400). It follows that studies are required not only on the blood levels of HCP itself but also in the relative blood concentrations of conjugated and unconjugated HCP resulting from HCP usage. Studies should also be conducted on the toxicity of the glucuronide itself.

Reproduction studies

Since HCP appears to cross the placenta (*Cited in F.C.T.* 1972, **10,** 114), it is of great importance to assess its teratogenic potential, and to relate this to in-use conditions. Gaines *et al.* (*loc. cit*). found that the survival of offspring was slightly reduced in the F_1 generation when the parent rats were fed HCP at a dietary level of 100 ppm (8 mg/kg/day). These offspring were mated after 54 or 166 days on the test diet and continued to receive this diet throughout mating, gestation and lactation. Litters in the resulting F_2 generation tended to be smaller than those in the preceding one, but the incidence of white-matter vacuolation decreased in successive generations. A dietary level of 20 ppm was found to have no effect on reproduction.

Since HCP is widely used in feminine-hygiene sprays, Kimmel *et al.* (*Lancet* 1972, **ii,** 765) attempted to determine whether absorption of HCP through the vaginal mucosa might interfere with embryonic development. In a preliminary study, 0·2 ml of a suspension containing 450 mg HCP/ml was inserted into the vagina of rats on days 7, 8, 9 and 10 of gestation and the animals were killed on day 20. Two out of 12 rats died after only 2 days of treatment with HCP. The foetal malformation rate in treated animals was 40% compared with 4% in control rats treated with the aqueous vehicle of suitable consistency, and the combined death plus resorption rate in the treated group (33%) was four times higher than that in controls. Abnormalities most frequently observed were microphthalmia, hydrocephalus and wavy ribs, but there were only two cases of cleft palate. Other studies employing lower levels of HCP comparable to those likely to arise from the use of commercial sprays, are to be published in due course and should provide a firmer basis for an assessment of the teratogenic potential of HCP when applied to mucous membranes. Nevertheless, when one considers that the proportion of HCP found in feminine deodorant sprays has been alleged in some cases to reach 1% as the propellant gas evaporates (*Sunday Times*, 12 November 1972), and in view of the fact that this phenol derivative is absorbed through the vaginal mucosa six times as readily as through the skin, such superfluous usage of a potential intoxicant can only be discouraged. Indeed the very basis for the inclusion of HCP in toothpastes, mouthwashes or vaginal deodorants is highly questionable, since *Staphyloccocus aureus* is rarely found in either mouth or vagina.

Conclusions

There are two major prerequisites for a clear assessment of the short-term hazard associated with topical application of consumer products containing HCP. Primarily, the pattern of daily use of specific products must be related to subsequent blood levels of the bactericide (regarded as an index of HCP toxicity). Secondly, a concentration of HCP at which no neurological effects occur must be defined, on the basis of epodemiological data and animal experimentation, in terms of both HCP application rate and blood levels. Some

considerable progress has now been made towards meeting the first requirement, and quantitative estimates of HCP absorption have been elaborated for infants bathed in HCP detergents (0·06–0·6 ppm) and for various HCP cleansing products (0·06–0·2 ppm). The second demand is not so easily satisfied. It may be argued that no neurological symptoms have been observed in individuals whose blood concentration of HCP exceeded 0·5 ppm, nor indeed in animals after histological examination of brain tissue. Nevertheless, this level is far too close to the 0·8 ppm in rat blood that has been associated with mild neurological effects to be dismissed lightly. Except for those medicinal products in which the known benefits of HCP incorporation exceed its ostensible hazard, a more realistic safety margin should be the aim. Thus, the routine use of 0·25% HCP soap by surgical personnel for hand-washing, giving rise to blood levels of the order of 0·06 ppm HCP, would not appear unjustified. In certain cases, however, such as in mouthwashes and vaginal deodorants, the potential risk associated with the use of this bactericide greatly outweighs its intended efficacy, leaving little justification for its continued use.

[J.J.-P. Drake—BIBRA]

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TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS

EMULSIFIERS AND STABILIZERS

2714. Carboxymethylcellulose sensitivity: A new phenomenon

Müller, Eva, Pevny, Irmgard u. Metz, G. (1973). Allergie gegenüber Carboxymethylcellulose, Bestandteil einer Steroidkristallsuspension. *Hautarzt* **24,** 317.

Sodium carboxymethylcellulose (CMC) has been shown to remain unabsorbed during passage through the gut and to have no systemic effects (Frawley *et al. Fd Cosmet. Toxicol.* 1964, **2,** 539; McElligott & Hurst, *ibid*, 1968, **6,** 449). No previous reports of any sensitizing potential of this compound have been located.

An urticarial reaction, complicated by the appearance of vascular oedema (Quincke's oedema) appeared in a woman, aged 29, who was given a second intralesional injection of a steroid formulation containing 0·75% CMC 4 wk after her initial dose of the same preparation. Intradermal tests with the components of the preparation showed that 0·75 and 0·375% CMC in saline provoked a + + reaction after 30 min, progressing to + + + after 45 min and + + + + after 60 min. In view of the widespread use of CMC both in pharmaceutical preparations and in food technology, this isolated report is considered significant, especially as the reaction occurred in the presence of triamcinolone acetonide, which might have been expected to suppress any allergic response.

PRESERVATIVES

2715. The complexity of sulphite excretion

Gunnison, A. F. & Palmes, E. D. (1973). Persistence of plasma *S*-sulfonates following exposure of rabbits to sulfite and sulfur dioxide. *Toxic. appl. Pharmac.* **24,** 266.

Sulphur dioxide and sulphites circulating in the blood are disposed of by formation of *S*-sulphoproteins, through the sulphitolysis of disulphide bridges (*Cited in F.C.T.* 1973, **11,** 504). The study reported here deals with the formation, persistence and clearance of *S*-sulphonates in the plasma of rabbits exposed to oral or iv-administered sulphite or inhaled sulphur dioxide.

When sulphite in a dose of 0·9 mmol/kg was injected iv, *S*-sulphonate formation was rapid, reaching a peak 20–30 min after the injection, by which time plasma sulphite had decreased almost to zero from the level of about 500 nmols/ml recorded at 10–15 min. Clearance of the sulphonate so formed was also rapid, about half of the total being cleared within a further 40–60 min. The peak *S*-sulphonate concentrations ranged from 110 to 180 nmols/ml. After exposure to 8·9 or 26 μmols sulphite/ml in drinking-water or to 10 ppm SO_2 in the atmosphere over several days, equilibrium levels of *S*-sulphonate were established in the rabbit plasma. Neither mode of administration led to any detectable free sulphite in the plasma, but *S*-sulphonate levels rose over the first 3–5 days and then maintained a roughly constant level until the exposure was terminated. On cessation of the

exposure, plasma clearance of *S*-sulphonate was initially exponential (to a level of about 10 nmols/ml above the endogenous concentration) and then levelled off for several days before falling again to the endogenous level. Clearance of exogenous *S*-sulphonate was significantly slower after inhalation of SO_2 than after sulphite ingestion, the mean half-lives being 4·1 and 1·3 days, respectively. Dialysis experiments showed that the diffusibility of exogenous *S*-sulphonate depended on the mode of its formation and therefore on the route of absorption of the sulphite giving rise to it. Evidence suggests that the clearance of plasma protein *S*-sulphonates is relatively slow and that the more rapid clearance is associated with the formation of compounds from other plasma components and probably specifically with that of cysteine *S*-sulphonate.

AGRICULTURAL CHEMICALS

2716. Reproduction effects of Delnav, toxaphene and Herban

Kennedy, G. L., Jr., Frawley, J. P. & Calandra, J. C. (1973). Multigeneration reproductive effects of three pesticides in rats. *Toxic. appl. Pharmac.* **25**, 589.

The insecticides Delnav and toxaphene have been investigated fairly thoroughly from a toxicological viewpoint (*Cited in F.C.T.* 1964, **2**, 500), the no-effect levels for their prolonged dietary administration to various animal species being well above the concentrations of these pesticides liable to be ingested by man. Comparatively little has been established, however, about the possible effects of these compounds on reproductive performance, and the authors cited above therefore undertook a study in rats, using feeding levels slightly above and below those that marginally failed to evoke any adverse effects in subacute feeding tests. The substituted-urea herbicide, Herban, was tested in the same manner. The technical-grade pesticide was used in each case.

Groups of weanling rats were fed 3 or 10 ppm Delnav in the diet for 79 days before mating. All the animals were continued on their test diet during mating and gestation, and two further generations were treated likewise, two litters being bred in each generation. Reproductive indices were calculated, body weights were recorded, haematology, blood-chemistry determinations and urine analyses were carried out on the F_2 generation parents, and complete gross and histopathological examinations were made after administration of Delnav for 42 wk to the F_0 parent generation and for 39 wk to the F_1 and F_2 parents. No measurable abnormalities occurred among either the parents or their offspring.

In the corresponding experiment with toxaphene, dietary levels of 25 and 100 ppm had no effect on litter size, pup survival or the body weights of weanlings, but the level of 100 ppm caused slight cytoplasmic fatty vacuolization of the liver in parent rats. There were no concomitant effects on growth, mortality, clinical parameters, organ weights or reproductive capacity. At a dietary level of 500 ppm, Herban had no untoward effects on any animals, but at 3000 ppm the growth of parental animals was depressed slightly, an effect attributed to the unpalatability of the test diet. Absolute liver weights and liver-to-body weight ratios were increased in all animals in this group.

The authors deduced no-effect levels of 10, 25 and 500 ppm in the diet of rats for Delnav, toxaphene and Herban, respectively. Reproduction was unaffected by these three pesticides at levels of 10, 100 and 3000 ppm, respectively, in these experiments.

2717. Tissue distribution of paraquat and diquat

Litchfield, M. H., Daniel, J. W. & Longshaw, Susan (1973). The tissue distribution of the bipyridylium herbicides diquat and paraquat in rats and mice. *Toxicology* **1,** 155.

The widely-used herbicides diquat and paraquat are well known for their acute toxic effects. This is particularly true of the severe, and frequently fatal, pulmonary changes that follow the accidental ingestion of paraquat by man (*Cited in F.C.T.* 1972, **10,** 700). Although in rats the major part of an oral dose of either herbicide is known to be eliminated in the faeces and most of the absorbed material is rapidly excreted in the urine (*ibid* 1967, **5,** 242), urinary excretion of small amounts of paraquat have been detected in man for up to a month after ingestion of paraquat solutions (*ibid* 1972, **10,** 701). This is presumably due to tissue binding, for which there is some evidence, but information on the tissue distribution of these bipyridylium compounds is sparse. The authors named above have investigated this aspect in mice, using the technique of whole-body autoradiography, and in rats by the colorimetric determination of tissue levels after the continuous feeding of the compound.

Mice were injected iv with either [^{14}C]paraquat (20 mg cation/kg) or [^{14}C]diquat (50 mg cation/kg) and the animals were killed at various intervals for autoradiographic examination. Within 10 min, both compounds were distributed throughout most tissues, with particular concentration in the cartilaginous and hepatic tissues. While paraquat was distributed throughout the liver parenchyma, diquat was largely restricted in the liver to the gall bladder. Other differences were that whereas low diquat levels were found in the brain and spinal cord, no paraquat was detected in the central nervous system (CNS) and the concentrations in cardiac muscle were substantially greater with paraquat. Elimination of diquat from all tissues except the intestine and bladder was complete within 24 hr, while low levels of paraquat remained in the lungs, skeletal muscle and CNS, although these were no longer visible in the 72-hr autoradiogram.

Analysis of brain, lung, liver, kidney, muscle, stomach and intestinal tissues from rats fed 50–250 ppm paraquat or 250 ppm diquat in the diet for up to 8 wk showed no accumulation of either compound. When surviving rats were returned to a normal diet for 7 days neither herbicide was detectable in the tissues studied.

In conclusion, the authors note that the uptake of paraquat by cardiac muscle and the persistence of this compound in lung tissue represent the most conspicuous differences between paraquat and diquat as regards their distribution in the body. They point out, for example, that feeding rats on diets containing paraquat concentrations of only twice the no-effect level of 120 ppm led to a level of the compound in the lungs some 5–10 times higher than that found after consumption of the 120 ppm diet. Such data should provide sufficient stimulus to provoke further investigation of the nature of the binding of this hazardous herbicide to lung tissue.

2718. 2,4,5-T and testosterone metabolism

Lloyd, J. W., Thomas, J. A. & Mawhinney, M. G. (1973). 2,4,5 T and the metabolism of testosterone-1,2-$^3H_2$ by mouse prostate glands. *Archs envir. Hlth* **26,** 217.

With the exception of DDT, few pesticides have been investigated in depth as regards their possible effects on male reproductive organs. The recent experiments of Lloyd *et al.* (cited above), who fed high doses of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) to mice, were aimed specifically at investigating the effects of this herbicide on the accumulation of androgen by the prostate gland.

2,4,5-T was administered to groups of mature male mice at levels of 6·25, 12·5 or 25 mg/kg daily for 10 days, by gastric intubation. This treatment was followed after a 24-hr interval by a single ip injection of [1,2-$^3H_2$]testosterone ([$^3H_2$]T) and the rats were killed 5 min later. The prostate gland from control animals metabolized about half of all assimilated [$^3H_2$]T within 5 min, the major metabolite being dihydrotestosterone. Treatment with 2,4,5-T markedly reduced the assimilation of [$^3H_2$]T by the prostate, but did not substantially affect the proportions of specific steroid metabolites produced. The reduced [$^3H_2$]T accumulation by the gland was not a dose-related effect and did not appear to be reflected in any increase in the hepatic formation of polar metabolites of [$^3H_2$]T or of dihydrotestosterone, androstanediol or androstenedione in the mice treated with the herbicide.

The authors suggest that 2,4,5-T may exert a direct effect on the prostate gland, the observed decreases in accumulation of the steroid reflecting changes in its binding to the target tissue.

[*In vitro* incubation of 2,4,5-T with fractions of prostate would provide a useful adjunct to this work. Similar experiments have recently been conducted with dieldrin, which inhibited the binding of dihydrotestosterone to fractions of rat ventral prostate gland, at least in the test-tube.]

FEED ADDITIVES

2719. Propylene glycol as a feed additive

Sauer, F. D., Erfle, J. D. & Fisher, L. J. (1973). Propylene glycol and glycerol as a feed additive for lactating dairy cows: An evaluation of blood metabolite parameters. *Can. J. Anim. Sci.* **53,** 265.

The viscous liquids, propylene glycol (PG) and glycerol, have achieved wide usage as solvents in the food industry, and are generally considered to be of low oral toxicity (*Cited in F.C.T.* 1963, **1,** 273; Weil *et al. Fd Cosmet. Toxicol.* 1971, **9,** 479; Gaunt *et al. ibid* 1972, **10,** 151). Some of the properties of these chemicals have been exploited in pharmaceuticals for human use, and some Canadian workers (cited above) have now discovered that they can be used to combat hyperketogenesis in lactating cows. In these animals, ketone-body formation is accentuated by the constant drain on low carbohydrate stores imposed by lactose synthesis and secretion. This effect can be counteracted by administration of glucose, but since glucose itself is not absorbed effectively from the gastro-intestinal tract, suitable gluconeogenic precursors have been examined. Of these, PG and glycerol were the most promising.

This study involved a total of 120 milking cows fed for 8 wk after the birth of their calves with either glycerol or PG at levels of 3 or 6% in the diet. PG was also given at 9%. Weekly blood analyses were used to classify the animals as either normal, clinically ketonic or subclinically ketonic. In cows not subjected to low concentrate intakes or in those with only average yields of milk, neither glycerol nor PG had much depressant effect on β-hydroxybutyrate, acetoacetate or free fatty acid (FFA). However, in conditions of stress due to reduced concentrate intake or adverse environmental factors, PG at all the dietary concentrations tested significantly reduced the levels of blood ketones and plasma FFA and increased blood-glucose levels. Similar improvements were seen with glycerol.

The authors conclude that the addition of 3–6% PG to the feeds of dairy cattle markedly decreases the incidence of clinical and subclinical ketosis during the critical period 4–8 wk after parturition. They point out that studies carried out elsewhere (Emery *et al. J. Dairy Sci.* 1964, **47,** 1074) showed that over 99% of administered PG was metabolized, with no detectable secretion into the milk, and that blood levels remained below 10 mg PG/100 ml when up to 2 kg PG was fed daily.

PROCESSING AND PACKAGING CONTAMINANTS

2720. Safety-in-use of a butylene glycol adipate plasticizer

Fancher, O. E., Kennedy, G. L., Jr., Plank, J. B., Lindberg, D. C., Hunt, W. H. & Calandra, J. C. (1973). Toxicology of a butylene glycol adipic acid polyester. *Toxic. appl. Pharmac.* **26,** 58.

Santicizer 334F® (Monsanto Co., St. Louis, Mo.; S-334F) is a 1,3-butylene glycol adipic acid polyester (mol wt 1700–2200) terminated with a 16% w/w mixture of myristic, palmitic and stearic acids. The compound is permitted in the United States as a plasticizer for PVC intended for food-contact use, the clearance having been based partly on the results of the study reported here.

S-334F was fed to rats at dietary levels of 1000, 5000 and 10,000 ppm for 2 yr. The treatment had no significant effects on the rate of body-weight gain, food consumption or mortality or on the results of haematological examinations, serum chemistry studies or urine tests. No consistent differences were observed in the absolute or relative organ weights (the latter being calculated on the basis of both body and brain weights). No gross or micropathological effects attributable to S-334F were detected.

Treatment of beagle dogs with the same dietary levels of S-334F for 2 yr similarly caused no adverse changes in any of the parameters examined.

In a three-generation reproduction study in rats, again using dietary levels of 1000, 5000 and 10,000 ppm S-334F, the rate of body-weight gain was slightly depressed in both males and females at the top dietary level, and in females given 5000 ppm. Survival was not affected, however. Both absolute and relative organ weights showed several significant differences, but in the absence of any pathological changes these were attributed to the smaller body weights of these animals. Mating indices, incidences of pregnancy and parturition and gestation times for treated animals compared favourably in all instances with the controls. The lactation indices [(number of viable pups at lactation day 21/number viable at day 5) × 100] were slightly lower for the first litters of the second generation fed

5000 ppm and of the first and second generations fed 10,000 ppm. This pattern did not persist into the second litter of the same generations or into either litter of the third generation. The numbers of offspring delivered and the numbers viable at various stages of the lactation period were not adversely affected at any dose level. The survival indices, body weights and general behaviour compared favourably with those of the progeny of control animals.

These results indicate a no-effect level of 10,000 ppm in both species. The results of extraction tests on 4 mil PVC film containing S-334F (50 phr) using water, heptane, 8% ethanol and 3% acetic acid as food simulants suggests that the maximum amount of S-334F likely to be extracted by food under anticipated conditions of use would allow a very large margin of safety for the plasticizer in view of the established no-effect level.

2721. A dubious study on phthalates

Nikonorow, M., Mazur, H. & Piekacz, H. (1973). Effect of orally administered plasticizers and polyvinyl chloride stabilizers in the rat. *Toxic. appl. Pharmac.* **26,** 253.

The authors of this paper describe four different experiments in the rat using di-(2-ethylhexyl) phthalate (DEHP), di-*n*-butyl phthalate (DBP), and two tin stabilizers, di-(*n*-octyl)-tin *S,S'*-bis(isooctyl mercaptoacetate) (DOTOM) and dibenzyltin *S,S'*-bis(isooctyl mercaptoacetate) (DBTOM). For each compound the work included a short-term toxicity study using oral intubation, a 12-month feeding study, and two reproduction studies, using female rats given the additives by oral intubation. In one of the latter studies, female rats were treated for 3 months before being mated, while in the other study treatment was confined to pregnancy.

The short-term study utilized two dose levels of each compound, 0·34 and 3·40 g DEHP, 0·12 and 1·2 g DBP, 0·02 and 0·2 g DOTOM and 0·018 and 0·18 g DBTOM/kg/day. There were fatalities at the top level of treatment of all the compounds, DOTOM and DEHP producing 100 and 75% mortality respectively. However the autopsy findings in several animals were purulent pneumonia and endometritis, suggesting the likelihood of bacterial infection, and "loss of mucosa in the stomach and some parts of the intestine", findings which could probably be attributed to autolysis.

The only other data recorded in the short-term study were body weights, limited haematology and results of serum-protein fractionation. Histopathology and organ-weight measurements were restricted to the liver, spleen and kidneys. The one significant finding reported was an increase in relative liver weight with all of the materials.

The dietary levels chosen for the 12-month study were 0·35%DEHP, 0·125% DBP, 0·02% DOTOM and 0·018% DBTOM. An increase in mortality was again seen in all groups, with the same primary causes of death. There was some body-weight reduction in the groups given DEHP and DOTOM and liver enlargement was reported with DEHP. No notable pathological changes were seen in the lungs, liver and spleen of any animals.

In rats treated for 3 months with 0·34 or 1·70 g DEHP, 0·12 or 0·60 g DBP, 0·02 or 0·04 g DOTOM or 0·018 or 0·09 g DBTOM/kg/day before mating, but strangely given no treatment during pregnancy, there were no effects on the number of young, the number of resorbed or dead young, foetal and placental weights or skeletal changes. However, when groups of female rats were treated only during pregnancy some effect was found, reduced

foetal weights being associated with either level of DEHP or DBTOM and with the higher level of DBP, but only with the lower level of DOTOM.

[This is yet another paper describing dubious findings in reproduction tests with phthalates. Careful study of the figures tabulated for the second reproduction study suggests that reductions in foetal weight were associated with the high doses of DEHP and DBP but that with the other groups, in all of which the mean foetal weights were similar, the reported effect might well have been a reflection of an abnormally high control value. On the other hand, the doses used must have caused maternal toxicity, and in fact 50% of the dams given the top level of DOTOM died. It is possible, therefore, that the lower foetal weights and increases in foetal deaths and resorptions were a direct result of maternal toxicity. In fact this study does not allow any assessment of the possible teratogenicity or foetotoxicity of these compounds and only indicates that a well-designed study is required to clarify this point once and for all.

The other tests were as limited in scope and achievement as the reproduction studies. Indeed, there seems little justification at all for the 12-month feeding study, since it was too short to investigate carcinogenicity and the observations made were no less limited than those in the shorter one. The opportunity the longer test might have offered for determining the reasons for the observed liver enlargement was not taken.

The paper as a whole is totally inadequate, none of the negative and few of the positive results being published. The commonly found situation in which only positive findings are published makes the overall assessment of a study difficult, as well as casting doubt on the limited results that are reported. Furthermore it is impossible to assess the significance of any findings in an experiment apparently conducted on badly infected animals and, in general, the dose levels used were so high that the results could make little or no contribution to any judgement on the safety of these plastics additives.]

THE CHEMICAL ENVIRONMENT

2722. Boric acid baldness

Stein, K. M., Odom, R. B., Justice, G. R. & Martin, G. C. (1973). Toxic alopecia from ingestion of boric acid. *Archs Derm.* **108,** 95.

This paper describes the case history of a 32-yr-old woman who suffered a diffuse hair loss over a period of many months. About 1 yr before admission to hospital the patient was suffering from weakness, loss of appetite, weight loss and scanty menstrual bleeding, and had noted some thinning of scalp, axillary and pubic hair. Her condition became progressively worse, with almost total hair loss, severe fatigue, anorexia and mental confusion. Her past history and extensive endocrine studies produced no information relevant to her disorder, until it was found that the patient used several bottles of mouthwash daily to relieve soreness of the tongue resulting from grand mal seizures, which had been occurring for about 4 yr. She gargled with large volumes of the mouthwash, which contained boric acid, and then swallowed it. Blood levels of boric acid were elevated to 3·2 mg/100 ml from the normal value of up to 0·3 mg/100 ml, calculated as boron. Avoidance of all borate-containing preparations led to regrowth of body and scalp hair, and blood levels of borate

returned to normal. The mechanism of this effect is not known, but boron does accumulate in the brain, liver, kidney and skin and may have collected in the hair follicles.

Hair loss has previously been reported as a result of borate intoxication in adults (*Cited in F.C.T.* 1971, **9,** 160), although the problems of boron toxicity more usually concern the mis-use on infants of products containing boric acid or borax (*ibid* 1971, **9,** 914; *ibid* 1974, **12,** 277). Obscure widespread alopecia in adults may be an important clue in the diagnosis of boron intoxication.

2723. Hypersensitivity to nickel

McConnell, L. H., Fink, J. N., Schlueter, D. P. & Schmidt, M. G., Jr. (1973). Asthma caused by nickel sensitivity. *Ann. intern. Med.* **78,** 888.

Millikan, L. E., Conway, F. & Foote, J. E. (1973). In vitro studies of contact hypersensitivity: Lymphocyte transformation in nickel sensitivity. *J. invest. Derm.* **60,** 88.

The ready permeability of the human dermis and epidermis to nickel (Ni) has led to the establishment of that metal as one of the commonest causes of contact dermatitis (*Cited in F.C.T.* 1969, **7,** 693), but dermatitis may not be the only manifestation of hypersensitivity to Ni, as appears from the first paper cited above. Within 1 wk of commencing work in a Ni-plating factory, a 24-yr-old man developed an itching vesicular rash on parts of his skin exposed to the plating solution. Protective covering improved his skin condition, but some 2 wk later he complained of a non-productive cough, tightness of the chest and wheezing. This occurred shortly after the start of the day's work and persisted for several hours after his return home. Dyspnoea increased and admission to hospital was necessary for treatment. On his return to work the patient again had dyspnoea and wheezing and had to discontinue handling the plating compounds. Experimental inhalation of Ni sulphate from a solution containing 10 mg/ml produced immediate hypoxia and bronchial obstruction, while scratch-testing with 1 mg Ni sulphate/ml provoked an immediate wheal and flare reaction, suggesting the IgE was concerned in the reaction. No passive transfer of reactivity could be demonstrated.

The second paper cited described an *in vitro* study of lymphocyte response using eight patients in whom Ni hypersensitivity had been confirmed by skin tests. The validity of the *in vitro* response was established by determination of the patients' normal phytohaemagglutinin (PHA) response. Lymphocytes from these patients, cultured for 6 days with an optimal concentration of Ni (between 40 and 120 μg/4-ml culture) showed an uptake of thymidine 5–10 times that of controls and correlating with the intensity of the patch-test results. In one patient undergoing prednisone treatment there was depression of the PHA response without apparent depression of the *in vitro* response to Ni.

2724. Pulmonary deposition of uranium oxides

Leach, L. J., Maynard, E. A., Hodge, H. C., Scott, J. K., Yuile, C. L., Sylvester, G. E. & Wilson, H. B. (1970). A five-year inhalation study with natural uranium dioxide (UO_2) dust—I. Retention and biologic effect in the monkey, dog and rat. *Hlth Phys.* **18,** 599.

Leach L. J., Yuile, C. L., Hodge, H. C., Sylvester, G. E. & Wilson, H. B. (1973). A five-year inhalation study with natural uranium dioxide (UO_2) dust—II. Postexposure retention and biologic effects in the monkey, dog and rat. *Hlth Phys.* **25,** 239.

Studies on the inhalation of insoluble uranium dusts have been carried out and have been used as a basis for the establishment of a threshold limit value (TLV) for uranium dusts in workroom atmospheres. However it has since been found that uranium dioxide (UO_2) and uranium octoxide (U_3O_8) can be retained in the lungs in relatively large quantities. Estimates of the extent of this accumulation have been made, but only on the basis of relatively few data. The investigators named above have therefore diverted their attention from the known renal effects of uranium (*Cited in F.C.T.* 1969, **7,** 695) to its storage in the body after inhalation, and to the possibility that this might constitute a radiological hazard.

Paper I cited above describes a study in which 110 pure-bred beagle dogs and 25 rhesus monkeys were exposed to 5·8 mg UO_2/m^3 (mass median particle diameter approximately 1·0 μm) for an average of 5·4 hr daily on 5 days/wk for up to 5 yr. Animals were killed at selected intervals for histopathological examination of all major organs, and for determination of uranium concentrations in the lung, liver, kidney, spleen, femur and tracheobronchial lymph nodes (TLN) by a fluorophotometric method. In addition, 200 rats were exposed to UO_2 and handled similarly in two studies each lasting for 1 yr. A few animals from all three species were retained after the exposure period for further observation, the results of which are described in Paper II.

Mortality during the exposure period was low and did not appear to be attributable to UO_2 inhalation. Weight changes were within normal limits, and blood parameters (measured bimonthly) showed no untoward changes. The uranium content of the tissues built up during the first year of exposure and then levelled off, except in the TLN in which the plateau was not reached until yr 4 in either the dog or monkey. The lungs and TLN were the principal sites of uranium accumulation, the steady-state values in the lung being about 2000 μg/g in the dog, 3600 μg/g in the monkey and 800 μg/g in the rat, and these were the only tissues showing histological changes. The commonest change was the presence of a granular black pigment, which to some extent increased with duration of exposure in dogs and monkeys but which was also found in smaller amounts in control animals. After long exposure some patchy pulmonary fibrosis was also seen in the monkeys, but it was not clear whether the changes were due to radiation or chemical activity. The absence of renal injury throughout the 5-yr period and the pattern of occurrence of the lesions found suggested that damage may have been due to radiation.

Natural UO_2 is an α-emitter of low specific activity, 25 μg uranium/g tissue giving a dose of about 0·03 rad/wk. This figure is very near the maximum permissible organ dose, which from the organ loads of uranium calculated, was exceeded in the lungs and TLN of both dogs and monkeys after the first few months of this study. Extrapolating from this, the authors suggest that there may be radiation hazards from the accumulation of uranium in the lung and TLN resulting from exposure to uranium levels equal to or less than the currently accepted TLV (250 $\mu g/m^3$).

In the second paper cited, animals observed in the post-exposure period of 6·5 yr were again killed at intervals and tissue histopathology and uranium content were investigated. The only abnormality observed in the surviving animals was a raised level of non-protein nitrogen (NPN) in the blood of older monkeys. Rats and dogs were not affected. Although the raised NPN levels were suggestive of kidney damage, none was noted.

During the post-exposure period, the lung content of uranium gradually fell in all species, but the changes in the TLN were more complex. These nodes appeared to continue to concentrate uranium for up to 1 yr after exposure in the dog and for 1 month in the rat, before the levels began to fall. Fibrosis observed in the lungs and TLN was consistent with radiological damage and confirmed the suggestion in the earlier paper that radiation damage was present. There was a very slow release of uranium from bone, so that although this tissue initially contained low levels of uranium, it acquired, as the post-exposure period continued, an increasing proportion relative to other tissues in which uranium depletion was more rapid.

A sobering finding was that of pulmonary neoplasia in four of the 13 dogs surviving for 75 months after the end of a 5-yr exposure, with six showing foci of atypical epithelial proliferation. Spontaneous pulmonary tumours are rare in dogs. Serious injury of this nature has not previously been linked with uranium exposure, although indications of pulmonary neoplasia have been observed in dogs after inhalation of other radiation sources ($^{238}PuO_2$ and $^{239}PuO_2$). The authors emphasize the importance of a long observation period as a factor in the appearance of these lesions.

Both of these papers suggest that there may be cause for concern over the exposure of workmen to the current TLV for UO_2. However, as noted in the first paper, the persuasive if fragmentary evidence provided by the absence of clinical and medical signs of injury in exposed workers is reassuring. A comprehensive programme for collecting industrial data would assist the clarification of the question of the TLV.

[It is also reassuring that a recent paper by Donoghue *et al.* (*Br. J. ind. Med.* 1972, **29,** 81) reports that the uranium content in the lungs and pulmonary lymph nodes of a workman, who was exposed to U_3O_8 and who died of natural causes at work, was significantly lower than the expected amounts estimated from environmental data using currently accepted parameters. Whether this can be attributed to a lower rate of deposition than anticipated or to a more rapid clearance is not altogether certain, but the former is considered the more likely. The importance of the size and nature of the particles in relation to their deposition cannot be overlooked, and this may be another useful avenue of further research.]

2725. Subcellular reactions of acrolein

Munsch, Nicole, de Recondo, Anne-Marie & Frayssinet, C. (1973). Effects of acrolein on DNA synthesis *in vitro*. *FEBS Lett.* **30,** 286.

Zollner, H. (1973). Inhibition of some mitochondrial functions by acrolein and methylvinylketone. *Biochem. Pharmac.* **22,** 1171.

The increasing industrial use of acrolein and its demonstrated presence in the vapour phase of cigarette smoke, together with recent indications of its formation during the oxidative degradation of the antitumour agent cyclophosphamide and during the metabolism of allyl compounds (*Cited in F.C.T.* 1973, **11,** 1139) have prompted several studies on the biological properties of this compound. In an early issue (*ibid* 1965, **3,** 850), we referred to an enhancement of liver alkaline-phosphatase activity after inhalation or ip injection of rats with acrolein, a finding ascribed to a non-specific stimulation of the pituitary-adrenal system. Workers at two European Research Institutes have now examined other

effects of acrolein at the molecular level, namely, its effects on DNA synthesis *in vitro* and on mitochondrial processes involved in energy transfer.

The inhibitory effect of acrolein on RNA synthesis, at least *in vitro*, is known to stem from a reaction with RNA polymerase and not with DNA template, and so a similar interference with DNA polymerase, possibly by reaction with the enzymatic thiol groups, might be expected to inhibit DNA synthesis. To investigate this possibility, Munsch *et al.* (cited above) incubated acrolein with nucleoside triphosphate substrates, DNA template and one of two DNA polymerases, prepared respectively from regenerating rat liver and *Escherichia coli*. Of these two enzymes, only that from rat liver contains a functional thiol group. When rat-liver DNA polymerase was used, polymerization of the four deoxyribonucleoside triphosphates, using calf-thymus DNA as template, was markedly reduced by the presence of acrolein, and was inversely proportional to the acrolein concentration. Enzyme inhibition occurred at acrolein concentrations between 8×10^{-5} and 8×10^{-4} M. It was concluded that an acrolein-enzyme interaction was responsible for the impaired replication of DNA *in vitro*. Since the *E. coli* enzyme was not affected, the inhibitory action of acrolein was ascribed to a reaction with thiol groups essential for the enzyme action. At very low acrolein concentrations, rat-liver polymerase was actually activated, a response thought to be due either to the oxidizing properties of the acrolein double bond or to the aldehyde moiety.

The authors speculate on the possibility that if acrolein plays a part in the carcinogenic process induced by tobacco, this role may be mediated through the action on mammalian nucleic acid polymerases.

The effect of acrolein and the related compound methylvinylketone (MVK) on mitochondrial function has been examined by Zollner (cited above), who incubated the compounds with rat-liver mitochondria, cofactors, and either glutamate, succinate or β-hydroxybutyrate as substrate. Respiration was inhibited by acrolein and by MVK with each of the three substrates, although sensitivity to acrolein varied according to the conditions. In particular, respiration with glutamate showed greater sensitivity to acrolein than that with succinate, and adenosine diphosphate-stimulated respiration was more sensitive than 2,4-dinitrophenol-stimulated respiration with respect to either substrate. Acrolein had least effect on the respiration of aged mitochondria with NADH.

The author postulates a three-fold action for acrolein, namely on glutamate transport, succinic dehydrogenase and transport of inorganic phosphate, the latter effect being competitive with respect to phosphate. The inhibitory action of MVK was similar to that of acrolein but was less potent because of substitution of the hydrogen of the aldehyde group.

2726. Background of acrylamide neuropathy

Hashimoto, K. & Ando, K. (1973). Alteration of amino acid incorporation into proteins of the nervous system *in vitro* after administration of acrylamide to rats. *Biochem. Pharmac.* **22,** 1057.

Rats pretreated with the liver microsomal-enzyme inducers DDT or phenobarbitone have been shown to be more resistant to acrylamide-induced neuropathy than controls, but whereas early functional neurological impairment was not associated with detectable peripheral neuropathy in rats treated only with acrylamide, severe nerve lesions appeared

in those pretreated with phenobarbitone (*Cited in F.C.T.* 1974, **12,** 281). Further clarification of this aspect of acrylamide toxicity is therefore welcome. The present study concerns the effect of acrylamide on the metabolism of nervous tissue.

Rats were fed 500 ppm acrylamide in the diet for 4 wk, and then given a normal diet without the amide for a further 4 wk. In treated rats there was greater incorporation of [^{14}C]lysine into spinal-cord protein than in controls. The increase continued until wk 6–8 and then tended to fall towards pretreatment levels. Lysine incorporation was more markedly increased in the lower part of the cord. In the sciatic-nerve protein there was a slight decrease in lysine incorporation at 2–3 wk, followed by a large increase after 4 wk and maximal incorporation at 6 wk. Acrylamide also significantly increased [^{35}S]methionine incorporation into spinal-cord and sciatic-nerve protein, but there was no early depression of methionine incorporation into the sciatic nerve as there was of lysine incorporation. Since incorporation of both amino acids was inhibited by potassium cyanide and 2,4-dinitrophenol, the process appears to be dependent on aerobic metabolic processes. Acrylamide failed to modify amino-acid incorporation into brain or liver tissue, its effect apparently being specifically directed to the spinal cord and peripheral nerves.

2727. Rapid excretion of fully fluorinated hydrocarbons

Modell, J. G., Tham, M. K., Modell, J. H., Calderwood, H. W. & Ruiz, B. C. (1973). Distribution and retention of fluorocarbon in mice and dogs after injection or liquid ventilation. *Toxic. appl. Pharmac.* **26,** 86.

Clark, L. C., Jr., Becattini, F., Kaplan, S., Obrock, Virginia, Cohen, D. & Becker, C. (1973). Perfluorocarbons having a short dwell time in the liver. *Science, N.Y.* **181,** 680.

Much has been said about fluorocarbon propellants and their inherent hazards when misused (see p.552 of this issue), but fluorocarbons can also appear in other guises. The group also includes highly fluorinated liquids, which can be used as oxygen-exchange and transport media and thus as potential blood substitutes (*Lancet* 1974, **I,** 126). Hence details of their toxicology, uptake, distribution and possible storage in the body are required.

The first authors cited above administered one of two fluorocarbons, Caroxin-D and FX-80, to mice in an ip or sc dose of 0·05 ml and observed the animals for 17–19 months before killing them to measure tissue levels of fluorocarbon. Growth rates were comparable in saline controls and the test animals. Most of the fluorocarbon absorbed was retained in the mice, and accidental overheating of the animals due to failure of the air conditioning led to the sc formation of bubbles of FX-80 vapour in a few. Analysis of the tissues after 17–19 months, taking care to avoid contamination, showed the presence of fluorocarbon in the brain, lung, liver and other organs, generally at levels of 0·1–0·2 mg/100 g tissue. The highest levels (0·5–0·6 mg/100 g) were found in the body fat.

The same authors also ventilated the lungs of dogs with Caroxin-D for 1 hr, prior to an observation period of 20–23 months. Fluorocarbon was again still present (about 0·1 mg/100 g in most tissues), and apart from the site of administration, the highest concentration was in the fat (1·9 mg/100 g). Although these substances appear to be inert (their persistent presence in the tissues being without any obviously toxic effect in this study), the fact that a small quantity can be absorbed, distributed and retained makes further study of their biochemical effects necessary before their general use can be considered.

The second paper cited above recognizes that many perfluorocarbons (in this context, compounds in which all hydrogen atoms are replaced by fluorine) may be deposited in the liver and spleen, where they are stored for life, but reports the discovery of closely related compounds which become deposited in the liver of the mouse immediately after iv injection but leave it again within a few days, to be excreted via the lungs and skin. Perfluorodecalin and perfluoromethyldecalin, given to mice iv in a dose of 2 ml/kg at a rate of 2 ml emulsion/min, had each left the liver within a few days, while other perfluorocarbons similarly administered were still present after a long period of time—certainly for months and probably for the lifetime of the animal. Perfluorinated cyclohexanes were also shown to leave the liver rapidly.

The authors suggest that some types of perfluorocarbon, notably those containing a C–O–C or C–N–C linkage, bind with parts of the liver tissue, while the fully fluorinated decalins are excreted comparatively rapidly either because they are not bound or because they are actively excreted. Such fluorocarbons, containing only carbon and fluorine, may thus represent a group which can possibly be used safely in intact animals.

2728. Bacterial build-up and breakdown of methylmercury

Vonk, J. W. & Sijpesteijn, A. K. (1973). Studies on the methylation of mercuric chloride by pure cultures of bacteria and fungi. *Antonie van Leeuwenhoek* **39,** 505.

The discovery of relatively large amounts of methylmercury in fish and shellfish in waters contaminated with mercury from industrial effluent has stimulated research into the agents responsible for the methylation of inorganic mercury in the environment. An earlier report (*Cited in F.C.T.* 1971, **9,** 140) on the methylation of mercuric chloride by lake and aquarium sediments suggested that micro-organisms were involved. Since then, there have been reports of methylmercury formation by the fungus *Neurospora crassa* (Landner, *Nature, Lond.* 1973, **230,** 452) and by the bacterium *Clostridium cochlearium* (Yamada & Tonomura, *J. Ferment. Technol., Osaka* 1972, **50,** 159).

Vonk & Sijpesteijn (cited above) have investigated the ability of a variety of micro-organisms, most of them common in water or soil, to methylate mercuric chloride. Aerobic cultures of all the micro-organisms, namely *Pseudomonas fluorescens, Mycobacterium phlei, Escherichia coli, Aerobacter aerogenes, Bacillus megaterium* and three fungi, *Aspergillus niger, Scopulariopsis brevicaulis* and *Saccharomyces cerevisiae,* were found to be capable of forming methylmercury from mercuric chloride at rates of some 300–1000 ng/litre of culture/7 days. These rates were comparable to those observed in the work on lake sediments (*Cited in F.C.T.* 1971, **9,** 140). It was noted that in *A. niger* most of the methylmercury formed was present in the mycelium, whereas in *Ps. fluorescens* most was found in the culture medium.

Under anaerobic conditions, methylation of mercury by *E. coli* and *A. aerogenes* was lower than that in aerobic cultures, and the authors point out that under natural anaerobic conditions formation of methylmercury may be prevented by precipitation of mercury as mercuric sulphide which, according to Yamada & Tonomura (*loc. cit.*), is not methylated to any appreciable extent.

[Clearly the conditions and factors affecting methylmercury synthesis by micro-organisms need to be investigated more thoroughly before the contribution of bacteria and fungi to methylmercury levels in the environment can be assessed. In this context it is

worth noting the work of Spangler *et al.* (*Science, N.Y.* 1973, **180,** 192), who have shown that if the incubation period is long enough (more than 50 days) the methylmercury formed in the presence of lake sediments is broken down again to inorganic mercury.]

2729. Toxicity of oxalate precursors

Richardson, K. E. (1973). The effect of partial hepatectomy on the toxicity of ethylene glycol, glycolic acid, glyoxylic acid and glycine. *Toxic. appl. Pharmac.* **24,** 530.

Several workers have speculated on the importance of biodegradation in determining the toxic response to ethylene glycol (EG), glycollate and glyoxylate in animals (McChesney *et al. Fd Cosmet Toxicol.* 1971, **9,** 21; Bachmann & Golberg, *ibid* 1971, **9,** 39; McChesney *et al. ibid* 1972, **10,** 655) with particular reference to the metabolic formation of oxalate and/or glycollate. To investigate this question further, the authors cited above have fed these oxalate precursors to rats previously subjected to partial hepatectomy, since the enzymes responsible for oxalate formation are all present in the liver.

After removal of either one-third or two-thirds of the liver, groups of male rats were orally intubated with glycine, sodium glycollate, EG or sodium glyoxylate, and urine was collected from each rat for 48 hr. Partial hepatectomy increased the toxicity of glyoxylate, but decreased the toxicity of EG and glycollate. Concomitantly, the urinary oxalate content was significantly affected only in rats fed glyoxylate, an increase being recorded in these animals. Liver regeneration was not altered by any of the compounds tested. In the isolated perfused liver, [U–^{14}C]oxalate biosynthesis from the [U–^{14}C]-labelled substrates decreased in the order glyoxylate, glycollate, EG and glycine.

The author inferred from the results that EG and glycollate toxicity are largely a function of metabolite production, notably of glyoxylate and oxalate, and that the liver is the major source of endogenous oxalate synthesis, at least in the rat.

2730. A black list of contact allergens

Baer, R. L., Ramsey, D. L. & Biondi, Ella (1973). The most common contact allergens 1968–1970. *Archs Derm.* **108,** 74.

This report lists the 24 substances which were responsible for contact allergy and elicited a positive patch-test result in more than 3% of patients seen at the New York University Skin and Cancer Unit during the period 1968–1970. Each allergen was tested on groups of at least 200 patients.

The highest percentage of positive reactions (22·2%) came from mercuric chloride, although many of the reactions to this compound were of low intensity. Mercaptobenzthiazole, thiram, diphenylguanidine and monobenzone (*p*-(benzyloxy)phenol), all additives commonly used in rubber technology, have attained increasing importance in recent years as causes of contact dermatitis. Sensitivity to *p*-phenylenediamine has proved to be twice as common in the USA as in Western Europe, possibly reflecting a higher exposure of the US population to cross-sensitizers of this structure. Ethylenediamine is a relatively new arrival among the important contact allergens and has probably acquired clinical importance because of its structural relationship to many other commonly encountered compounds, such as antihistamines.

Nickel sensitivity has remained remarkably constant over the past 35 yr. An apparent reduction in the incidence of sensitivity to formaldehyde (22·1% in 1961 to 8·7% in the present study) may be due to the fact that test concentrations used clinically have been reduced during that period to 2%. The 5% concentration used earlier probably led to the false inclusion of cases of primary irritancy. The weak reactions to turpentine, formaldehyde, diphenylguanidine and Peruvian balsam often encountered probably indicate a level of sensitivity to these substances below that which normally induces clinical symptoms. The other substances featuring in the list are potassium dichromate, Bismarck brown, resorcinol, epoxy resins and hardeners, ethyl aminobenzoate, neomycin, chrysoidine brown, acrylic monomer, 2-naphthyl benzoate, hexachlorophene, pyrethrum and poison ivy oleoresin.

NATURAL PRODUCTS

2731. The vicious circle of drinker's anaemia

Lockner, D. & Ericson, U. (1973). Haematological effects of chronic ethanol administration and folic acid deficiency in mice. *Acta haemat.* **49,** 242.

Ethanol may suppress the production of blood cells by a direct action on folate metabolism (*Cited in F.C.T.* 1970, **8,** 438), and chronic alcoholics who suffer liver damage are commonly anaemic. Evidence is now adduced that ethanol acts in concert with folic acid deficiency to cause anaemia.

Groups of mice were given 10% (v/v) aqueous ethanol, or drinking-water containing a small amount of methotrexate (to induce folate deficiency), or 10% ethanol plus a little methotrexate, for periods of 5–52 wk. A control group was given tap-water. No haematological defects appeared in the groups given either agent alone, but in the mice given both agents, moderate anaemia appeared within 5 wk and severe anaemia was demonstrable after 18 wk. The anaemia was of the hyperchromic-macrocytic type, and there was also a significant decrease in the white-cell count. This group showed a reduction in body weight and in liver and kidney weights and an increase in spleen weight.

This study also provided some support for the direct involvement of ethanol with folic acid metabolism, since blood levels of folic acid were somewhat lower in animals treated only with ethanol than in the controls, although in this study the difference was not significant.

2732. Structure and hepatotoxicity of substituted furans

Seawright, A. A. & Mattocks, A. R. (1973). The toxicity of two synthetic 3-substituted furan carbamates. *Experientia* **29,** 1197.

The furanosesquiterpene essential oils are found in the foliage of various species of Myoporaceae and are quite commonly responsible for the poisoning of livestock in Australia and New Zealand. The best known essential oil in this group is ngaione. In sheep, it causes hepatocellular necrosis, which may be either centrilobular or periportal. In mice, a similar

hepatic lesion is produced in the mid-zonal region. The location of the lesion can be varied by appropriate alteration of the levels of microsomal mixed-function oxidases. Stimulation of these oxidases by phenobarbitone shifts the lesion to a periportal location while their depression by pretreatment with SKF 525A transfers the lesion to the centrilobular area.

These facts suggest that metabolic conversion to an active intermediate probably accounts for the toxic properties of ngaione, but they give no clue to the part of the molecule involved. There are indications, however, from studies of a group of related furan derivatives that produce the same type of liver lesion in mice, that the furan ring with a substituent side chain at position 3 may be important. Preliminary studies showed that simple analogues such as 3-hydroxymethylfuran, 3-carboxyfuran and the methyl ester of the latter produced centrilobular necrosis in mice when given ip at the LD_{50} level (150–380 mg/kg). The site of the necrosis could not be shifted by altering the level of mixed-function oxidase activity. The *N*-ethylcarbamate derivative of 3-hydroxymethylfuran (with an LD_{50} of only 32 mg/kg in mice) produced severe extrahepatic lesions, notably pulmonary oedema and renal tubular necrosis, while hepatic involvement was slight or absent.

After the activity of mixed-function oxidases had been increased by phenobarbitone pretreatment, mice could tolerate as much as 50–60 mg/kg. Centrilobular liver necrosis was consistently produced at these dose levels, but the extrahepatic lesions were less severe. Depression of the oxidase activity by carbon disulphide pretreatment did not affect liver injury, but lung and renal lesions occurred at higher doses.

The *N,N*-diethylcarbamate of 3-hydroxymethylfuran produced extensive centrilobular necrosis in a dose of 60 mg/kg (the LD_{50}), while higher doses (100 mg/kg) produced more rapid death associated with pulmonary oedema. Stimulation of mixed-function oxidases changed the location of the liver lesion to the periportal zone, but did not reduce the level of toxicity. Depression of these enzymes by carbon disulphide markedly reduced the liver lesion.

These findings suggest that the furan ring substituted in position 3 is basically responsible for the hepatotoxic effect exhibited by the series of substituted furans, although the changes produced depend on the side-chain structure. It seems that hepatic microsomal metabolism has a role to play in the mechanism of the hepatotoxicity and possibly also in the development of the extrahepatic lesions produced by these compounds.

2733. Skin reactions among the lilies

Bleumink, E. & Nater, J. P. (1973). Contact dermatitis to garlic; cross-reactivity between garlic, onion and tulip. *Arch. dermatol. Forsch.* **247,** 117.

Some time ago we described the case of a man who showed hypersensitivity to garlic and onion, with weak reactivity to tulip, the phenomenon being attributed to a heat- and acid-labile hydrophilic allergen (*Cited in F.C.T.* 1973, **11,** 520). A more extensive study has now been reported involving the patch testing of 63 patients with ulcera cruris and 168 with contact dermatitis. Of the first group, 11% gave positive reactions to aqueous and/or ethanolic extracts of garlic and to between one and five of the common test allergens in a standard series. Of the second group, 4·2% gave a positive reaction to garlic extracts. In neither group was any patient who was sensitive to garlic also sensitive to onion extracts, although both types of extract were prepared by a similar method. One of eight

patients reacting to garlic gave a positive reaction to tulip extract, while one who had previously shown contact dermatitis attributed to tulip failed to react when tested with garlic extracts.

Gas chromatography showed that tuliposide-A (the glucose ester of α-methylene-γ-butyrolactone), the allergenic component of tulip bulbs, was absent from extracts of garlic and onion, so that the possibility of cross-reactivity due to this factor was excluded. Garlic extracts contained tuliposide-B (the glucose ester of α-methylene-β-hydroxy-γ-butyrolactone), a compound which readily reacts with thiol groups (e.g. in cysteine), but since incubation of garlic with cysteine did not reduce skin reactivity, this compound was unlikely to be involved in epicutaneous reactions to garlic.

The active compound in garlic allergy was concluded to be a low-molecular-weight, hydrophilic, heat- and acid-labile substance, which could be expected to be inactivated during normal cooking processes. Many persons who reacted to garlic reacted positively to some or all of the common allergens, coal tar, wood tar, Peruvian balsam, wool alcohols and colophony, and the presence of some cross-reacting substance common to these materials cannot be ruled out.

2734. Gossypol locks up the iron

Herman, D. L. & Smith, F. H. (1973). Effect of bound gossypol on the absorption of iron by rats. *J. Nutr.* **103,** 882.

It has been reported that diets containing 10% cottonseed protein containing bound gossypol at levels up to 0·68% had little effect on the growth of weanling rats, but higher concentrations of bound gossypol impaired weight gain, probably by reducing the availability of essential amino acids (*Cited in F.C.T.* 1973, **11,** 695). The toxic effects of gossypol have been reduced or eliminated by the addition of divalent iron to the diet of non-ruminant animals, and the finding that iron would form a complex with gossypol *in vitro* prompted an investigation into the importance of this reaction in the gastro-intestinal tract.

In rats fed 0·005 (control), 0·12, 0·24 or 0·35% bound gossypol in the diet, the absorption of divalent iron, added to the feed at levels of 400 and 1600 ppm, decreased linearly with increasing gossypol concentration. When dietary iron (as ferrous sulphate) was increased, the absolute quantity of iron absorbed increased, although there was a reduction in absorption expressed as a proportion of the total ingested. The levels of free and bound gossypol in the animals' livers rose as the feed content was increased, but diminished with any increase in iron intake. It is suggested that if a gossypol-iron complex is formed in the liver rather than in the gut, biliary excretion of this complex and its consequent loss in the faeces could explain the apparent reduction in iron absorption in gossypol-fed rats. This study indicates, however, that bound gossypol in the diet complexes with iron in the intestinal tract with a consequent reduction in the absorption of both components.

2735. Tannic acid and vitamin B_{12} absorption

Carrera, G., Mitjavila, S. et Derache, R. (1973). Influence de l'acide tannique sur la disponibilité digestive de la vitamine B_{12} chez le rat. *Ann. Nutr. Alim.* **27,** 73.

Previous studies by these authors (*Cited in F.C.T.* 1973, **11,** 1156) have shown that tannic acid (TA) depresses the absorption of dietary nitrogenous material, such as methionine, and of vitamin B_{12}. This paper describes a study of the bioavailability of vitamin B_{12} after its interaction with glycoprotein-TA complexes in the gastro-intestinal tract.

The excretion of an intragastric dose of 0·5 μg of radiolabelled B_{12} (specific activity, 0·25 μCi) was investigated in groups of four Sprague–Dawley rats dosed 2 hr previously by gavage with either normal saline or 250 or 500 mg TA/kg body weight. Faeces were collected every 24 hr for 3 days and on day 3 the animals were killed and the gut contents of each was added to the faeces collection for that day. The results of this work are expressed in terms of the 'digestive utilization coefficient' of vitamin B_{12}, which was utilized more effectively by the saline-treated controls than by the TA-treated groups, although no significant difference was found between the two TA levels of treatment.

Verzar's *in situ* technique was used to examine the effect of TA–glycoprotein complexes on vitamin B_{12} absorption, 30 anaesthetized rats being catheterized near the pylorus and the ileocaecal junction, with ligation of this section of the gut. Either 6 ml Ringer–Locke solution or 2 ml TA (125 mg/ml) plus 4 ml gastric juice, or the latter mixture centrifuged to remove the TA–glycoprotein complex was introduced into the ligated part of the gut. Labelled vitamin B_{12} was added to all three solutions, before their introduction. After perfusion for 1·5 hr the rats were killed for removal of the ligated gut, the contents and washings were centrifuged and the complexed B_{12} was measured by scintillation counter. The highest level of B_{12} complex was found in the group given TA and gastric juice, without centrifugation. Initial removal of the TA–glycoprotein complex by centrifugation of the other TA mixture reduced the formation of a B_{12} complex by half and accordingly increased B_{12} availability.

It is also possible for TA to complex with vitamin B_{12} directly under certain conditions of pH and concentration (Carrera *et al. Trav. Soc. Pharm. Montpellier*, in press). TA–B_{12} dissociates above pH 5, the initial pH of the gut section used, and as the pH rises to 7 (the pH of the distal section) a complex containing increasing quantities of mucin is produced to give a stable TA–B_{12}–glycoprotein complex, which reduces the bioavailability of vitamin B_{12}.

2736. New mycotoxins characterized

Eppley, R. M. & Bailey, W. J. (1973). 12,13-Epoxy-Δ^9-trichothecenes as the probable mycotoxins responsible for stachybotryotoxicosis. *Science, N.Y.* **181,** 758.

Although first associated with horses, stachybotryotoxicosis has since been shown to affect a wide variety of domestic and farm animals. This mycotoxicosis results from the ingestion of feed contaminated with certain strains of *Stachybotrys atra* (also known as *S. alternans*) and occurs in two fairly distinct forms. The acute or 'shock' form is characterized principally by nervous disorders, which may terminate in death, while the other 'typical' form develops through three stages, of which the main signs are stomatitis and necrosis of oral fissures, thrombocytopaenia and an increase in clotting time, and finally an increase in thrombocytopaenia and leucopaenia, total failure of the clotting mechanism, an increase in body temperature and development of further areas of necrosis, frequently accompanied by bacterial infection. In these cases, death usually ensues within 1–6 days.

Cultures of *S. atra*, grown for 4 wk at 20–22°C on oats, produced five compounds which were toxic to the larvae of the brine shrimp (*Artemia salina*). The compounds were designated satratoxins C, D, F, G and H, according to their distribution on thin-layer chromatograms. From 2 kg oats were isolated 2 mg satratoxin C, 3 mg D, 2 mg F, 8 mg G and 12 mg H. Satratoxin D was found to be identical with the known fungal metabolite roridin E, a macrocyclic sesquiterpene. Satratoxins G and H, with molecular weights of 544 ($C_{28}H_{32}O_{11}$) and 528 ($C_{29}H_{36}O_9$), respectively, both yielded verrucarol on alkaline hydrolysis in methanol. In this respect, they resembled the roridins and verrucarins. The satratoxins thus appear to be members of the 12,13-epoxy-Δ^9-trichothecene group, closely related to the toxins produced by various species of Myrothecium.

2737. Another fungal toxin

Hutchison, R. D., Steyn, P. S. & van Rensburg, S. J. (1973). Viridicatum-toxin, a new mycotoxin from *Penicillium viridicatum* Westling. *Toxic. appl. Pharmac.* **24,** 507.

One of the relatively recent additions to the seemingly endless list of toxigenic fungi is *Penicillium viridicatum*, which is known to cause tubular degeneration in the pig kidney and liver lesions in rats and guinea-pigs (*Cited in F.C.T.* 1971, **9,** 759). It has also been shown to have some carcinogenic effect (Zwicker *et al. Fd Cosmet. Toxicol.* 1973, **11,** 989). A substance which was apparently the sole acutely toxic component of maize cultures of this fungus has now been characterized.

Cultures of a toxigenic *P. viridicatum* strain were subjected to several chloroform and methanol extractions and the final methanol-soluble product was fractionated by column chromatography on formamide-impregnated cellulose powder. The toxin so obtained was reported to be a yellow pigment with a molecular formula $C_{30}H_{31}NO_{10}$. The acute oral LD_{50} in male rats was 122·4 mg/kg. Deaths were generally sudden, with no preceding clinical signs, and the time of occurrence was dose related, the first deaths occurring within 24 hr in the group given the highest dose (500 mg/kg) and the last on day 7 among rats given the lowest dose (50 mg/kg). Post-mortem examination revealed hyaline-droplet degeneration of the myocardium in all treated animals, renal tubular necrosis in those that died and a frequent incidence of hepatocyte changes and splenic atrophy.

COSMETICS, TOILETRIES AND HOUSEHOLD PRODUCTS

2738. Investigations into the fate of chlorhexidine

Winrow, M. J. (1973). Metabolic studies with radiolabelled chlorhexidine in animals and man. *J. periodont. Res.* **8,** (Suppl. 12), 45.

Although chlorhexidine has been used for some 20 yr as a topically applied bactericide, interest in its metabolism, absorption and excretion has come to the fore only recently, prompted no doubt by its increasing use in oral hygiene, by its potential as a substitute for hexachlorophene and, on the practical side, by the increasing availability of radiolabelled compounds. We reported last month (*Cited in F.C.T.* 1974, **12,** 433) on an autora-

diographic study of the tissue distribution of [^{14}C]chlorhexidine given orally to mice, and the paper cited above reports further on the fate of the compound in several species.

Chlorhexidine labelled with ^{14}C in either the aromatic ring or central hexamethylene chain was administered orally, as the digluconate salt, in varying amounts to the rat, mouse, dog, marmoset and rhesus monkey. Urinary excretion of chlorhexidine was extremely low in all species, over 90% of the administered dose being recovered, mainly from the faeces, within 72 hr. Except in the mouse (in which the figure was 3–7%), less than 1% of the administered dose was recovered in the urine over a period of about 7 days. Less than 0·25% of the administered dose was found in the liver and kidney of the rodents, while the brain, lungs, heart, spleen, fat and muscle were devoid of radioactivity (the limit of detection being 0·01 μg chlorhexidine/g). Similar results were obtained in the other animal species examined.

In the single human volunteer studied, only 82% of the dose of 0·07 mg/kg, administered as a solution of the digluconate in a gelatin capsule, was recovered in the 6 days of the experiment. It was thought, however, that quantitative recovery would have been obtained if sampling had continued longer. No radioactivity was found in the blood or saliva.

Urine samples, including those from the volunteer, were examined by thin-layer chromatography (TLC), particularly for *p*-chloroaniline, an established chemical breakdown product of chlorhexidine known to be rapidly excreted by the kidney after oral administration. No *p*-chloroaniline was detected in any of the samples examined. Ring- and chain-labelled chlorhexidine produced essentially the same radioactivity distribution on the TLC plates, showing that metabolic cleavage was minimal. Only unchanged chlorhexidine was detected in the faecal extracts.

The contention that the high faecal content of chlorhexidine was due to extremely low absorption rather than to absorption followed by biliary excretion was supported by the finding that in rats with implanted biliary cannulae, 50–70% of an iv dose was excreted in the bile within 48 hr, while only 0·2% was recovered in that time after oral dosing. TLC examination of the bile of iv-dosed rats revealed only polar metabolites and a small amount of chlorhexidine.

A mouth-rinse study, using 10 ml of a 0·2% [^{14}C]chlorhexidine solution, was conducted on a volunteer to investigate uptake and retention in the human mouth. After rinsing with the solution for 1 min, the subject removed about 67% of the radioactivity by ejecting the solution and subsequently rinsing his mouth with water. Levels of ^{14}C in plaque and saliva were then monitored for 24 hr. The amount in saliva fell steadily, while plaque levels remained more constant.

METHODS FOR ASSESSING TOXICITY

2739. Serum urocanase in toxicity testing

Trip, J. A. J., van Dam, J., Eibergen, R. & Que, G. S. (1973). Investigations on correlations between serum enzymes and histological findings in liver disease. With special reference to transaminases and urocanase. *Acta med. scand.* **193,** 113.

The value of serum enzyme studies in toxicity testing has been mentioned in these pages recently (*Cited in F.C.T.* 1973, **11,** 930). It was reported that although microscopic methods

are more sensitive in determining toxic effects, it is possible to quantitate the release of certain enzyme proteins into the blood, and thus provide an early indication of minimal organ damage of a specific type to augment, rather than supplant, histopathological studies at autopsy.

In a search for a specific enzyme suitable for detecting liver-cell damage, Trip *et al.* (cited above) examined the correlation between urocanase levels in the serum and histological findings in needle-biopsy specimens from 186 patients with liver disease or some other disease affecting liver function. A variety of such diseases was encountered, ranging from cirrhosis, hepatitis and cholestasis to liver conditions of unknown aetiology. Because urocanase is an enzyme specific to the liver, it might be anticipated that its appearance in the serum would be a reflection of liver-cell necrosis.

Good agreement with histological findings was indeed found for serum urocanase levels in about 75% of patients, but similar correlations were also established for serum glutamic–oxalacetic and glutamic–pyruvic transaminases, which are not liver-specific enzymes. It was felt that an enhanced degree of correlation might have resulted from a more representative mode of biopsy, but this was not considered likely to be the major explanation since in most cases where a lack of correlation was found it concerned only one of the enzymes. Since the results with no single enzyme were clearly superior to those with the other two, even with respect to individual diseases, and since urocanase determination is a fairly time-consuming procedure, it was not judged to be an appropriate choice for the routine laboratory screening of liver damage.

2740. Improving on Draize

Marzulli, F. N. & Ruggles, D. I. (1973). Rabbit eye irritation test: Collaborative study. *J. Ass. off. analyt. Chem.* **56,** 905.

Many studies have aimed at establishing the reliability and reproducibility of tests for irritancy using the rabbit eye. Scores obtained in different laboratories have shown considerable variation, however, consistently high or low figures being associated with certain laboratories (Weil & Scala, *Toxic. appl. Pharmac.* 1971, **19,** 276).

Ten laboratories collaborated in evaluating the Draize rabbit-eye irritation test, using the same range of sample materials and 70% isopropanol as the common standard. The materials were 12% aqueous potassium oleate, 100% polyoxyethylene nonylphenol, 1% zinc pyrithion in dimethylsulphoxide, 5% aqueous polyoxyethylene(20) oleyl ether, 8·3% aqueous silicic acid, and undiluted 1,1,1-trichloroethane. Of these borderline substances, five were agreed irritants and two were non-irritants by the criteria and techniques adopted. Observation periods of 24, 48 and 72 hr and 7 days were adopted and criteria of corneal changes, iris changes, conjunctival redness and chemosis were applied by means of a simple pass/fail procedure.

Adherence to this procedure yielded reliable and reproducible results, with different laboratories distinguishing between irritants and non-irritants with a high degree of consistency. Only where a single parameter was used, rather than all four, was the consistency of the results inadequate. Additional studies to identify and eliminate the remaining sources of variability will be necessary, however, before a reproducible assessment of the degree of irritancy of a substance can be obtained. Fluorescein staining appeared to be

a potentially useful adjunct in the estimation of corneal damage, and it is suggested that its application be further explored.

PATHOLOGY

2741. Inert particles in the alveoli

Heppleston, A. G. & Young, A. E. (1973). Uptake of inert particulate matter by alveolar cells: An ultrastructural study. *J. Path.* **111**, 159.

Inhaled mineral particles less than 5 μm in size penetrate into the alveoli, from where they may be transported back into the bronchi as free particles and subsequently discharged with mucus by means of ciliary action. Alternatively they may be ingested by alveolar macrophages, which then emigrate into the lumen. The extent to which phagocytosis by the alveolar epithelial cells may also play a part in disposing of particulate matter has provoked much controversy. The study cited above looked into this question and particularly into the specific functions of the type I and type II (granular pneumocyte) epithelial cells.

The particles used in this study were relatively small, a suspension of India ink containing carbon particles below 50 nm in size being diluted with saline and instilled into the trachea of mice. The treatment was repeated on five successive days and animals were killed at intermals of 3–4 days after the final treatment, up to day 24. Most of the carbon particles were found within macrophages lying in the alveolar spaces. A few were readily identified in a minority of type I epithelial cells, but none were detected in the type II cells. The latter cells were concluded to be not phagocytic but secretory, providing the pulmonary surfactant.

The type I epithelial cells may transfer particles of the size used in this experiment from the alveolus to the interstitial space between the epithelium and the blood vessels. Larger particles, such as are commonly inhaled under conditions of industrial exposure, may not be transportable in phagosomes through the type I epithelium but may reach an interstitial location as a result of the growth of epithelium over the macrophages and deposition of reticulin. If this process is repeated in a number of adjacent alveoli, an area of consolidation will result and this may gradually develop into dust lesions of the type frequently found in coal workers.

2742. Morphological background to DMNA-induced ascites

Bhathal, P. S. & Hurley, J. V. (1973). An electron microscope study of the production of ascites in acute dimethylnitrosamine (DMN)-induced liver injury. *J. Path.* **111**, 103.

A single high dose of dimethylnitrosamine (DMNA) administered ip to rats produces hepatocellular necrosis within 1 day and a rise in intrahepatic portal pressure and ascites within 3–5 days. A similar condition may occur in several disease processes, including liver cirrhosis and congestive heart failure. The mechanism by which the raised intrahepatic pressure results ultimately in the leakage of excess fluid through the liver capsule into the

peritoneal cavity, where it may accumulate, is well known, and the paper described here clarifies the morphological changes accompanying this movement of fluid, at least as far as the liver response to DMNA is concerned.

The morphological features of the mesothelial layer covering the liver were studied during ascites formation both ultrastructurally and by a silver impregnation technique, livers being taken for examination at intervals up to 11 days after DMNA treatment. During days 3–5, when ascites formation was maximal, the mesothelium on the liver surface became hypertrophied and developed increasing numbers of microvilli and cytoplasmic organelles (mitochondria, Golgi vesicles and phagolysosomes). Gaps up to 1 μm across were present between the mesothelial cells during this period. These gaps were revealed relatively infrequently by the electron microscope, but they were demonstrable by silver impregnation as dense localized deposits or small gaps and were seen to occur in large numbers. The ascitic fluid is thought to escape through these gaps. There was no evidence that the peritoneal surface covering other viscera contributed to the formation of ascites.

During the period of ascites formation there was widespread oedema beneath the capsule of the liver. The basement membrane of the capsule appeared to offer little hindrance to the passage of plasma protein, a situation which accounted for the high protein content of the ascitic fluid.

CANCER RESEARCH

2743. Asbestos properties and mesothelioma production

Stanton, Mearl F. & Wrench, Constance (1972). Mechanisms of mesothelioma induction with asbestos and fibrous glass. *J. natn. Cancer Inst.* **48**, 797.

Wagner, J. C., Berry, G. & Timbrell, V. (1973). Mesotheliomata in rats after inoculation with asbestos and other materials. *Br. J. Cancer* **28**, 173.

Mesothelioma of the pleura, comparable to that found in man, can readily be induced in the rat by direct intrapleural application of asbestos. This offers a means for investigating the carcinogenic mechanisms involved. In an effort to determine the importance of the physical form of the fibre in relation to its carcinogenic potential, three types of asbestos (one of them in five different forms), six types of fibrous glass, two types of silica and two types of metal particles were applied on gelatine-saturated fibrous glass pledgets directly to the pleura of rats. The pledgets were from a single source of fibrous glass of the type used in building construction for thermal insulation. The strand diameters were 3–10 times greater than those of the asbestos fibres, with a mean of 5 μm but an upper level of over 25 μm. They acted as a scaffold to retain the asbestos and to distribute it uniformly over a large area. The pledgets also acted as an irritant, which additionally served as a control on the specificity of the carcinogenic response. In preliminary experiments, the glass pledgets did not appear to be carcinogenic or to increase the incidence of asbestos-induced mesotheliomas.

Each material was implanted in this way in the pleura of 30 Osborne–Mendel rats (11–16 wk old), having been applied to the pledget suspended in warm gelatine. The animals were observed daily for 2 yr, only moribund rats being killed in the interim. Amosite, chrysotile and crocidolite, conforming to UICC reference standards and all applied in a dose

of 40 mg, were potently carcinogenic, producing a mesothelioma incidence in the 58–75% range. South African crocidolite and partially pulverized standard UICC crocidolite were equally potent. Another type of crocidolite, which was hand-milled so that it was not exposed to extraneous oils or metallic milling, gave a tumour response comparable to those of a standard reference milled crocidolite. Reducing the dose of the implanted asbestos decreased the tumour incidence. More important, however, was the reduction in tumour incidence associated with implantation of a standard crocidolite reduced by excessive milling to submicroscopic fibrils (1–2·5 μm in diameter).

Other types of materials implanted in the pleura in the same way as asbestos produced fewer tumours. Thus microspheres of noncrystalline silica (15–150 nm in diameter) produced a single mesothelioma among 48 rats. Fibrous glass of the type used as the vehicle in these studies, and two other types of fibrous glass with mean diameters ranging from 5 to 10 μm produced a mesothelioma incidence of about 4% when reduced to short fibrous fragments before application. Two forms of an especially fine fibrous glass, ranging from 0·06 to 3 μm in diameter and further milled to a length approaching that of asbestos fibres, resulted in moderately high incidences of mesotheliomas in the 12–18% range.

Thus the carcinogenicity of asbestos and fibrous glass seems to be related primarily to the particle size and shape of these materials rather than to their physico-chemical properties.

In another series of experiments (Wagner *et al.* cited above), samples of different forms of asbestos (both normal and benzene-extracted) from various sources, and certain other materials (such as barium sulphate, ceramic and glass fibres, glass powder and aluminium oxide) were administered by intrapleural inoculation to rats, mainly in doses of 20 mg. SFA chrysotile and crocidolite were also administered in five different doses between 0·5 and 8 mg.

A high proportion of most asbestos-treated groups developed mesothelioma, a total of 386 tumours occurring in 1112 rats examined. Crocidolite was the most carcinogenic, followed in descending order by amosite, anthophyllite and chrysotile. The risk of developing a mesothelioma at a given time after injection was found to be approximately proportional to the dose for both SFA chrysotile and crocidolite. Brucite was found to be about as carcinogenic as Canadian chrysotile. A few (three) mesotheliomas occurred in 31 rats treated with the ceramic fibre and single tumours occurred in groups of 30–35 rats treated with barium sulphate, glass powder or aluminium oxide.

Types of asbestos with very different chemical compositions produced comparable numbers of mesotheliomas. The removal of oils by benzene extraction had no significant effect on the carcinogenicity of the samples, nor had large differences in their trace-metal content.

When the samples were examined by electron microscopy and classified according to the number of "significant" fibres (less than 0·5 μm in diameter and greater than 10 μm in length), good agreement with the reported order of carcinogenicity was found.

2744. The urinary factor in bladder cancer

Chapman, W. H., Kirchheim, D. & McRoberts, J. W. (1973). Effect of the urine and calculus formation on the incidence of bladder tumors in rats implanted with paraffin wax pellets. *Cancer Res.* **33,** 1225.

It is widely held that bladder tumours are caused by carcinogens excreted by the kidney. Although there is little to support this belief in the case of human bladder tumours, there are at least two sets of animal experiments which provide some support. In dogs fed β-naphthylamine, Scott & Boyd (*J. Urol.* 1953, **70,** 914) diverted the urine from the bladder to the sigmoid colon and prevented the appearance of bladder tumours, while McDonald & Lund (*ibid* 1954, **71,** 560) isolated the top half of the bladder from the urinary stream by a constriction and found that tumours developed only in the intact lower half, the only part in contact with the urine.

Further studies have now been carried out on the role of the urine in the development of urinary tumours and on the role of 3-hydroxyanthranilic acid (OHAA), which is thought to be responsible for the induction of some bladder tumours in man. The bladder of inbred Fischer 344 rats was divided into a double pouch by a midline ligature and a paraffin-wax pellet, with or without OHAA, was implanted in each part. The lower pouch remained in contact with the urine in all the animals, while the upper was completely isolated and kept free of urine in about one-third. In roughly another one-third of the rats, the upper and lower pouches remained in communication, so that the urine had access to the upper part, while in the remainder the constricting suture eroded through the bladder wall, so that the bladder reverted to a single cavity ("single pouch"). All rats surviving for over 40 wk were included in the final assessment.

No tumours formed in the 99 isolated upper pouches, while 49 formed in the 119 communicating upper pouches, which were in contact with urine, and 79 formed in the 102 single pouches. The presence of 20% OHAA in the pellets did not affect the incidence of tumours. Stone formation was evident in half of the pouches that were in contact with urine and the incidence of tumours approximately doubled in the pouches with stone formation. No tumours were produced in areas that were not in contact with urine, even when foreign bodies were present.

2745. A touch of the sun

Black, H. S. & Douglas, D. R. (1973). Formation of a carcinogen of natural origin in the etiology of ultraviolet light-induced carcinogenesis. *Cancer Res.* **33,** 2094.

The aetiology of carcinogenesis of the skin induced by ultraviolet (UV) irradiation is not yet clear. Cholesterol α-oxide (CαO), a product of the photochemical reaction of sterols, has been shown to be carcinogenic (Bischoff *et al. Fedn Proc. Fedn Am. Socs exp. Biol.* 1955, **14,** 183) and to be formed *in vitro* in human skin exposed to irradiation (Black & Lo, *Nature, Lond.* 1971, **234,** 306). It has also been found in the skin of hairless mice exposed to such irradiation (Black & Douglas, *Cancer Res.* 1972, **32,** 2630).

In an attempt to establish whether the formation of this compound may be a factor in the development of UV-induced skin cancer, these same authors exposed two groups of hairless mice to sub-erythemic doses of UV radiation from a mercury arc lamp for 5 days/wk. One of the groups was retained as a control to establish the time of tumour formation, while animals from the second group were killed at fortnightly intervals during exposure for skin analysis. Two further groups were maintained in parallel with the first two, but were not exposed to irradiation. An area of the dorsal skin was excised from the killed animals and analysed using thin-layer chromatography, followed—after esterification of CαO with acetic anhydride[^{14}C]—by gas-liquid radiochromatography.

The levels of CαO in the skin of the non-irradiated control mice remained roughly constant throughout the study within the range of 0·3–1·0 μg/g skin. This was also true of the test animals during the first 6 wk of irradiation, but the level then rose sharply reaching at wk 10 a peak equivalent to about 16 μg CαO/animal (from the base level of about 2 μg/animal). The level then began to fall again and soon after the first squamous cell carcinomas appeared, the incidence being about 25% at wk 16 and 90% by wk 24.

Bischoff (*Adv. Lipid Res.* 1969, **7**, 161) reported only 20–50% tumour induction with an sc dose of 20 mg CαO, a dose far greater than the low level of CαO found here, and the tumours produced were sarcomas. However, there is probably little justification for comparisons between the effects of a compound administered sc and those resulting from its formation within the target tissue. The success of the present study in demonstrating increased levels of CαO in association with a high tumour incidence in the skin of UV-irradiated mice suggests that this may be a line of investigation worth pursuing.

TERATOGENESIS

2746. The subtler side of hydroxyurea

Butcher, R. E., Scott, W. J., Kazmaier, Kathy & Ritter, E. J. (1973). Postnatal effects in rats of prenatal treatment with hydroxyurea. *Teratology* **7**, 161.

Previous work by some of these authors has suggested that hydroxyurea administered to rats on day 12 of gestation is bound to embryonic tissues (*Cited in F.C.T.* 1973, **11**, 169). In these experiments, embryotoxicity involving the neural tube did not lead to observable malformations of the central nervous system. That this picture may not be adequate appears from the present paper.

Rats were injected ip on day 12 of gestation with 375 or 500 mg hydroxyurea/kg. After 5 hr, these animals were anaesthetized and the numbers of implantation sites were counted. A few embryos were removed for microscopic examination and hydroxyurea determination. The rats were then allowed to deliver normally and all litters were examined at birth and weaned 25 days later. Between 30 and 40 days after birth, open-field exploratory activity of the offspring was tested, and at 50 days the animals were weighed and swimming ability and the capacity to learn the escape route from a water-barred maze were assessed. An appreciable amount of hydroxyurea was identified in 12-day embryos from the treated animals, and microscopic examination of the neural tube revealed evidence of cytotoxicity, the severity of which was dose-related. Litter size was comparable in treated and control animals, and at birth there was no external evidence of malformation in either group given hydroxyurea. However, in the group given the higher dose a reduction in weight was apparent in 50-day-old offspring and in both groups the open field tests showed a failure of normal neuromuscular control of the hind limbs. By this stage of the study, too, kinking of the tail was apparent in 47% of the offspring of the group given 500 mg hydroxyurea/kg compared with 6% in the group given 375 mg/kg, none in the control group and only 3% in 20-day foetuses of dams given the higher dose level in the earlier study. Swimming ability

was not significantly affected, but maze-learning was impaired in hydroxyurea-treated animals. This effect was apparently dose-related and was not modified when the young were reared from birth by control dams.

These findings indicate that standard teratological studies may fail to detect significant physical and behavioural consequences of prenatal treatment with hydroxyurea.

Fd Cosmet. Toxicol. Vol. 12, p. 597. Pergamon Press 1974. Printed in Great Britain

MEETING ANNOUNCEMENT

HEALTH EFFECTS OF CHEMICALS IN FOODS

To commemorate the centennial anniversary of the introduction of legislation pertaining to the safety of foods in Canada, the Health Protection Branch will host an international symposium on food safety on 12–14 May 1975 in Ottawa, Canada.

The symposium will be entitled "Health Effects of Chemicals in Foods". Subjects to be discussed include an assessment of world food needs and future trends in food production, a survey of the public health implications of chemicals (both natural and synthetic) in foods, newer methodological approaches to the epidemiological and toxicological evaluation of potential toxicants in food with special emphasis on the assessment of subtle and irreversible toxic effects, methods that may be used to extrapolate experimental animal data to human population groups and the benefit/risk analysis of the use of chemicals in foods, particularly in relation to regulatory controls. A generous amount of time has been allotted in the programme to the exchange of informed opinion regarding approaches to the evaluation of food safety.

The symposium will be held in the auditorium of the National Library, 395 Wellington Street, Ottawa. The registration fee is $50.00. Details of the programme and information on accommodation may be obtained from Mrs. Jean R. Renaud, Head, Technical Secretariat, Room 1–8, Health Protection Branch Building, Health & Welfare Canada, Ottawa, Ontario, Canada KIA OL2 (Telephone: 996–4613).

Fd Cosmet. Toxicol. Vol. 12, p. 599. Pergamon Press 1974. Printed in Great Britain

FORTHCOMING PAPERS

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

Long-term toxicity studies of carmoisine in mice. By P. L. Mason, I. F. Gaunt, K. R. Butterworth, Joan Hardy, Ida S. Kiss and P. Grasso.

Short-term toxicity of cyclohexylamine hydrochloride in the rat. By I. F. Gaunt, M. Sharratt, P. Grasso, A. B. G. Lansdown and S. D. Gangolli.

Accumulation of ingested sulphite- and sulphate-sulphur and utilization of sulphited proteins by rats. By W. B. Gibson and F. M. Strong.

The short-term toxicity of quillaia extract in rats. By I. F. Gaunt, P. Grasso and S. D. Gangolli.

Two-year feeding and multigeneration studies in rats on five chemically modified starches. By A. P. de Groot, H. P. Til, V. J. Feron, Harriet C. Dreef-van der Meulen and Marian I. Willems.

Development and reversibility of pancreatic acinar cell changes in the rat produced by tebuthiuron (1-(5-*tert*-butyl-1,3,4-thiadiazol-2-yl)-1,3-dimethylurea). By W. J. Griffing and G. C. Todd.

Study on the carcinogenicity of lead arsenate and sodium arsenate and on the possibly synergistic effect of diethylnitrosamine. By R. Kroes, M. J. van Logten, Johanna M. Berkvens, T. de Vries and G. J. van Esch.

Carcinogenic effects of low dietary levels of aflatoxin B_1 in rats. By G. N. Wogan, S. Paglialunga and P. M. Newberne.

The aetiology of caecal enlargement in the rat. By D. C. Leegwater, A. P. de Groot and Marina van Kalmthout-Kuyper.

Sanguinarine in the blood and urine of cases of epidemic dropsy. By I. S. Shenolikar, C. Rukmini, K. A. V. R. Krishnamachari and K. Satyanarayana. (Short Paper).

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

Chemical carcinogens in the environment and in the human diet: Can a threshold be established? By G. Claus, I. Krisko and Karen Bolander. (Review Paper).

Contents continued]

Aims and Scope

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

Instructions to Authors

General. Authors from the United Kingdom should send *Original Papers and Reviews* to the Assistant Editor. All other papers and reviews should be sent to the appropriate Regional Editor. All 'Letters to the Editor' should be sent to the Editor and must be signed before they can be considered for publication.

Submission of a paper to the Editor will be held to imply that it reports unpublished original research, that it is not under consideration for publication elsewhere and that if accepted for the Journal, *Food and Cosmetics Toxicology*, it will not be published again, either in English or in any other language, without the consent of the Editor.

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

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

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

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

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

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

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

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

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

e.g. 1943a, 1943b or (1943a, b).

Footnotes. These as distinct from literature references should be avoided as far as possible. Where they are essential, reference is made by the symbols * † ‡ § ‖ ¶ in that order.

Illustrations and Diagrams. These should be kept to a *minimum* and they should be numbered and marked on the back with the author's name. Legends accompanying illustrations should be typewritten on *separate* sheets. Diagrams and graphs must be drawn in Indian ink on good quality paper or tracing linen. The following standard symbols should be used on line drawings since they are easily available to the printers:

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Other Nomenclature, Symbols and Abbreviations. In general, authors should follow the recommendations published in the *Handbook for Chemical Society Authors* (1961), p. 164 and in the *I.U.P.A.C. Information Bulletin*, No. 13, p. 64, Appendix B (1961). In the title and summary, abbreviations should be avoided; in the Introduction, Results and Discussion they should be used sparingly.

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