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Food and Cosmetics Toxicology

An International Journal published for the
British Industrial Biological Research Association

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The various features of the Journal are distinguished by use of the following superscripts inserted after the appropriate page number:

P = Papers (original); M = Monographs; R = Review articles; L = Letters to the Editor.

F.C.T.—B

Index of Subjects*

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INFORMATION SECTION

ARTICLES OF GENERAL INTEREST

TOXICOLOGY: ABSTRACTS AND COMMENTS

F.C.T. 11/4—A

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Research Section

Long-term Toxicity Studies of Erythrosine. I. Effects in Rats and Dogs

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(*Received 1 March 1973*)

Abstract—Groups of 12 male and 12 female weanling Osborne–Mendel rats were fed diets containing erythrosine (FD & C Red No. 3) at levels of 0, 0·5, 1·0, 2·0 or 5·0% for 2 yr. Growth depression was observed in the rats given the 5·0% level. Spleen weights, expressed as organ-to-body weight ratios, were decreased in the male rats given the 0·5, 2·0 and 5·0% dose levels and in female rats given the 5·0% dose level. Slight caecal distension occurred in rats given erythrosine at 1·0% and the condition increased with increasing dose levels. The histology of the distended caeca was normal. Chronic nephritis was approximately doubled in incidence in the 0·5% group compared with that in the control group, but this was probably due to chance variation.

Weekly sc injections of erythrosine (approximately 12 mg/rat) were given to a group of 18 rats for 2 yr. Injection-site ulcerations were produced but no tumours were observed. In an acute oral toxicity study in rats, the LD_{50} of erythrosine was found to be 1840 mg/kg.

In another long-term study, erythrosine was fed at dietary levels of 0, 0·5, 1·0 or 2·0% to groups of three male and three female beagle dogs. All dogs survived the 2-yr period and no effects attributable to the ingestion of erythrosine were observed.

INTRODUCTION

Erythrosine (tetraiodofluorescein; FD & C Red No. 3), a coal-tar dye, is one of the seven colourings originally permitted under the Food and Drug Act of 30 June 1906 (Calvery, 1942). It is widely used as a colouring agent in drugs, cosmetics and foods, including candy, chewing gum, cookies, ice cream, cake frosting and fruit cocktail. Approximately 238,000 lb were certified for use in the fiscal year 1972.

Waliszewski (1952) reported that erythrosine in an oral dose of 2·5 g/kg body weight or an iv injection of 0·37 g/kg was lethal to mice; iv injection also produced haemolytic disorders. Lu & Lavelle (1964) administered erythrosine to six male adult rats by intubation at a dose level of 2 g/kg and concluded that the acute oral LD_{50} value exceeded that dose.

In a short-term toxicity study in rats, Umeda (1956) gave 20 rats weekly sc injections of 1 ml of a 5·0% aqueous solution of erythrosine. Tumours were not observed in any rats and

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seven rats survived for 300 days or more. Graham & Allmark (1959) administered erythrosine sc to five female rats twice daily for 3 days at a level of 250 mg/kg body weight and concluded that the compound had no oestrogenic activity.

In a long-term toxicity study in mice, Waterman & Lignac (1958) fed erythrosine to a total of 122 male and female mice of mixed breeding from five different strains. The mice, between 50 and 100 days old, were given a diet providing 1 mg erythrosine/day for each animal. A group of 168 mice served as negative controls. Positive control groups were given *o*-aminoazotoluene and dimethylaminoazobenzene, both of which induced signs of liver tumours after approximately 200 days. The incidence of tumours in mice given erythrosine was not significantly greater than that in the negative controls, and it was concluded that this colouring had no carcinogenic action.

Willheim & Ivy (1953) fed erythrosine at a level of 4·0% of the diet to five male and five female rats for periods up to 1 yr. They observed gross staining in the glandular stomach and small intestine and granular deposits in the stomach, small intestine and colon. Hepatic cirrhosis was noted in one rat of the four that lived to 12 months. Fifty control animals observed for 20 months or more failed to develop tumours or hepatic cirrhosis.

Bowie, Wallace & Lindstrom (1966) observed a reduction in haemoglobin and red blood cells and an elevation in protein-bound iodine and total iodine in rats administered erythrosine orally. Hansen, Davis, Graham, Perry & Jacobson (1973) confirmed the elevated protein-bound iodine values but did not observe the effects on haemoglobin and red blood cells.

To study the metabolic and excretory patterns of erythrosine in adult rats, Webb, Fonda & Brouwer (1962) administered the compound by stomach-tube in log-spaced doses from 0·5 to 500 mg/kg body weight. Within 5 days, 102% of the dye was recovered in the excreta. Urine and bile were collected for the initial 2–4 hr after iv injections of 3 mg/kg had been given; an average of 55% of the administered dose was found in the bile and 1·3% was found in the urine. Daniel (1962) also gave erythrosine to rats by stomach-tube in a dose of 500 mg/kg and found that from 55 to 72% of the dye was excreted in the faeces within 3 days.

The present study reports the acute oral LD_{50} and the effects of 2-yr oral administration of erythrosine to rats and dogs. Results of weekly sc injections of erythrosine to rats, previously reported in abstract form (Nelson & Hagan, 1953), are also presented.

EXPERIMENTAL

Materials. Erythrosine, obtained commercially, was certified as FD & C Red No. 3. The dyes used to study the acute toxicity in rats and the long-term toxicity in rats and dogs contained 95, 92 and 96% pure dye, respectively. The diets were prepared by adding erythrosine to ground Purina Laboratory Chow (Ralston Purina Co., St. Louis, Mo.) and blending in a food mixer.

Studies in rats

Acute toxicity. The acute oral toxicity of erythrosine was determined in young adult Osborne–Mendel rats. Erythrosine was administered by stomach-tube as a 10·0% (w/v) aqueous solution to groups of five male and five female rats. The rats were fasted overnight before treatment, and their fasted weights ranged from 230 to 275 g for the males and from 190 to 235 g for the females. The surviving rats were observed for approximately 3 wk after treatment. The LD_{50} was calculated by the method of Litchfield & Wilcoxon (1949).

Long-term toxicity. Five groups of 24 litter-mate Osborne-Mendel rats, evenly divided by sex, were fed erythrosine at 0, 0·5, 1·0, 2·0 or 5·0% dietary levels for 2 yr. The rats were housed individually and were allowed food and water *ad lib.* Weekly records were kept of animal weights, mortalities and clinical observations. At approximately 3, 6, 14 and 21 months, blood samples were collected from the tail vein of 10 rats in each group, and haemoglobin, haematocrit and total and differential leucocyte counts were determined. At the end of 2 yr, the survivors were killed by decapitation and autopsied and the weights of heart, liver, spleen, kidneys and testes were recorded. Rats that died before the end of the experiment were autopsied but organ weights were not recorded.

Of the 120 rats started on the experiment, 109 were submitted for pathological examination, the rest being discarded chiefly because of advanced post-mortem autolysis. Sixty-one rats were sectioned microscopically, 21 in detail and 40 with sections limited to kidney, liver, testes, tumours (if present) and other organs with abnormalities. The haematoxylin–eosin stained paraffin sections from the rats studied in detail included sections of lung, heart, liver, spleen, pancreas, stomach, small intestine, colon, caecum, kidney, adrenal, thyroid, testis and prostate (or ovary and uterus), urinary bladder, leg bones with voluntary muscle and marrow, and any tumour or other tissue appearing abnormal. The parathyroid was often included with the thyroid.

Carcinogenicity tests involving sc injection. Groups of 18 Osborne-Mendel rats, approximately 4 wk old and evenly divided by sex, were started on weekly sc injections of 1 ml of 2·0% aqueous erythrosine or of 0·9% saline (controls). During the 2-yr study, the erythrosine concentration was decreased to 1·5% and finally to 0·75% because of ulcerations at the injection site.

Studies in dogs

Long-term toxicity. Twenty-four young beagle dogs, divided into four groups of three males and three females, were fed laboratory chow diets containing erythrosine at levels of 0, 0·5, 1·0 or 2·0% for 2 yr. Periodic haematological examinations, including haemoglobin and haematocrit determinations and white cell counts, were performed before and during the test period, and records of both body weights and food consumption were kept throughout the test period. At the conclusion of the test, the dogs were killed and autopsied. Sections of liver, kidney, heart, lung, gall bladder, spleen, pancreas, adrenal, thyroid, parathyroid, stomach, small and large intestines, brain (four levels), rib bone and marrow, testis, prostate, ovary, uterus, submaxillary salivary gland, lymph node and skeletal muscle from the six dogs given the 2·0% erythrosine diet and from the six control dogs were examined microscopically. Although these 12 dogs received the more thorough microscopic examination, tissues from other dogs were also examined.

In addition, from about half of the dogs, paraffin sections of Zenker-fixed liver and kidneys stained with haematoxylin–eosin and frozen sections of formalin-fixed liver and kidney stained with Oil Red O were examined. Approximate myeloid-erythroid cell ratios were determined on Wright–Giemsa-stained bone-marrow smears from 23 dogs.

RESULTS

Acute toxicity in rats

The oral LD_{50} of erythrosine was calculated to be 1840 mg/kg with 95% confidence limits of 1614–2098 mg/kg; the slope function was 1·36 with 95% confidence limits of 1·1–1·7. Deaths occurred from 3 hr to 3 days. The effects produced by erythrosine were

depression, diarrhoea with dye in the faeces, and pink ears, tail, paws and nose. In rats that died shortly after administration of the compound, the internal organs, muscle and fat were also pink. Rats killed at the end of the observation period still had dye-stained fur in the anal region and dye-stained tails, but organs and fat were normal in appearance.

Long-term toxicity in rats

Growth, mortality, haematology and organ weights. No effects (to $P > 0{\cdot}05$) on mortality, haematology or weights of liver, heart, kidney or testes were observed in rats given erythrosine at dietary levels up to 5·0%, but there were significant reductions in spleen weights. The average spleen-to-body weight ratios (g/kg) for the control males and for the males given the 5·0, 2·0 and 0·5% dietary levels of erythrosine were 2·51, 1·64 ($P < 0{\cdot}02$), 1·54 ($P < 0{\cdot}01$) and 1·71 ($P < 0{\cdot}05$), respectively, while the ratios for the control females and for females given the 5·0% dietary level were 2·44 and 1·78 g/kg ($P < 0{\cdot}02$) respectively. On an absolute organ-weight basis, only the spleen weights of the males given the 5·0 and 2·0% levels of erythrosine were significantly lower than those of the controls ($P < 0{\cdot}01$ and $P < 0{\cdot}05$, respectively).

Significant growth inhibition occurred in both sexes only at the 5·0% level. After 1 yr of the experiment, the mean body weights (g) were 450 for control males and 380 for males given the 5·0% diet ($P < 0{\cdot}001$) and 213 for control females and 177 for females given the 5·0% diet ($P < 0{\cdot}01$). Weights of both male and female rats were also significantly depressed at 12, 27 and 104 wk ($P < 0{\cdot}05$).

Gross pathology. The only gross lesion that could be attributed to erythrosine treatment was caecal distension (ranging from very slight to moderately marked). The incidence of caecal distension and of certain spontaneous conditions in rats given the various dosage levels is shown in Table 1. Moderate and slight distension of the caecum occurred in the rats fed 5·0% erythrosine and slight distension occurred in those fed the 2·0 and 1·0% levels; none was observed at the 0·5% and control levels.

Table 1. *Gross pathological changes in rats fed 0–5% erythrosine in the diet for 2 yr*

Dietary level (%)	Distended caecum (no. of rats affected)	Tumours			Kidney granularity (no. of rats affected)	Testicular atrophy (no. of rats affected)
		No. of rats affected	Total no.	No. of mammary tumours		
0	0	7	9	7	6	3
0·5	0	4	6	4	12	2
1·0	3	3	4	1	4	1
2·0	2	7	8	2	4	1
5·0	12	3*	3*	0	2	2

*Including one rat with a thyroid adenoma discovered on microscopic examination.
Numbers given refer to groups of 12 male and 12 female rats at each dietary level.

Microscopic pathology. No effects were observed microscopically that were attributable to erythrosine. Grossly distended caeca showed normal histology on microscopic examination. The most common tumours were mammary adenocarcinomas and fibroadenomas (Table 2). No mammary tumours were observed in the rats fed the 5·0% level, but one male survivor given this level had a thyroid colloid adenoma. The common incidental lesions were pneumonia (mostly chronic) of varying degrees and chronic nephritis of the usual

Table 2. *Tumour incidence in rats fed 0–5% erythrosine in the diet for 2 yr*

		No. of tumours									
		Males					Females				
Type of tumour	Dietary level (%).....	0	0·5	1·0	2·0	5·0	0	0·5	1·0	2·0	5·0
	No. of rats with tumours*.	2	0	2	5	3	5	4	1	2	0
Mammary adenocarcinoma		0	0	0	0	0	2	2	0	1	0
Mammary fibroadenoma		0	0	0	0	0	5	2	1	1	0
Pulmonary lymphosarcoma		1	0	1	1	1	0	1	0	0	0
Abdominal lymphosarcoma		0	0	1	0	0	0	0	0	0	0
Fibrosarcoma		0	0	0	3	0	0	0	0	0	0
Endometrial sarcoma		0	0	0	0	0	0	1	0	0	0
Thyroid colloid adenoma		0	0	0	0	1	0	0	0	0	0
Splenic reticulum-cell sarcoma		0	0	0	0	1	0	0	0	0	0
Squamous cell carcinoma		0	0	0	1	0	0	0	0	0	0
Adrenal cortical carcinoma		0	0	0	1	0	0	0	0	0	0
Ovarian thecal-cell tumour		0	0	0	0	0	0	0	1	0	0
Caecal adenoma		1	0	0	0	0	0	0	0	0	0

*Numbers of rats out of groups of 12 males or 12 females at each dietary level. Since some rats had more than one tumour the total number of tumours is greater than the number of affected rats.

types seen in our older rats. One rat given the 5·0% diet had one testis showing marked-to-extreme atrophy and sections from the other testis showed slight-to-moderate patchy atrophy. There was marked testicular atrophy in one of the controls, but the remaining testis sections showed no more than slight atrophy. Other major incidental lesions included a splenic haematoma and moderate spontaneous ulcerative caecitis in a male rat fed the 1·0% diet, a 1–2 mm perforation on the greater curvature of the stomach of a female rat fed the 0·5% diet, a 0·35 mm ulcer of the stomach of a male rat given the 5·0% diet, and a large chronic tubo-ovarian abscess in one rat given the 1·0% diet. Examination of the organs revealed no difference in the degree or incidence of pathological changes in the groups, except that the incidence of chronic nephritis was about doubled in the group given the 0·5% level compared with that in the control group, a finding probably due to chance variation.

Carcinogenicity tests involving sc injection

Erythrosine given as weekly sc injections in doses of approximately 12 mg/rat produced no effects except local irritation with small punched-out ulcers. Table 3 shows the results of weekly injections for a 2-yr period. No injection-site tumours were produced.

Table 3. *Mortality and tumour incidence in rats injected sc with erythrosine*

			Survivors at month				
			18		24		Rats with tumours at injection site
Group	Approximate dose (mg/rat/wk)	No. of rats at start	No.	(%)	No.	(%)	
Erythrosine	12	18	17	94·4	8	44·4	0
Control	0	18	16	88·9	7	38·9	0

Table 4. *Body weights and organ weights of dogs fed 0–5% erythrosine in the diet for 2 yr*

Dietary level (%)	Dog no.*	Body weight (kg)		Organ weight (g)							
		Starting	Terminal	Brain	Heart	Liver	Kidney	Spleen	Thyroids	Adrenals	Testes
0	382	8·4	8·3	79	70	228	39	56	0·9	1·1	24
	397	5·9	7·0	71	61	220	31	37	0·8	1·1	10
	370	8·0	9·7	88	78	265	34	36	1·2	1·3	21
	618	5·5	8·5	78	61	331	44	32	0·8	1·7	—
	627	7·5	8·7	74	70	275	48	27	0·9	1·5	—
	628	6·5	12·0	—	68	409	55	35	1·5	2·5	—
0·5	380	8·9	10·5	69	88	356	58	89	0·9	1·5	22
	383	7·6	8·6	79	77	250	47	59	1·0	1·3	20
	396	7·5	8·9	85	80	287	44	85	0·9	1·3	15
	617	5·7	7·5	65	58	215	42	32	0·9	2·1	—
	623	6·9	10·9	76	70	390	52	26	1·1	1·4	—
	626	6·9	4·7	66	39	149	28	20	0·6	1·6	—
1·0	379	7·3	10·2	73	94	303	54	57	1·3	1·9	20
	385	5·9	5·8	81	60	232	39	27	0·6	1·5	16
	395	6·4	7·2	86	70	242	33	32	1·1	1·0	26
	620	5·8	13·7	69	79	338	52	57	1·4	1·4	—
	622	5·5	6·5	73	69	361	56	44	1·1	1·6	—
	625	6·8	7·8	74	71	249	51	32	1·5	1·5	—
2·0	378	8·5	9·6	70	81	360	61	52	1·4	1·5	21
	384	8·1	8·6	93	78	290	63	53	2·0	1·4	22
	394	7·1	6·5	74	56	232	36	27	0·5	1·3	11
	619	7·0	8·5	72	64	273	40	63	1·0	1·6	—
	621	6·1	7·0	78	59	254	38	13	1·0	1·9	—
	624	6·1	8·0	71	62	262	37	26	1·2	1·5	—

*Those of the 300 series were male and of the 600 series female.

Long-term toxicity study in dogs

No effects attributable to ingestion of erythrosine were observed in these studies. Table 4 shows dosage levels, initial and final body weights and organ weights of dogs given erythrosine. No haematological effects attributable to ingestion of erythrosine were noted in the periodic examinations.

No compound-related gross pathology was noted. A control male had a congenital absence of the right kidney and emaciation was observed in one dog in each of the groups given the 1·0 and 0·5% diets, the dog fed the 0·5% diet being the more emaciated of the two. Obesity was also seen in one female in each of these groups. No intestinal worms were found in these 24 dogs.

There were no compound-related histological effects, and only minor incidental abnormalities were seen. Slight chronic thyroiditis was noted in one male and one female given the 2·0% erythrosine diet and the male also showed a slight decrease in the myeloid-erythroid ratio in the bone marrow. Another male in the group given the 2% erythrosine diet had slight testicular atrophy and the third male in this group had cystic mucoid glands in the gall bladder. One control female had slight cystitis of the urinary bladder. No other gross or microscopic pathology was seen.

DISCUSSION

Erythrosine can be considered slightly toxic when given acutely. The effects observed in the feeding study in rats were limited to the dietary levels of 1·0% and higher, and involved only growth depression and caecal enlargement. No effects were attributed to treatment at levels up to 2·0% in the dog study. No injection-site tumours were observed after repeated sc administration to rats.

Rat and dog studies continue to serve as the main support for estimating the maximum acceptable daily intake of erythrosine for man. The acceptable daily intake for man is 2·5 mg/kg or 100 ppm of the total daily diet, which is based on a 100-fold safety factor and no-effect levels of 0·5% in rats and 2·0% in dogs.

The average intake of erythrosine, used as a colouring agent, from food, dietary supplements, drugs and cosmetics is estimated to be 34·1 mg/day. For a 60-kg human this would be approximately 0·6 mg/kg, which is well below the acceptable daily intake for man of 2·5 mg/kg.

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Etude de la toxicité à long terme de l'érythrosine. I. Effets chez le rat et le chien

Résumé—Deux groupes de rats Osborne-Mendel sevrés, 12 mâles et 12 femelles, ont consommé pendant 2 ans de l'érythrosine (FD & C Red No. 3) à raison de 0, 0,5, 1,0, 2,0 ou 5,0% du régime. Un ralentissement de la croissance a été observé chez les rats soumis au régime à 5,0%. Le poids de la rate par rapport au poids du corps avait diminué chez les mâles qui avaient été soumis aux régimes à 0,5, 2,0 et 5,0% et chez les femelles qui avaient été soumises au régime à 5,0%. Une légère distension du caecum a été constatée chez les rats qui avaient consommé 1,0% d'érythrosine; elle était d'autant plus marquée que la dose administrée avait été plus forte. L'histologie des caecums distendus était normale. La fréquence des néphrites chroniques avait à peu près doublé, par rapport au groupe témoin, dans le groupe soumis au régime à 0,5%, mais ceci n'était probablement qu'un effet de hasard.

On a administré de l'érythrosine pendant 2 ans, en injections sous-cutanées hebdomadaires (environ 12 mg par animal), à un groupe de 18 rats. Des ulcérations se sont produites aux sites d'injection, mais on n'y a pas observé de tumeurs. Une étude de la toxicité orale aiguë chez le rat a révélé que la DL_{50} d'érythrosine était de 1840 mg/kg.

Dans une autre étude à long terme, on a donné de l'érythrosine à raison de 0, 0,5, 1,0 ou 2,0% du régime à des groupes de chiens bigles, 3 mâles et 3 femelles. Tous les chiens ont survécu à l'essai, qui a duré 2 ans, et on n'a observé aucun effet imputable à l'ingestion d'érythrosine.

Langzeitige Toxizitätsuntersuchungen mit Erythrosin. I. Wirkungen bei Ratten und Hunden

Zusammenfassung—Gruppen von 12 männlichen und 12 weiblichen abgesetzten Osborne-Mendel-Ratten erhielten 2 Jahre lang Futter mit 0, 0,5, 1,0, 2,0 oder 5,0% Erythrosin (FD & C Red No. 3). Wachstumsverminderung wurde bei den Ratten beobachtet, die 5,0% erhielten. Das Milzgewicht, ausgedrückt als Verhältnis des Organgewichts zum Körpergewicht, war bei männlichen Ratten, die 0,5, 2,0 und 5,0% erhielten, und bei weiblichen Ratten, die 5,0% erhielten, verringert. Leichte caecale Distension trat bei Ratten auf, die 1,0% Erythrosin erhielten, und der Zustand nahm mit steigender Dosierung zu. Die Histologie der distendierten Caeca war normal. Die Häufigkeit der chronischen Nephritis war in der Gruppe mit 0,5% annähernd verdoppelt, verglichen mit der in der Kontrollgruppe, aber dies war wahrscheinlich auf Zufallsvariation zurückzuführen.

Eine Gruppe von 18 Ratten erhielt 2 Jahre lang wöchentliche sc Injektionen von Erythrosin (etwa 12 mg/Ratte). An den Injektionsstellen wurden Ulcerationen hervorgerufen, aber es wurden keine Tumoren beobachtet. In einer Untersuchung der akuten oralen Toxizität in Ratten wurde die LD_{50} von Erythrosin mit 1840 mg/kg festgestellt.

Bei einer anderen langzeitigen Untersuchung wurde Erythrosin in den Konzentrationen 0, 0,5, 1,0 oder 2,0% im Futter an Gruppen von drei männlichen und drei weiblichen Beaglehunden verfüttert. Alle Hunde überlebten die 2-Jahres-Periode, und es wurden keine Wirkungen beobachtet, die sich der Verfütterung von Erythrosin zuschreiben liessen.

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Long-term Toxicity Studies of Erythrosine. II. Effects on Haematology and Thyroxine and Protein-bound Iodine in Rats*

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Abstract—Erythrosine (FD & C Red No. 3) was administered to male and female Osborne–Mendel rats either by intubation twice weekly for 85 wk or continuously in the diet for 86 wk. No consistent differences in red blood cell counts, haematocrit, haemoglobin, reticulocyte counts or other indications of anaemia were observed. Increased values for protein-bound iodine were attributed to circulating erythrosine in the blood serum; these values had returned to normal 16 wk after erythrosine administration ceased. Thyroxine-iodine levels were not affected in treated rats. No gross nor microscopic pathology was attributed to administration of this dye.

INTRODUCTION

The toxicity of erythrosine (FD & C Red No. 3) has been studied in rats, mice and dogs; most results have been negative. In 2-yr feeding studies in rats and dogs conducted in our laboratories, the only adverse effects noted were growth inhibition in rats given a 5·0% erythrosine diet and distended caeca in rats given dietary levels of 1·0% or higher (Hansen, Zwickey, Brouwer & Fitzhugh, 1973). Other investigators reported that haemolytic changes occurred in mice (Waliszewski, 1952), and that anaemia and elevated protein-bound iodine (PBI) values were produced in rats administered erythrosine (Bowie, Wallace & Lindstrom, 1966).

The present studies were done primarily to evaluate the effects of erythrosine on the red blood cell count, haematocrit, haemoglobin and clotting times of rats, to determine whether erythrosine could be detected in the blood serum of the animals and to determine any possible effect of liberated iodine on thyroid function.

EXPERIMENTAL

Test material. Erythrosine, certified as FD & C Red No. 3 and containing 95% pure dye, was used in these studies.

Animals and diet. Osborne–Mendel rats (100 days old at the start of the tests) were housed

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individually and given food and water *ad lib.* The basal diet was Purina Laboratory Chow (Ralston Purina Co., St. Louis, Mo.).

Treatment. Five groups of 100-day-old Osborne–Mendel rats were fed diets containing erythrosine at 0, 0·5, 1·0, 2·0 or 4·0% for 86 wk. Each group consisted of 25 males and 25 females except for the control (0%) group, which consisted of 50 males and 50 females. Additional groups of 25 male and 25 female 100-day-old Osborne–Mendel rats were intubated twice weekly with erythrosine at dose levels of 0, 100, 235, 750 or 1500 mg/kg for 85 wk. Solutions were prepared in distilled water at concentrations to give a uniform dose volume of 10 ml/kg body weight. At the end of the treatment periods, the animals were fed the control diet until the experiment was terminated at 2 yr.

Tests and observations. Weekly records were kept of body weights, food intake, deaths and clinical observations. Prior to treatment and at 3, 6, 12 and 18 months, blood samples were taken from the tail veins of ten males and ten females for haemoglobin and haematocrit determinations and red blood cell, white blood cell, differential cell and reticulocyte counts. Clotting times were determined at 3, 6 and 12 months. Whenever possible the same animals were bled at each interval. At other time periods, blood samples were collected by cardiac puncture for determinations of protein-bound iodine (PBI), thyroxine (T_4) by the method of Murphy & Pattee (1964) and prothrombin times. A procedure was developed to determine whether erythrosine was present in the serum of the treated animals, thus serving as a source of iodine which would falsely elevate PBI values. Known amounts of erythrosine were added to serum from control animals, and the dye was extracted and determined colorimetrically (M. Singh and C. Graichen, Food and Drug Administration, personal communication 1967). Recovery of erythrosine by this method was 91–101%.

Post-mortem studies. At autopsy, organs appeared normal and weights were not recorded. All rats were autopsied and some without gross lesions were discarded. In some cases, pathological examination was limited to the trunk and limbs, but in the majority of the rats the entire body, including the head, was examined. Formalin-fixed tissues from 268 of the 300 rats fed erythrosine and 234 of the rats intubated with erythrosine were subjected to gross and microscopic examination. Paraffin sections of all gross lesions (except middle-ear infections) in these animals were stained with haematoxylin and eosin and examined microscopically. In addition, at the end of the 2-yr study a fairly complete series of tissues from some rats in the 4%, 1500 mg/kg and control groups were examined microscopically for any minor morphological changes which could be attributed to long-term toxicity. Typically, these tissues included the brain, eye, extraorbital lachrymal gland, salivary gland, thyroid, trachea, oesophagus, squamous and glandular stomach, duodenum, jejunum, ileum, caecum, colon, heart, lung, liver, pancreas, spleen, adrenal, kidney, urinary bladder, bone, bone marrow, skeletal muscle, and either ovary and uterus or testes and prostate. Sections of the parathyroid, Harderian gland, salivary lymph node, caecal lymph node and normal mammary gland of some animals were also studied. Approximately 1310 pieces of tissue from the diet-fed animals and 1325 pieces of tissue from the intubated animals were blocked, sectioned, stained and examined microscopically.

Statistical analysis. The data on the haematocrit and haemoglobin determinations, RBC counts, and PBI and T_4 estimations were analysed by Student's *t* test. The Mann–Whitney U-test (Siegel, 1956) was applied to the data on clotting times and reticulocyte cell counts.

RESULTS

Body weights

Growth inhibition occurred only in animals given the two highest dietary levels of erythrosine. At the end of 1 yr, male and female rats given the 4·0% diet and female rats given the 2·0% diet showed significant growth inhibition. The mean weights (g) were: control males, 544; males given the 4·0% diet, 501 ($P<0·01$); control females, 351; females given the 4·0% diet, 293 ($P<0·001$); females given the 2·0% diet, 326 ($P<0·01$). Diarrhoea was observed in the animals fed at the 4·0% level.

Haematology and serum analyses

Tables 1 and 2 show the mean values and standard errors for red blood cell counts and haemoglobin and haematocrit determinations for the diet-fed and intubated animals, respectively, before treatment and at 3, 6, 12 and 18 months. Statistically significant differences were observed in each parameter but not at each sampling time. Most of the differences from control values were increases, whereas if anaemia had occurred, decreases would have been expected. Total and differential counts of white blood cells were not affected by erythrosine administration.

Coagulation times and reticulocyte counts are shown in Tables 3 and 4. Statistically significant differences were observed at different times in both the coagulation times and reticulocyte counts, but there was no consistent pattern when the values for the diet-fed animals were compared with those of the animals that received erythrosine by intubation. At 12 months, the coagulation times of the male animals fed erythrosine in the diet were longer than those of the controls, whereas there were no differences in the females. Only sporadic differences were observed in the males and females of the intubated groups. When significant differences occurred, the reticulocyte counts of the intubated animals were higher than those of the controls, whereas in the diet-fed animals the counts were usually lower. At 18 months, the counts for intubated controls were exceptionally low.

After the rats had been fed the diets for 84 wk, prothrombin times were determined on the male and female controls and on the males and females fed the 4·0% diet. No differences between the groups were observed.

After erythrosine administration for approximately 62 wk, blood samples were collected from animals on the control, 0·5 and 4·0% diets for determination of the amount of erythrosine in the serum and the PBI values (Table 5). Since the erythrosine molecule contains a theoretical value of 56·5% iodine, the elevated PBI values were attributed to erythrosine circulating in the serum.

Additional blood samples were collected between wk 79 and 83 of administration of the dye for T_4 determinations, from 6 to 13 samples being obtained from each of the groups treated with 0, 0·5 or 4·0%, or 100 or 1500 mg/kg. No differences were observed between the control values and those of any treated group (Table 5).

All animals surviving after the treatment period of 85–86 wk were fed the control diet for the rest of the 2 yr. After the control diet had been fed for 16 wk, the PBI values were determined in the serum of 6–10 animals from the groups given 0, 0·5 or 4·0% or 100 or 1500 mg/kg. These values were within normal limits in both the diet-fed and intubated animals (Table 5).

Gross and microscopic pathology

The lesions observed during gross examination appeared to be randomly distributed.

Table 1. *Red blood cell count, haemoglobin and haematocrit in rats fed 0–4% erythrosine in the diet*

Sex and dietary level (%)	Haematological data at month				
	0†	3	6	12	18
			Red blood cells ($\times 10^6$/mm^3)		
Males					
0·0	7·60 ± 0·12	7·97 ± 0·39	8·59 ± 0·17	7·94 ± 0·24	6·83 ± 0·38
0·5	7·16 ± 0·33	8·66 ± 0·57	9·14 ± 0·14*	8·12 ± 0·29	6·24 ± 0·52
1·0	7·03 ± 0·25	7·54 ± 0·29	7·60 ± 0·17(*)	7·81 ± 0·41	7·16 ± 0·18
2·0	6·95 ± 0·33	8·30 ± 0·21	8·26 ± 0·13	8·14 ± 0·13	8·20 ± 0·27*
4·0	7·13 ± 0·29	7·81 ± 0·17	8·12 ± 0·16	8·42 ± 0·29	8·39 ± 0·28*
Females					
0·0	7·03 ± 0·21	7·71 ± 0·34	8·15 ± 0·50	6·44 ± 0·41	6·09 ± 0·36
0·5	6·93 ± 0·19	8·20 ± 0·29	8·35 ± 0·26	7·17 ± 0·17	5·97 ± 0·27
1·0	6·42 ± 0·30	6·57 ± 0·20(*)	6·90 ± 0·14(*)	7·12 ± 0·15	6·28 ± 0·11
2·0	6·70 ± 0·15	7·49 ± 0·22	7·40 ± 0·15	7·57 ± 0·12*	6·97 ± 0·14*
4·0	6·72 ± 0·13	7·87 ± 0·44	7·39 ± 0·14	7·37 ± 0·10	6·87 ± 0·55
			Haemoglobin (g/100 ml)		
Males					
0·0	15·2 ± 0·25	15·8 ± 0·26	15·1 ± 0·27	15·5 ± 0·26	14·5 ± 0·63
0·5	15·0 ± 0·35	15·3 ± 0·23	15·8 ± 0·25	15·4 ± 0·42	14·0 ± 1·05
1·0	15·2 ± 0·20	14·6 ± 0·13(*)	14·9 ± 0·21	14·6 ± 0·66	15·0 ± 0·46
2·0	15·7 ± 0·32	15·7 ± 0·42	15·7 ± 0·22	15·2 ± 0·19	15·4 ± 0·29
4·0	14·9 ± 0·21	16·2 ± 0·35	16·3 ± 0·19*	16·3 ± 0·36	15·4 ± 0·34
Females					
0·0	14·1 ± 0·20	15·4 ± 0·33	14·6 ± 0·25	14·1 ± 0·68	14·1 ± 0·50
0·5	14·1 ± 0·16	15·2 ± 0·26	15·3 ± 0·12*	14·9 ± 0·17	15·0 ± 0·31
1·0	14·1 ± 0·24	14·4 ± 0·29(*)	15·1 ± 0·25	14·9 ± 0·18	15·1 ± 0·30
2·0	13·8 ± 0·20	15·0 ± 0·21	15·2 ± 0·19	15·1 ± 0·18	15·1 ± 0·32
4·0	14·4 ± 0·20	16·4 ± 0·44	15·4 ± 0·33	15·2 ± 0 29	15·5 ± 0·41*
			Haematocrit (%)		
Males					
0·0	51·1 ± 0·69	51·5 ± 1·33	46·9 ± 0·59	50·4 ± 0·88	46·4 ± 2·01
0·5	51·9 ± 1·23	50·9 ± 1·02	49·2 ± 1·01	49·8 ± 1·39	47·0 ± 2·89
1·0	52·1 ± 1·16	49·8 ± 1·00	49·4 ± 0·88*	48·9 ± 1·72	48·3 ± 1·12
2·0	53·6 ± 0·99	50·9 ± 0·62	50·8 ± 0·63*	49·6 ± 0·85	50·3 ± 0·87
4·0	51·4 ± 0·82	51·2 ± 0·33	49·9 ± 1·04*	51·3 ± 0·76	49·1 ± 0·80
Females					
0·0	47·8 ± 0·66	48·5 ± 0·88	48·6 ± 0·93	45·0 ± 2·10	44·0 ± 1·02
0·5	49·8 ± 1·16	51·6 ± 0·70*	51·2 ± 0·51*	47·9 ± 0·75	46·0 ± 1·29
1·0	47·5 ± 0·93	47·3 ± 0·93	47·6 ± 0·62	47·0 ± 0·92	45·8 ± 1·06
2·0	47·5 ± 0·65	49·3 ± 0·67	48·0 ± 0·58	48·3 ± 0·63	48·4 ± 0·83*
4·0	49·2 ± 0·93	50·0 ± 0·92	49·9 ± 0·74	46·7 ± 0·76	42·2 ± 4·89

†Before start of treatment.

Values are means ± SEM for groups of ten rats and those marked with an asterisk are significantly ($P < 0·05$) higher [*] or lower [(*)] than the control value.

Table 2. *Red blood cell count, haemoglobin and haematocrit in rats given erythrosine by intubation twice weekly*

Sex and dose level (mg/kg)	Haematological data at month				
	0†	3	6	12	18
Red blood cells ($\times 10^6$/mm^3)					
Males					
0	7·73 ± 0·36	7·81 ± 0·17	7·07 ± 0·50	7·96 ± 0·24	7·51 ± 0·20
100	7·70 ± 0·23	7·49 ± 0·17	8·64 ± 0·29*	8·35 ± 0·27	7·34 ± 0·50
235	7·77 ± 0·26	7·85 ± 0·38	8·30 ± 0·14*	7·91 ± 0·26	8·24 ± 0·36
750	7·30 ± 0·21	8·01 ± 0·22	7·93 ± 0·21	8·14 ± 0·17	7·00 ± 0·30
1500	7·35 ± 0·45	7·75 ± 0·29	7·95 ± 0·19	7·74 ± 0·37	7·59 ± 0·29
Females					
0	6·50 ± 0·13	6·87 ± 0·25	6·44 ± 0·23	6·55 ± 0·22	6·59 ± 0·13
100	6·80 ± 0·13	6·85 ± 0·15	7·47 ± 0·17*	6·85 ± 0·40	5·79 ± 0·40
235	6·66 ± 0·16	6·79 ± 0·14	7·00 ± 0·18	6·92 ± 0·20	6·59 ± 0·29
750	6·78 ± 0·11	7·19 ± 0·12	7·10 ± 0·13*	6·95 ± 0·11	6·10 ± 0·21
1500	7·05 ± 0·20*	7·12 ± 0·23	7·16 ± 0·13*	6·34 ± 0·16	7·15 ± 0·24
Haemoglobin (g/100 ml)					
Males					
0	14·9 ± 0·28	14·7 ± 0·25	14·3 ± 0·91	15·5 ± 0·28	15·6 ± 0·41
100	15·6 ± 0·28	14·8 ± 0·18	15·8 ± 0·39	14·8 ± 0·53	15·2 ± 0·99
235	15·3 ± 0·30	14·7 ± 0·22	15·7 ± 0·34	15·6 ± 0·36	15·7 ± 0·56
750	15·2 ± 0·32	14·8 ± 0·21	15·5 ± 0·20	15·0 ± 0·24	14·0 ± 0·52(*)
1500	14·7 ± 0·29	14·2 ± 0·20	15·6 ± 0·25	15·1 ± 0·36	14·4 ± 0·46
Females					
0	13·6 ± 0·16	13·7 ± 0·25	14·4 ± 0·21	14·6 ± 0·26	15·3 ± 0·34
100	14·4 ± 0·42	14·5 ± 0·10*	15·1 ± 0·15*	13·8 ± 0·53	14·7 ± 0·81
235	14·3 ± 0·26*	14·0 ± 0·17	15·2 ± 0·22*	14·6 ± 0·29	15·0 ± 0·33
750	14·6 ± 0·26*	14·3 ± 0·25	15·3 ± 0·18*	14·7 ± 0·16	14·8 ± 0·30
1500	14·8 ± 0·27*	14·5 ± 0·15*	15·3 ± 0·09*	14·4 ± 0·51	15·0 ± 0·48
Haematocrit (%)					
Males					
0	49·8 ± 1·28	49·7 ± 0·86	46·6 ± 2·82	49·5 ± 0·50	48·8 ± 1·19
100	51·3 ± 1·13	50·3 ± 0·68	51·0 ± 0·77	49·3 ± 1·29	47·9 ± 3·41
235	50·5 ± 1·22	51·8 ± 0·95	52·1 ± 0·72	51·0 ± 1·43	49·1 ± 1·47
750	51·4 ± 1·23	50·9 ± 0·93	51·1 ± 0·53	49·6 ± 0·70	46·1 ± 1·47
1500	50·5 ± 1·16	47·6 ± 1·06	49·8 ± 1·26	50·6 ± 1·12	44·9 ± 2·21
Females					
0	46·4 ± 0·76	46·8 ± 0·92	45·3 ± 0·96	46·2 ± 0·83	45·9 ± 1·43
100	46·9 ± 0·92	48·4 ± 0·70	46·7 ± 1·18	45·0 ± 1·32	44·1 ± 1·83
235	49·6 ± 0·96*	48·9 ± 0·66	48·8 ± 0·68*	47·7 ± 1·20	45·6 ± 0·63
750	48·1 ± 1·24	46·6 ± 1·16	48·8 ± 0·57*	47·1 ± 0·41	46·7 ± 0·97
1500	49·5 ± 0·96*	45·4 ± 1·05	48·0 ± 0·52*	46·6 ± 1·23	48·5 ± 1·01

†Before start of treatment.

Values are means ± SEM for groups of ten rats (between six and ten at 18 months). Those marked with an asterisk are significantly ($P < 0·05$) higher [*] or lower [(*)] than the control value.

Table 3. *Coagulation times and reticulocyte counts in rats fed 0–4% erythrosine in the diet*

Sex and dietary level (%)	Values at month				
	0†	3	6	12	18
	Coagulation time (min)				
Males					
0	2·41 ± 0·09 (1·85–2·84)	2·46 ± 0·15 (1·58–3·05)	1·96 ± 0·14 (1·30–2·57)	2·33 ± 0·09 (2·00–2·87)	
0·5	2·39 ± 0·14 (1·30–3·02)	2·38 ± 0·15 (1·72–3·17)	2·07 ± 0·08 (1·67–2·42)	2·72 ± 0·11* (2·15–3·28)	
1·0	2·21 ± 0·11 (1·57–2·62)	2·33 ± 0·18 (1·55–2·97)	2·41 ± 0·16* (1·58–3·30)	3·00 ± 0·17* (2·25–3·88)	
2·0	2·57 ± 0·15 (1·95–3·30)	2·48 ± 0·13 (1·45–2·99)	2·59 ± 0·15* (1·77–3·17)	2·70 ± 0·14* (1·92–3·45)	
4·0	2·25 ± 0·09 (1·72–2·77)	2·33 ± 0·14 (1·49–2·93)	1·96 ± 0·11 (1·50–2·58)	2·79 ± 0·13* (2·25–3·75)	
Females					
0	2·40 ± 0·08 (2·07–2·87)	2·48 ± 0·15 (1·67–3·10)	1·97 ± 0·10 (1·33–2·43)	2·78 ± 0·13 (2·18–3·50)	
0·5	2·04 ± 0·13(*) (1·02–2·43)	2·31 ± 0·24 (1·30–3·73)	2·25 ± 0·10 (1·83–2·83)	2·51 ± 0·13 (1·88–3·07)	
1·0	2·15 ± 0·13 (1·33–2·75)	2·08 ± 0·14 (1·42–2·65)	2·25 ± 0·15 (1·50–2·92)	2·52 ± 0·13 (1·87–3·12)	
2·0	2·30 ± 0·08 (1·97–2·75)	2·37 ± 0·15 (1·82–3·20)	2·36 ± 0·20 (1·25–3·42)	2·66 ± 0·14 (1·75–3·33)	
4·0	2·09 ± 0·12 (1·42–2·44)	2·22 ± 0·20 (1·30–3·47)	2·01 ± 0·12 (1·42–2·53)	2·47 ± 0·15 (1·83–3·20)	
	Reticulocyte count (% of red blood cells)				
Males					
0	0·17 ± 0·07 (0·00–0·63)	0·94 ± 0·19 (0·08–1·77)	0·32 ± 0·09 (0·00–0·95)	0·86 ± 0·21 (0·00–2·53)	2·95 ± 1·17 (0·43–11·00)
0·5	0·48 ± 0·23 (0·00–2·03)	0·23 ± 0·08(*) (0·00–0·73)	0·72*‡ (0·00–0·72)	3·94 ± 3·05 (0·26–31·33)	4·24 ± 1·92 (0·00–18·9)
1·0	0·33 ± 0·13 (0·00–1·23)	0·57 ± 0·12 (0·00–1·16)	0·52 ± 0·13 (0·00–1·08)	0·55 ± 0·15 (0·13–1·81)	1·43 ± 0·58 (0·00–6·43)
2·0	0·19 ± 0·07 (0·00–0·57)	0·60 ± 0·25 (0·00–1·82)	0·76 ± 0·13* (0·24–1·31)	0·28 ± 0·13(*) (0·00–1·23)	0·61 ± 0·17(*) (0·00–1·56)
4·0	1·44 ± 1·35 (0·00–13·58)	0·53(*)‡ (0·00–0·53)	0·41 ± 0·10 (0·00–1·07)	0·34 ± 0·19(*) (0·00–1·92)	0·41 ± 0·16(*) (0·00–1·54)
Females					
0	0·73 ± 0·39 (0·00–3·76)	1·06 ± 0·19 (0·17–1·91)	0·38 ± 0·14 (0·00–1·30)	1·19 ± 0·28 (0·55–3·54)	2·67 ± 0·78 (0·34–7·00)
0·5	0·37 ± 0·10 (0·00–0·83)	0·38 ± 0·08(*) (0·14–0·83)	0·40 ± 0·19 (0·00–1·85)	0·55 ± 0·12(*) (0·00–1·32)	2·76 ± 1·69 (0·00–17·82)
1·0	0·20 ± 0·13 (0·00–1·20)	0·78 ± 0·16 (0·10–1·85)	0·78 ± 0·15 (0·00–1·40)	2·67 ± 1·93 (0·00–20·03)	1·17 ± 0·32 (0·00–3·47)
2·0	0·14 ± 0·05 (0·00–0·39)	0·32 ± 0·15(*) (0·00–1·13)	1·03 ± 0·21* (0·30–2·39)	0·50 ± 0·17(*) (0·00–1·31)	0·85 ± 0·28(*) (0·00–3·13)
4·0	0·58 ± 0·20 (0·00–1·94)	0·09 ± 0·06(*) (0·00–0·52)	0·56 ± 0·12 (0·00–1·46)	0·33 ± 0·12(*) (0·00–1·06)	1·92 ± 1·18 (0·00–11·88)

†Before start of treatment.

‡Reticulocyte count in only one of ten males.

Values are means ± SEM for groups of ten rats, with ranges in parentheses. Those marked with an asterisk are significantly ($P < 0·05$) higher [*] or lower [(*)] than the control value.

Table 4. *Coagulation times and reticulocyte counts in rats given erythrosine by intubation twice weekly*

Sex and dose level (mg/kg)	Values at month				
	0†	3	6	12	18
	Coagulation time (min)				
Males					
0	2·47 ± 0·11 (1·85–2·92)	2·38 ± 0·17 (1·58–3·05)	2·21 ± 0·11 (1·38–2·55)	2·58 ± 0·15 (1·70–3·23)	
100	2·47 ± 0·13 (1·94–3·12)	2·60 ± 0·19 (1·43–3·38)	2·01 ± 0·11(*) (1·66–2·80)	2·87 ± 0·13 (2·25–3·50)	
235	2·34 ± 0·13 (1·72–2·90)	2·29 ± 0·23 (1·41–4·07)	2·05 ± 0·07 (1·63–2·36)	2·52 ± 0·13 (1·66–2·91)	
750	2·42 ± 0·15 (1·65–3·08)	2·34 ± 0·09 (1·85–2·78)	2·20 ± 0·07 (1·96–2·58)	1·98 ± 0·39 (1·08–2·83)	
1500	2·78 ± 0·17 (2·01–3·56)	2·25 ± 0·09 (1·76–2·61)	2·12 ± 0·12 (1·33–2·50)	2·25 ± 0·23 (1·03–3·38)	
Females					
0	2·40 ± 0·10 (1·84–2·80)	2·73 ± 0·16 (2·17–3·57)	2·14 ± 0·11 (1·50–2·70)	2·45 ± 0·18 (1·45–3·30)	
100	2·13 ± 0·11 (1·53–2·77)	2·47 ± 0·18 (1·72–3·42)	1·97 ± 0·08 (1·53–2·38)	2·50 ± 0·14 (1·50–3·00)	
235	2·63 ± 0·08 (2·30–3·03)	2·01 ± 0·07(*) (1·43–2·43)	2·06 ± 0·15 (1·45–3·08)	2·24 ± 0·15 (1·25–3·13)	
750	2·42 ± 0·10 (1·67–2·73)	2·32 ± 0·13 (1·65–3·10)	2·09 ± 0·07 (1·66–2·36)	2·19 ± 0·14 (1·20–2·80)	
1500	2·33 ± 0·14 (1·92–3·23)	2·11 ± 0·08(*) (1·56–2·46)	2·02 ± 0·11 (1·40–2·50)	2·05 ± 0·16 (1·45–2·98)	
	Reticulocyte count (% of red blood cells)				
Males					
0	0·32 ± 0·16 (0·00–1·30)	0·21 ± 0·10 (0·00–0·92)	4·30 ± 3·42 (0·16–35·15)	0·47 ± 0·21 (0·00–1·66)	0·37‡ (0·00–0·37)
100	0·28 ± 0·13 (0·00–1·30)	0·06 ± 0·04 (0·00–0·33)	0·70 ± 0·15 (0·00–1·51)	0·48 ± 0·21 (0·00–2·08)	1·36 ± 0·72* (0·00–7·69)
235	0·45 ± 0·13 (0·00–1·00)	0·07 ± 0·05 (0·00–0·49)	0·56 ± 0·15 (0·09–1·37)	0·33 ± 0·16 (0·00–1·59)	0·39 ± 0·09 (0·00–0·70)
750	0·61 ± 0·28 (0·00–2·84)	0·04 ± 0·03 (0·00–0·27)	0·81 ± 0·21 (0·00–2·37)	0·88 ± 0·33 (0·00–3·59)	1·03 ± 0·39 (0·00–2·78)
1500	0·71 ± 0·19* (0·00–1·76)	0·48 ± 0·62 (0·00–1·58)	1·07 ± 0·78 (0·37–2·49)	1·05 ± 0·23* (0·19–2·10)	0·81 ± 0·23* (0·00–2·04)
Females					
0	0·47 ± 0·17 (0·00–1·30)	0·37 ± 0·21 (0·00–2·03)	0·90 ± 0·21 (0·22–2·38)	0·77 ± 0·20 (0·00–2·01)	0·06 ± 0·05 (0·00–0·46)
100	0·76 ± 0·24 (0·11–2·30)	0·15 ± 0·07 (0·00–0·49)	1·06 ± 0·15 (0·49–2·08)	9·56 ± 8·87 (0·00–89·39)	0·94 ± 0·26* (0·19–2·47)
235	0·85 ± 0·22 (0·00–2·40)	0·08 ± 0·04 (0·00–0·25)	0·82 ± 0·21 (0·00–2·34)	0·54 ± 0·19 (0·00–1·39)	0·80 ± 0·20* (0·17–1·73)
750	0·63 ± 0·21 (0·00–2·36)	0·17 ± 0·08 (0·00–0·78)	0·91 ± 0·14 (0·40–1·69)	0·91 ± 0·21 (0·00–2·24)	0·75 ± 0·16* (0·00–1·36)
1500	0·63 ± 0·16 (0·00–1·38)	0·31 ± 0·10 (0·00–0·87)	1·15 ± 0·19 (0·10–2·20)	1·31 ± 0·24 (0·00–2·53)	0·90 ± 0·35* (0·00–3·48)

†Before start of treatment.
‡Reticulocyte count in only one of ten males.
Values are means ± SEM for groups of ten rats with ranges in parentheses. Those marked with an asterisk are significantly ($P < 0·05$) higher [*] or lower [(*)] than the control value.

Table 5. *Erythrosine, thyroxine (T_4) and PBI in the blood of rats treated with erythrosine*

Dose level	Blood levels of: Erythrosine at *c.* 62 wk* (μg/100 ml)	PBI (μg/100 ml): At *c.* 62 wk*	PBI (μg/100 ml): After treatment†	T_4 at 79–83 wk‡ (μg/100 ml)
0§	1·5 ± 0·38	2·4 ± 0·21		
0	2·2 ± 0·91	3·1 ± 0·23	1·7 ± 0·08	4·4 ± 0·25
In diet (%)				
0·5	405 ± 62·5	257 ± 36·0	2·6 ± 0·45	4·5 ± 0·33
4·0	131 ± 21·9	161 ± 20·7	2·3 ± 0·11	4·9 ± 0·51
By intubation (mg/kg)				
100			2·7 ± 0·22	3·7 ± 0·20
1500			1·7 ± 0·15	4·2 ± 0·31

*Values are means ± SEM of 3–9 samples, with duplicate determinations on each.
†Rats were given erythrosine for 85–86 wk and then fed the control diet for the following 16 wk. Values are means ± SEM for 6–10 animals.
‡Values are means ± SEM for 6–13 animals.
§These control rats were from a colony kept in a room distant from those fed the erythrosine diets.

Slight caecal distension occurred in some rats but was not as pronounced as that reported in the previous study by Hansen *et al.* (1973). The rats in the present study received the dye for 85 or 86 wk and treatment was withheld for the rest of the 2 yr, whereas those in the previous study were fed the diet for the entire 2 yr. This may account for the different results.

Table 6. *Summary of pathological data for rats given erythrosine by stomach tube or in the diet*

Parameter	Data for rats given erythrosine: By stomach tube in twice-weekly doses (mg/kg) of: 0	100	235	750	1500	In the diet at a level (%) of: 0·0	0·0	0·5	1·0	2·0	4·0
Nos of rats/group											
At start of test	50	50	50	50	50	50	50	50	50	50	50
Surviving at 1 yr	40	45	47	46	43	42	44	45	46	47	47
at wk 78	22	29	20	29	23	28	37	37	36	37	43
Killed at 2 yr	2	2	7	8	4	6	8	7	11	10	5
Discarded	1	1	3	4	7	5	6	2	9	5	5
Examined, including heads*	38	37	25	42	29	33	31	37	34	38	40
Pathological lesions											
Pituitary adenoma	11	8	3	8	1	4	9	3	7	7	6
Pituitary adenocarcinoma	0	3	0	0	0	2	0	1	0	0	0
Meningioma	0	0	0	0	0	0	0	0	1	0	0
Lymphosarcoma	0	6	3	2	1	7	5	3	4	5	5
Myelogenous leukaemia	0	0	0	0	0	1	1	0	0	0	0
Fibrosarcoma	4	0	1	1	2	1	0	0	1	0	1

*Entire animals examined. Examination of other rats not discarded was limited to the trunk and limbs.

Table 6.—*Continued.*

Parameter	Data for rats given erythrosine										
	By stomach tube in twice-weekly doses (mg/kg) of					In the diet at a level (%) of					
	0	100	235	750	1500	0·0	0·0	0·5	1·0	2·0	4.0
Pathological lesions—*continued*											
Fibroma	0	1	1	0	0	0	0	0	0	0	0
Epidermoid or squamous carcinoma	1	0	0	2	1	0	1	0	1	0	0
Mammary fibroadenoma	5	2	9	2	4	6	6	9	4	8	2
Mammary adenocarcinoma	0	2	0	4	0	1	0	0	2	1	1
Salivary gland adenocarcinoma	0	0	0	0	0	0	0	0	0	0	1
Lipoma	0	0	1	0	0	0	0	0	0	0	0
Liposarcoma	0	1	0	0	0	0	0	0	0	0	0
Haemangiosarcoma	0	1	0	0	0	0	0	1	0	0	0
Mesothelioma	0	0	1	0	0	0	1	1	0	1	0
Thyroid adenoma	0	0	0	0	1	0	1	0	1	0	0
Parathyroid adenoma	0	0	0	0	0	0	0	0	0	1	0
Adrenal adenoma	0	0	0	0	0	0	0	0	1	1	0
Hepatoma	0	0	0	0	0	0	0	0	0	1	0
Interstitial cell tumour of testis	0	0	1	0	1	1	1	0	0	0	1
Testicular atrophy	3	3	3	1	1	3	3	2	1	4	4
Preputial gland adenocarcinoma	0	0	0	0	0	0	0	0	0	0	1
Prostatitis	0	0	0	0	0	0	2	0	0	0	0
Ovarian adenoma	0	0	0	0	0	0	0	1	0	0	0
Haemangiopericytoma of ovarian bursa	0	0	0	0	0	0	0	0	0	1	0
Endometrial sarcoma	0	0	0	0	0	0	1	0	0	1	2
Endometrial adenocarcinoma	0	0	0	0	0	0	0	0	0	0	1
Endometrial polyp	0	0	0	0	0	4	0	0	3	2	0
Uterine polyp	0	2	1	3	0	0	0	0	0	0	0
Papillary carcinoma of caecum	0	0	1	0	0	0	0	0	0	0	0
Intestinal leiomyosarcoma	0	0	0	0	0	0	0	0	0	1	0
Abdominal adenocarcinoma	0	0	0	0	0	0	0	0	0	0	1
Pneumonia	29	34	38	32	26	27	30	36	24	24	26
Peribronchial lymphocytic cuffing	5	5	5	4	4	2	8	5	4	6	3
Myocardial fibrosis	6	1	0	1	7	5	6	0	3	1	4
Ventricular thrombosis	0	0	0	0	0	0	1	0	0	0	0
Atrial thrombosis	0	1	0	1	0	0	0	0	0	0	0
Pericarditis	2	0	0	0	0	0	0	0	0	0	0
Mesenteric arteritis	0	1	0	0	0	0	0	0	0	0	0
Hepatic necrosis/hepatitis	0	0	0	0	0	2	0	2	2	0	0
Cystic degeneration of liver	0	0	0	0	0	0	0	1	0	0	0
Nephritis/nephrosis	5	7	5	5	6	6	6	4	5	8	4
Foot-pad granuloma	3	6	4	6	6	7	8	16	7	5	6
Abscess	3	6	2	0	2	2	0	5	1	1	4
Ear granuloma	0	0	0	1	0	0	0	0	0	0	0
Peritonitis	0	0	0	0	0	0	0	0	1	0	0
Dermatitis	0	0	0	0	0	0	0	1	0	0	0
Cataract	5	3	1	0	1	0	4	2	3	2	6
Middle-ear infection†	2	1	0	2	1	8	18	28	20	18	15
Trichosomoides sp. parasites	1	0	3	0	1	0	1	1	1	7	1
Ectoparasites (mites)	3	2	1	3	1	3	5	5	4	5	6
Rats with benign tumour(s) only	10	6	12	6	6	7	12	10	8	12	8
Rats with malignant tumour(s)	5	12	6	9	4	11	9	6	8	9	12

†Gross diagnosis, tissue not sectioned.

Table 6 summarizes the pathology observed in rats receiving erythrosine by stomach tube and in the diet. It should be noted that the 100 control animals in the feeding study were considered as two separate groups for pathology. The animals were treated identically and show the random distribution of tumours that occur in ageing control rats.

The lesions seen in both studies were considered to be unrelated to the administration of erythrosine. No effect on thyroid morphology was observed.

DISCUSSION

Andersen, Keiding & Nielsen (1964) reported that drugs and foods coloured red cause a false elevation of serum PBI and Skanse (1962) has also shown that PBI levels can be influenced by the administration of a variety of iodine-containing substances. Under certain circumstances, erythrosine can be degraded to fluorescein with the liberation of iodine (Dickinson & Raven, 1962).

Danowski & Greenman (1949) have drawn attention to the fact that high concentrations of inorganic iodide in the blood can produce an increase in PBI levels that is unconnected with any effect on or by the thyroid. In addition, Saxena, Chapman & Pryles (1962) reported that no toxic effects occurred when 100 mg iodide was administered daily to children over a course of years. Andersen *et al.* (1964) gave human subjects erythrosine in daily doses of 16 mg for 10 days and found an almost twofold increase in PBI levels; values reached a peak at 15–20 days and then declined sharply during the following 10 days. These subjects required almost 3 months for the PBI to return to the initial value. In our studies, the elevated PBI values were attributed to erythrosine circulating in the blood serum and the thyroxine–iodine levels were not affected by the administration of erythrosine. Our results do not confirm the previous report of Bowie *et al.* (1966) that the administration of erythrosine produces anaemia in rats.

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Etude de la toxicité à long terme de l'érythrosine. II. Effets sur l'hématologie et sur l'iode lié à la thyroxine et aux protéines chez le rat

Résumé—On a administré de l'érythrosine (FD & C Red No. 3) à des rats Osborne–Mendel, mâles et femelles, soit par intubations bihebdomadaires répétées pendant 85 semaines, soit par addition continue à la nourriture pendant 86 semaines. On n'a pas observé de différences cohérentes dans les numérations érythrocytaires, l'hématocrite et les nombres de réticulocytes ni pour d'autres indices d'anémie. L'augmentation des taux d'iode fixé aux protéines a été attribuée à l'érythrosine circulant dans le sérum sanguin; ces taux étaient redevenus normaux 16 semaines après l'arrêt de l'administration d'érythrosine. Les taux d'iode de la thyroxine n'ont pas été influencés chez les rats traités. Aucun indice pathologique macroscopique ou microscopique n'a été imputé à l'administration du colorant.

Langzeit-Toxizitätsuntersuchungen von Erythrosin. II. Einflüsse auf die Hämatologie und auf thyroxin- und proteingebundenes Jod bei Ratten

Zusammenfassung—Erythrosin (FD & C Red No. 3) wurde männlichen und weiblichen Osborne–Mendel-Ratten entweder 85 Wochen lang zweimal wöchentlich mit der Schlundsonde oder 86 Wochen lang dauernd in der Nahrung verabreicht. Es wurden keine konsistenten Unterschiede hinsichtlich der Zahl der roten Blutkörperchen, des Hämatokritwertes, des Hämoglobins, der Reticulocytenzahlen oder anderer Anzeichen von Anämie beobachtet. Erhöhte Werte proteingebundenen Jods wurden dem zirkulierendem Erythrosin im Blutserum zugeschrieben; diese Werte kehrten 16 Wochen nach Einstellung der Erythrosinanwendung zur Normalität zurück. Die Thyroxinjodkonzentrationen wurden bei den Ratten, die Erythrosin erhielten, nicht beeinflusst. Keine makro- oder mikroskopische Pathologie wurde der Anwendung dieses Farbstoffs zugeschrieben.

Fd Cosmet. Toxicol. Vol. 11, pp. 547–553. Pergamon Press 1973. Printed in Great Britain

Modification of the Acute Toxicity of Mutagenic and Carcinogenic Chemicals in the Mouse by Prefeeding with Antioxidants

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Abstract—Butylated hydroxytoluene (BHT) fed to male mice for 4 wk gave significant protection against mortality caused by ethyl methanesulphonate (EMS), *n*-propyl or isopropyl methanesulphonate, ethylene dibromide, diethylnitrosamine and cyclophosphamide. It did not protect male mice against X-rays, methyl methanesulphonate (MMS), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine or dipropylnitrosamine, but female mice were protected from the lethal effects of MMS. Other agents, such as butylated hydroxyanisole, 1,2-dihydro-6-ethoxy-2,2,4-trimethyl quinoline and sodium phenobarbitone also gave protection against EMS toxicity. The protection, when it occurred, may have been due to the induction of drug-metabolizing enzymes.

INTRODUCTION

Antioxidants are commonly added to human and animal food as preservatives for unsaturated lipids and other materials subject to spoilage by oxidation. There is increasing evidence that many of these compounds produce a variety of physiological effects in mammals, but the data are not yet sufficient to indicate whether the long-term consequences of ingesting particular antioxidants are good, bad or insignificant.

Consequences observed in animals fed antioxidants include a reduction in growth rate and body weight (Brown, Johnson & O'Halloran, 1959; Deichmann, Clemmer, Rakoczy & Bianchine, 1955; Frawley, Kohn, Kay & Calandra, 1965; Johnson & Hewgill, 1961), an increased rate of urinary excretion of ascorbic acid (Gaunt, Feuer, Fairweather & Gilbert, 1965; Gaunt, Gilbert & Martin, 1965), elevated serum cholesterol (Day, Johnson, O'Halloran & Schwartz, 1959; Frawley *et al.* 1965; Gaunt *et al.* 1965; Johnson & Hewgill, 1961), and a hyperfunctional enlargement of the liver with concomitant increases in the amount of smooth endoplasmic reticulum and in the mitotic activity of the hepatocytes (Botham, Conning, Hayes, Litchfield & McElligott, 1970; Brown *et al.* 1959; Deichmann *et al.* 1955; Feuer, Gaunt, Golberg & Fairweather, 1965; Gilbert & Golberg, 1965; Kerr, Lefevre, Lane & Lieber, 1966; Lane & Lieber, 1967). Along with liver hypertrophy there is an increase in the activity levels of at least seven liver enzymes (Creaven, Davies & Williams, 1966; Gilbert & Golberg, 1967; Gilbert, Martin, Gangolli, Abraham & Golberg, 1969; Magour & Nievel, 1971) and possibly of many others. A decrease in three hepatic enzyme activities has also been reported (Feuer *et al.* 1965; Nievel, 1969).

It has also been claimed that the feeding of two of the compounds discussed below increases the mean lifespan of mice (Comfort, Youhotsky-Gore & Pathmanathan, 1971; Harman, 1957, 1961, 1968a,b; Kohn, 1971).

This report is concerned with the relationship of antioxidant pretreatment to the acute lethal effects of radiation and of several mutagenic and carcinogenic compounds.

EXPERIMENTAL

Animals, diets and pretreatments. These experiments utilized (C3H × 101)F_1 hybrid (C31F_1) mice, which were 10–12 wk old at the time of treatment. In the main experiment, pretreatment consisted of feeding the mice for approximately 30 days *ad lib.* with pelleted Purina Lab Chow (Ralston Purina Company, St. Louis, Mo.) containing 0·75% (w/w) butylated hydroxytoluene (BHT). Mice from alternate pens of breeding production were kept on regular Purina Lab Chow but were otherwise handled similarly to the pretreated mice and served as controls. The effect of feeding this BHT diet for shorter periods was also studied. In some of the earlier experiments, both experimental and control groups of animals were fed Purina Lab Chow in meal (powdered) form. In these cases, meal was presented *ad lib.* in meal feeders, and the meal for the experimental groups was mixed with 1% BHT or butylated hydroxyanisole (BHA). Other pretreatments studied were BHA or 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline (ethoxyquin; DEQ) in olive oil given by stomach-tube in a dose of 500 mg/kg/day on five consecutive days, or four daily ip injections of sodium phenobarbitone in doses of 50–100 mg/kg dissolved in Hanks' balanced salt solution. In each case, controls were given the vehicle only, in the same number of doses and by the same route. The single dose of the test substance was administered 24 hr after completion of the pretreatment.

Experimental procedures. X-rays were given at a dose rate of 78 R/min with a G.E. Maximar 250 kVp machine. Of the mutagenic and carcinogenic chemicals studied, the methyl, ethyl, *n*-propyl and isopropyl methanesulphonates (MMS, EMS, PMS and IMS, respectively), diethylnitrosamine (DEN), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and cyclophosphamide (cytoxan; CYC) were dissolved in Hanks' balanced salt solution, and ethylene dibromide (EDB) and dipropylnitrosamine (DPN) in olive oil. All were injected ip. Control animals were injected with an equal volume of either Hanks' solution or olive oil.

Materials. EMS, MMS, EDB, DEN and DPN were obtained from Eastman Organic Chemicals, Rochester, N.Y. PMS, IMS and MNNG were obtained from Koch-Light Laboratories Ltd., Colnbrook, England. CYC was supplied by Mead Johnson & Co., Evansville, Ind., and DEQ by the Monsanto Co., St. Louis, Mo.

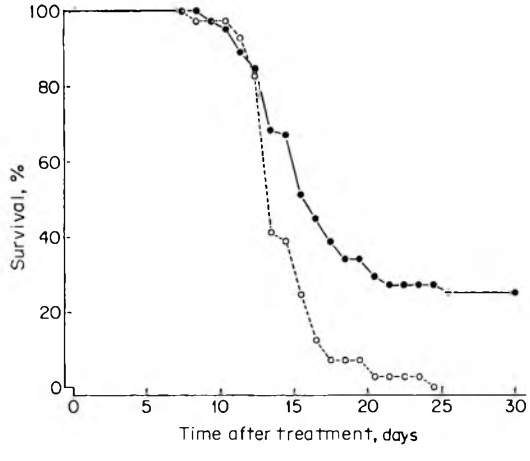

FIG. 1. Survival rate after whole-body X-ray treatment (725 R) in C31F_1 male mice fed Purina Lab Chow meal containing 1% BHT for 28 days before X-ray treatment (pretreated group; ○) or fed the same meal without added BHT (control group; ●).

RESULTS

The end point of these experiments was 30-day survival, though other differences in relative toxicity in the experimental and control groups, such as general condition and mean body weight, were sometimes apparent.

Prefeeding with 1% BHT in meal for 30 days offered no measurable protection from

Table 1. *The effect of prefeeding 0·75% BHT in pelleted food on 30-day survival of mice after treatment with various toxic agents*

Treatment				No. of mice		
Agent	Dose (mg/kg)	Sex	Group†	Treated	Surviving at 30 days	Survival (%)
EMS	525	M	P	32	28	87·5**
			C	31	0	0·0
EMS	425	F	P	48	48	100·0**
			C	48	7	14·6
MMS	175	M	P	48	14	29·2
			C	49	15	30·6
MMS	185	M	P	39	3	7·7
			C	40	1	2·5
MMS	175	F	P	38	29	76·3**
			C	40	8	20·0
MMS	185	F	P	22	13	59·1**
			C	24	3	12·5
PMS	900	M	P	42	40	95·2**
			C	48	5	10·4
PMS	900	M	P	56	48	85·7**
			C	56	3	5·4
IMS	300	M	P	42	37	88·1**
			C	32	0	0·0
IMS	325	M	P	50	30	60·0**
			C	54	5	9·3
EDB	200	M	P	47	11	23·4*
			C	47	3	6·4
MNNG	125	M	P	60	5	8·3
			C	59	3	5·1
MNNG	150	M	P	44	0	0·0
			C	48	4	8·3
DEN	150	M	P	24	24	100·0**
			C	24	12	50·0
DEN	175	M	P	24	24	100·0**
			C	24	0	0·0
DEN	200	M	P	33	21	63·6**
			C	35	0	0·0
DPN	600	M	P	22	5	22·7
			C	22	3	13·6
CYC	550	M	P	32	18	56·2**
			C	32	0	0·0

EMS, MMS, PMS and IMS = Ethyl, methyl, *n*-propyl and isopropyl methanesulphonates, respectively
EDB = Ethylene dibromide MNNG = *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine
DEN and DPN = Diethyl and dipropyl nitrosamines CYC = Cyclophosphamide

†Each experiment involved a pretreated group and a matched control group, marked "P" and "C" respectively in this table.

Asterisks indicate that the value for a pretreated group differs significantly from the corresponding control value: $*P < 0{\cdot}05$; $**P < 0{\cdot}001$.

acute mortality due to X-rays; on the contrary, there was evidence that this pretreatment potentiated the X-ray damage (Fig. 1). A dose of 725 R of X-rays caused 100% mortality in the group prefed with BHT, while in the control group (treated with X-rays only) approximately 25% of the animals survived for longer than 30 days. The difference was statistically significant ($P < 0{\cdot}001$).

Prefeeding with 0·75% BHT in the diet did, however, dramatically reduce the 30-day mortality caused by a number of alkylating chemicals. $C31F_1$ males were protected against the lethal effects of EMS, PMS, IMS, EDB, DEN and CYC (Table 1). Males of this strain were not protected from the effects of MMS, MNNG and DPN. Females were protected from EMS and in contrast to the males, from MMS.

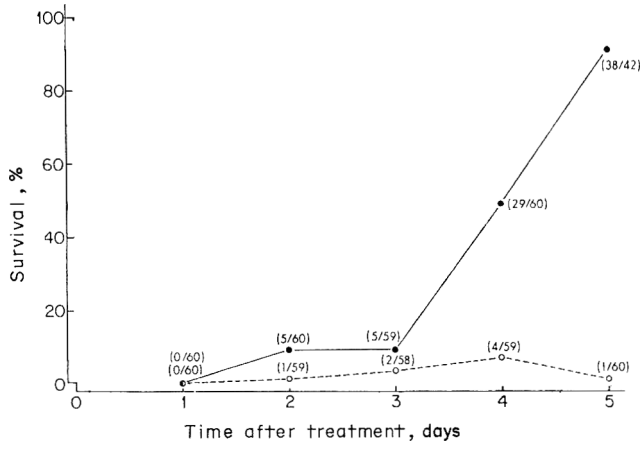

FIG. 2. Survival 30 days after treatment with 525 mg ethyl methanesulphonate/kg in groups of $C31F_1$ male mice previously fed for different periods of time on Purina Lab Chow pellets containing 0·75% BHT (●) or on the standard diet (control group; ○). Numbers in parentheses give the survivors and number treated for each group.

The protective action of BHT pretreatment against the lethal effects of EMS was not effective from the start of feeding. In an experiment in which groups of animals were fed 0·75% BHT for different periods before receiving a dose of 525 mg EMS/kg, no protection was noted after 24 hr of feeding (Fig. 2). The protection, if any, was minimal on days 2 and 3, rose dramatically on day 4, and approached a maximum on day 5. As noted previously (Table 1) the protective effect of BHT remained high throughout at least 30 days of feeding.

Several other pretreatments, besides 0·75% BHT pelleted in food, were effective in protecting against mortality in mice treated with EMS. Other effective antioxidant treatments included BHT or BHA fed at a level of 1% in meal and BHA or DEQ given in olive oil by stomach tube (Table 2). Sodium phenobarbitone protected in doses of 75 and 100 mg/kg/day, but not in doses of 50 mg/kg/day (Table 2). EMS given in repeated doses of 25 mg/kg/day as a pretreatment did not protect against a subsequent acute dose of EMS (525 mg/kg).

DISCUSSION

Several observations suggest that the protective effect of BHT prefeeding is probably related to enzyme induction in the liver microsomes. First, BHT is known to be a strong enzyme inducer (Creaven *et al.* 1966; Gilbert & Golberg, 1965 & 1967; Gilbert *et al.* 1969).

Table 2. *The effects of various pretreatments on the 30-day survival of C31F$_1$ male mice after administration of 525 mg EMS/kg*

Preteatment		No. of mice		
Agent	Dose	Treated	Surviving at 30 days	Survival (%)
Phen	4 × 50†	24	2	8·3
Control	—	27	0	0·0
Phen	4 × 75†	24	9	37·5**
Control	—	27	0	0·0
Phen	4 × 100†	34	14	41·2**
Control	—	33	0	0·0
BHT	1%/meal‡	40	38	95·0**
Control	—	35	0	0·0
BHA	1%/meal‡	32	25	78·1**
Control	—	32	0	0·0
DEQ	5 × 500§	20	18	90·0**
BHA	5 × 500§	16	10	62·5**
Control	—	18	0	0·0
EMS	4 × 25†	31	0	0·0
Control	—	29	0	0·0

Phen = Sodium phenobarbitone BHT = Butylated hydroxytoluene
BHA = Butylated hydroxyanisole DEQ = Ethoxyquin
EMS = Ethyl methanesulphonate

†Four ip doses (mg/kg/day) injected on consecutive days.
‡Fed *ad lib.* at a level of 1% (w/w) in Purina Lab Chow in meal form for 30 days.
§Five doses (mg/kg/day) in olive oil given by stomach-tube on consecutive days.
Asterisks indicate that the value for a pretreated group differs significantly from the corresponding control value: $^{**}P < 0{\cdot}001$.

Magour & Nievel (1971) have shown that, for one of the compounds tested in these experiments (DEN), BHT induces a de-ethylating enzyme in the liver microsomes of the rat and that this enzyme activity is inversely correlated with DEN toxicity. Secondly, other general enzyme inducers which are not antioxidants, such as phenobarbitone, confer similar protection against EMS toxicity. Thirdly, the induction of protection is correlated in time (Fig. 2) with the induction of some enzymes and not with BHT tissue levels (Gilbert & Golberg, 1965). And finally, the specificity of the protective effect, seen for example in the great differences between reactions to closely related agents such as EMS and MMS, suggests an enzymatic mechanism.

The relationship of the results reported here to carcinogenesis and mutagenesis needs to be further clarified. We have shown (R.B. Cumming and Marva F. Walton, report in preparation, 1973) that when the toxic properties of EMS are reduced by feeding with BHT, the mutagenic capacity of this same compound is likewise reduced. It is well known that certain general enzyme inducers will disarm specific carcinogens with respect to their capacity to induce tumours (Meechan, McCafferty & Jones, 1953; Miller, Miller, Brown & McDonald, 1958; Miyaji, Moskowski, Senoo, Ogata, Odo, Kawai, Sayama, Ishida & Matsuo, 1953; Tawfic, 1965). However, it is also well documented that enzyme induction can potentiate the effects of some agents (Kato, 1961; Murphy & DuBois, 1958), so caution should be used in suggesting the use of enzyme induction to counteract toxic agents.

The possible relationship of antioxidant feeding to the increase in lifespan in laboratory

rodents, as reported by Harman (1957, 1961 & 1968a,b), Comfort *et al.* (1971) and Kohn (1971), remains obscure. The evidence available suggests that any such effect is due to some factor other than inhibition of free radicals. We have found no protective effect against agents such as X-rays, whose damage may be partially due to free-radical reactions, but great protection is conferred against damage by agents, such as EMS, whose action does not involve free radicals. Feeding with antioxidants at levels that are effective in increasing the mean lifespan does produce a variety of physiological effects. Since restriction in body weight and specific liver-enzyme patterns have already been correlated with increased lifespan in laboratory animals (Ross, 1969), free-radical inhibition need not be invoked to explain the life-lengthening effects reported to date.

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Modification de la toxicité aiguë de produits chimiques mutagènes et carcinogènes chez la souris par la consommation préalable d'antioxydants

Résumé—Consommé pendant 4 semaines par des souris mâles, l'hydroxytoluène butylé (BHT) a protégé significativement ces animaux contre la mortalité imputable au méthanesulfonate d'éthyle (EMS), au méthanesulfonate de *n*-propyle ou d'isopropyle, au dibromure d'éthylène, à la diéthylnitrosamine et au cyclophosphamide. Il n'a pas protégé les souris mâles contre les rayons X, le méthanesulfonate de méthyle (MMS), la *N*-méthyl-*N'*-nitro-*N*-nitrosoguanidine et la dipropylnitrosamine, mais a protégé les souris femelles contre les effets létaux du MMS. D'autres produits, tels que l'hydroxyanisol butylé, la 1,2-dihydro-6-éthoxy-2,2'-triméthylquinoline et le phénobarbital sodique ont également conféré une protection contre la toxicité de l'EMS. On suppose que, dans les cas où elle s'est manifestée, la protection était due à l'induction d'enzymes qui métabolisaient les produits.

Modifikation der akuten Toxizität mutagener und carcinogener Chemikalien in der Maus durch vorangehende Verfütterung von Antioxydantien

Zusammenfassung—Butyliertes Hydroxytoluol (BHT), das 4 Wochen lang an männliche Mäuse verfüttert worden war, ergab einen signifikanten Schutz gegen die durch Äthylmethansulfonat (ÄMS), *n*-Propyl- oder Isopropylmethansulfonat, Äthylendibromid, Diäthylnitrosamin und Cyclophosphamid verursachte Mortalität. Es schützte nicht männliche Mäuse gegen Röntgenstrahlen, Methylmethansulfonat (MMS), *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidin und Dipropylnitrosamin, aber weibliche Mäuse wurden vor der letalen Wirkung von MMS geschützt. Andere Mittel wie butyliertes Hydroxyanisol, 1,2-Dihydro-6-äthoxy-2,2,4-trimethylchinolin und Natriumphenobarbiton schützen auch gegen die Toxizität von ÄMS. Die Schutzwirkung, falls sie eintrat, könnte auf die Induktion medikamentmetabolisierender Enzyme zurückzuführen gewesen sein.

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Studies on Carrageenan and Large-bowel Ulceration in Mammals

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Abstract—The administration of carrageenan in either native or degraded form produces ulceration in the large intestine of the guinea-pig and rabbit. Ulceration may be acute or chronic according to the dose given. Pre-ulcerative changes consist principally of accumulation of macrophages in the lamina propria and they are associated with the presence of carrageenan in the sub-epithelial tissues. None of the above changes were observed in the rat, hamster, squirrel monkey or ferret. Limited observations in man suggest that he is also resistant to this effect, indicating that carrageenan is unlikely to be involved in the aetiology of human ulcerative colitis.

INTRODUCTION

Carrageenan is a sulphated polygalactoside that occurs in many species of Rhodophyceae (Stoloff, 1959). For many years aqueous extracts have been obtained from seaweeds, particularly from *Chondrus crispus* and *Eucheuma spinosum* (Anderson, 1967; Rose, 1972). When obtained in this way it is known as native carrageenan and consists of a heterogeneous mixture of polyanions, which differ from one another principally in their proportions of galactose, anhydrogalactose and sulphated galactose units. They are divided broadly into κ- and λ-carrageenan on the basis of their solubility in potassium chloride solution (Anderson, 1967; Rees, 1972).

The molecular weight of native carrageenan is in the range 100,000–800,000 (Masson & Caines, 1954) and this form is used extensively in the food and toiletry industries as a gelling agent (Stoloff, 1959). Mild acid hydrolysis has been found to produce a partially degraded carrageenan of molecular weight *c.* 30,000 from *Eucheuma spinosum*, which has found use in some parts of the world as an antipeptic agent (Anderson, 1967).

The Joint FAO/WHO Expert Committee on Food Additives (1970) examined the data available prior to 1969 on the biological effects of carrageenan and recommended an unconditional acceptable daily intake of up to 50 mg/kg, equivalent to 3·5 g/day for an adult man. Some studies reported since that time have thrown doubt on the safety of native and degraded carrageenan. Marcus & Watt (1969) reported the occurrence of ulcerative lesions (histologically resembling those of ulcerative colitis) in the caecum, colon or rectum of rats, guinea-pigs and rabbits. Mice were said to develop minimal changes consisting of focal cellular infiltration of the colonic mucosa. More detailed descriptions of the lesions occurring in guinea-pigs and rabbits given degraded carrageenan for as little as 7–14 days

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have since been published by these authors (Watt & Marcus, 1969 & 1970a,b), but details of the changes in mice have not been made available. They found ulceration of the colon in four of 12 female rats which had received for 6 months a 5% aqueous solution of degraded carrageenan in place of drinking-water, their parents having received the same treatment during pregnancy. The relevance of these results is difficult to judge. Ulceration caused by a direct toxic effect of carrageenan on the gut mucosa cannot be differentiated from ulceration due to persistent diarrhoea. The latter could occur as an osmotic phenomenon, induced by many substances including carrageenans. Other workers have reported the production of caecal and colonic ulcers in guinea-pigs but have failed to confirm that rats and mice develop ulceration when fed on carrageenan (C. Shirlaw, personal communication 1970; Maillet, Bonfils & Lister, 1970). No colonic ulceration was found in mice and rats fed on native carrageenan, extracted from *Chondrus crispus* and *Gigartina mamillosa*, at dietary levels supplying up to 12·5 g carrageenan/kg/day (Nilson & Wagner, 1959) or in rats and mice given degraded carrageenan prepared from *Eucheuma spinosum* in gastrically intubated doses of up to 4 g/kg/day for 6 months (Maillet *et al.* 1970).

Since all reports of the oral administration of native or degraded carrageenan to guinea-pigs have described the occurrence of caecal ulceration, most of our observations were made on this species. These involved a study of the changes preceding ulceration as well as the role played by neomycin-sensitive intestinal flora in the development of the lesions. Additional experiments were carried out in order to determine whether the ulcerations in the guinea-pig were reversible and whether other species were susceptible to the development of ulceration by carrageenan.

EXPERIMENTAL

Animals and diet. White guinea-pigs and New Zealand white rabbits, obtained from commercial breeders, were fed *ad lib.* on SG1 diet (Joseph Rank Ltd., Robertsbridge, Sussex). SPF-derived albino Wistar rats and hamsters were fed on Spillers' Laboratory Small Animal Diet. Ferrets of mixed origin were fed on a mixture of powdered Spillers' Laboratory Small Animal Diet, Spratts ZF6 diet and milk in a proportion of 4:4:1, by weight). Squirrel monkeys of mixed origin were fed diet 41B (Joseph Rank Ltd.) and mixed fruits. Drinking-water was freely available to all species except when substituted by degraded carrageenan solutions.

Materials. Both native and degraded carrageenans were obtained from Laboratoires Glaxo, Paris. Native carrageenan was a fine cream-coloured powder prepared commercially from *Eucheuma spinosum* (moisture content, 6%; viscosity of a 0·5% aqueous solution at 25°C, 22 cP; total heavy metals, 20 ppm). Degraded carrageenan was a fine cream-coloured powder prepared commercially by controlled hydrolysis of native carrageenan extracted from *Eucheuma spinosum* (moisture content, 5%; viscosity of a 0·5% aqueous solution at 25°C, 7 cP; sulphate content, 33·2%; total heavy metals, 20 ppm). Sodium sulphate (anhydrous) was obtained from Hopkin & Williams Ltd., Chadwell Heath, Essex and sodium cyclamate, the sodium salt of cyclohexylsulphamic acid (99% pure), from Abbott Laboratories, Queensborough, Isle of Sheppey.

Experimental procedures

The design and procedural details of the carrageenan experiments are summarized in Table 1. In addition, groups of 12 male guinea-pigs (initial body weight 400–500 g) were

Table 1. *Details of experiments*

Experiment no.	Species	No. of animals	Sex	Body weight (g)	Dose level (%)	Type of carrageenan	Vehicle	Duration of treatment
1	Guinea-pig	7	F	120–160	2	Degraded	Water	21–45 days
		3	F		2	Degraded	Water*	21–45 days
		7	F		5	Native	Diet	21–45 days
		5	F		5	Native	Diet*	21–45 days
2	Guinea-pig	20	M	400–500	5	Degraded	Water*	5–9 days
		20	M	400–500	1	Degraded	Water*	Groups of 2–3 killed every 3–4 days up to 42 days
		21	M	150–200	5	Native	Diet*	do.
3	Guinea-pig	28	M	350	1	Degraded	Water*	Treatment stopped at wk 3. Groups of 4 killed at wk 1, 2, 3, 4, 7, 11 and 15
4	Ferret	3	F	800	1·5†	Degraded	—	28 days
	Squirrel monkey	2	F	800	1·5†	Degraded	—	28 days
	Rabbit	3	F	3000	1·5†	Degraded	—	28 days
	Hamsters	8	F	100	5	Degraded	Diet	6 months
	Rat	10	M	150	1	Degraded	Water	56 days
		10	M	150	5	Degraded	Water	56 days
		10	M	150	5	Native	Diet	56 days

*Neomycin (0·1%) added to the diet.
†Doses (expressed as g/kg/day) were given by gastric intubation.

given a 2% aqueous solution of sodium sulphate or sodium cyclamate instead of drinking-water (Experiment 5).

In all the experiments, animals were killed by an overdose of barbiturate (Nembutal). The entire gastro-intestinal tract was dissected out, opened and washed with normal saline. It was examined under a dissecting microscope for any signs of ulceration. Samples from each section of gut and any obvious lesions were fixed in buffered formalin. Paraffin-wax sections were prepared and stained with haematoxylin and eosin, with toluidine blue and with a carrageenan-specific staining procedure using Alcian Blue (Cangolli, Wright & Grasso, 1973).

For the demonstration of lysosomal enzymes, 1 cm^2 pieces of caecum were immersed in cold formol–sucrose (4% formaldehyde and 7·5% sucrose in 0·07 M-phosphate buffer, pH 7·4) for 20–24 hr, blotted and then left overnight in 1% gum acacia in 30% sucrose. All fixatives were kept at 2–4°C. Sections, 8 μm thick were cut perpendicular to the mucosal surface on a freezing microtome, and free-floating sections were used for staining. Lysosomal acid-phosphatase activity was demonstrated by incubating sections in a Gomori (1952) medium with β-glycerophosphate as substrate for 30 min at 37 C. The incubation mixture was warmed and filtered before use (Novikoff, 1963). Control sections were incubated in substrate-free media.

Caecal ulcers were excised for electron microscopy in 2 mm^2 pieces containing the minimum of surrounding tissue and fixed by immersion for 1 hr in 1% aqueous osmium tetroxide solution buffered to pH 7·2 with 0·1 M-sodium cacodylate. After dehydration through graded alcohols, the tissue was embedded in Epon 812. For orientation by light microscopy, 1 μm thick embedded sections were stained with toluidine blue (Ito & Winchester, 1963). Ultra-thin sections were stained with uranyl acetate and lead citrate.

RESULTS

Effect of neomycin on the development of ulceration in the guinea-pig (Experiment 1)

Guinea-pigs given 5% native carrageenan in the diet or 2% degraded carrageenan in the drinking-water developed multiple pin-point caecal and colonic ulcerations after 3–5 wk of treatment. Histologically, the ulcers consisted of extensive macrophage infiltration at the base, over which lay a thin layer of fibrin. Polymorphonuclear cells and lymphocytes were present in appreciable numbers. The epithelium around the ulcer was heavily infiltrated by macrophages, polymorphs and lymphocytes. In some animals, heavy polymorphonuclear infiltration occurred, leading to the formation of micro-abscesses close to the ulcerated areas (Fig. 1).

The addition of neomycin to the diet markedly reduced the population of polymorphonuclear cells in the ulcers but did not affect the apparent incidence of ulcers or the time of their appearance.

Course of development of caecal ulcers in guinea-pigs (Experiment 2)

Animals receiving 5% degraded carrageenan developed severe diarrhoea and began to lose weight after day 1. Occult blood was detected in the faeces from day 4 onwards and the condition of the animals deteriorated rapidly until they were killed between days 5 and 9. At autopsy, haemorrhagic areas were present in the wall of the caecum and proximal colon of all animals. Histopathological investigation revealed these to be large areas of

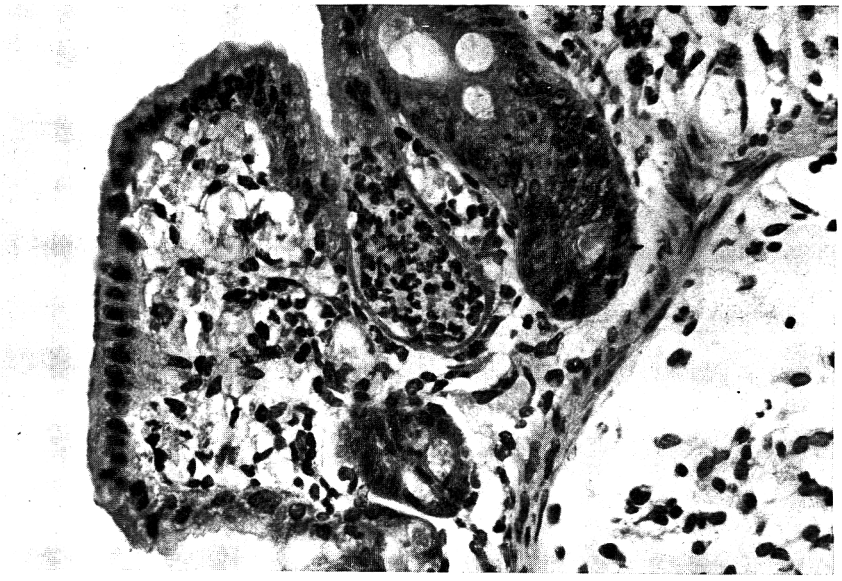

FIG. 1. Micro-abscess in the caecum of a guinea-pig given 2% degraded carrageenan in the drinking-water for 4 wk. This animal had ulcers in the caecum and colon. Haematoxylin and eosin ×400.

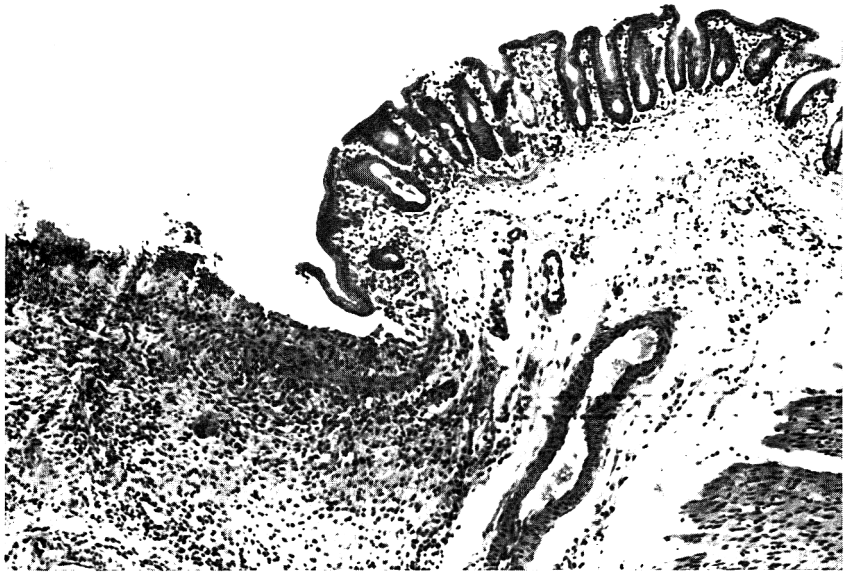

FIG. 2. Massive ulceration in the caecal mucosa of a guinea-pig after treatment with 5% degraded carrageenan in the drinking-water for 7 days. Haematoxylin and eosin ×40.

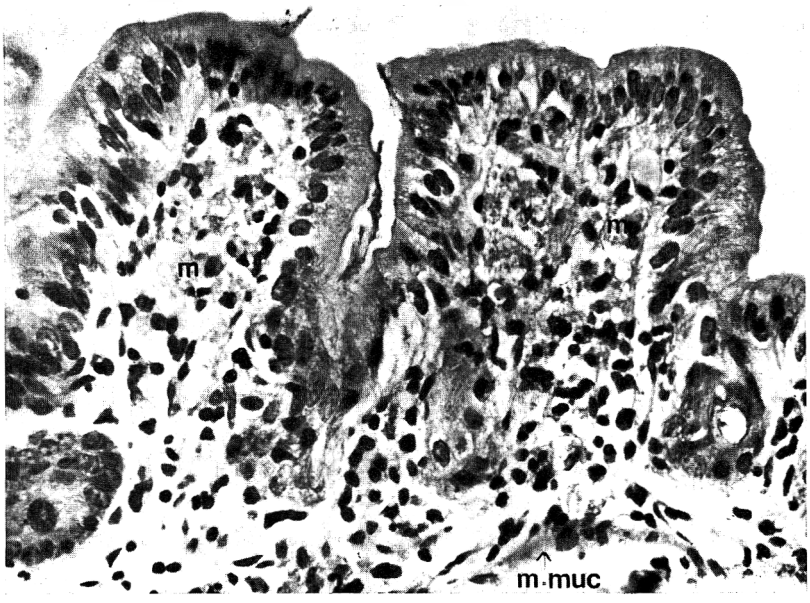

FIG. 3. Macrophage infiltration in the lamina propria of the caecal mucosa of a guinea-pig given 1 % degraded carrageenan in the drinking-water for 2 wk (m—Macrophage; m. muc—Muscularis mucosae). Haematoxylin and eosin ×400.

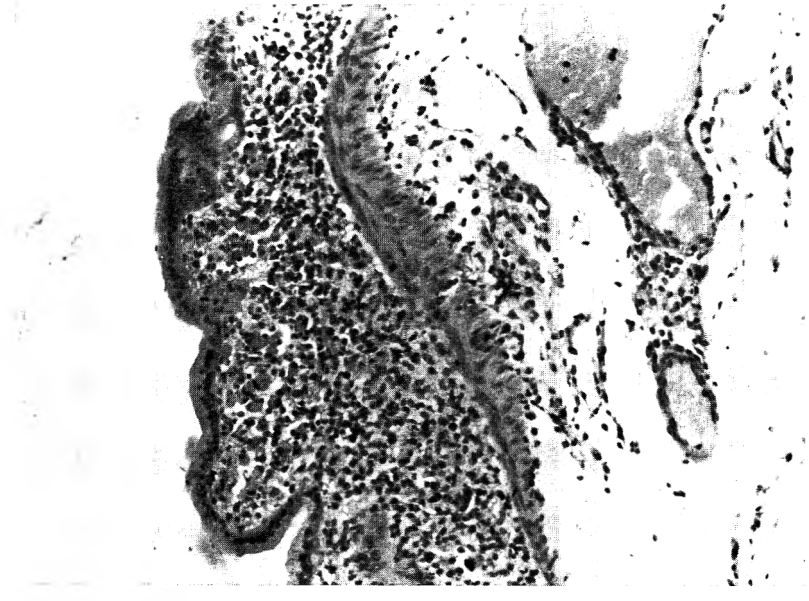

FIG. 4. Extensive macrophage infiltration from one of the pale raised areas seen at autopsy in a guinea-pig given 1 % degraded carrageenan for 3 wk. Mucosal crypts are not identifiable, presumably because of expansion of the lamina propria. Haematoxylin and eosin ×100.

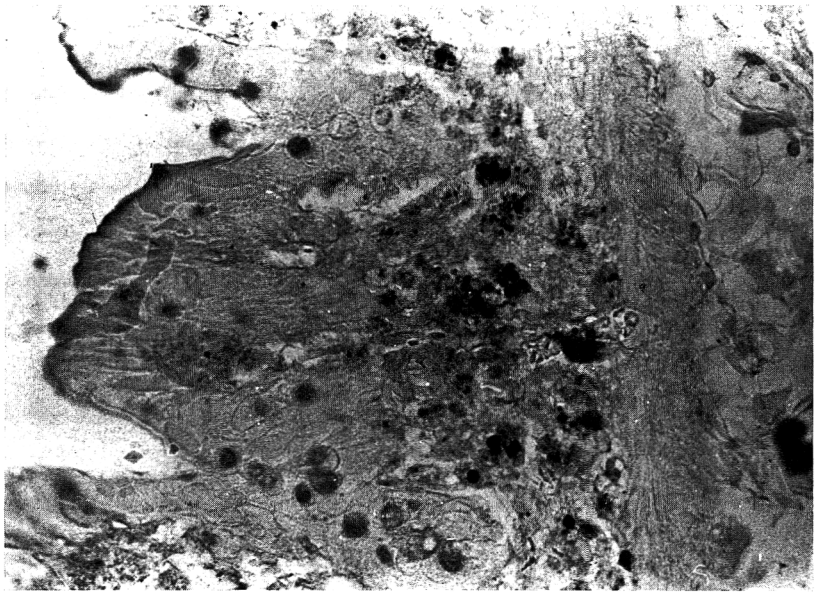

FIG. 5. Carrageenan within macrophages forming an early granuloma in the caecum of a guinea-pig given 1% degraded carrageenan in the drinking-water for 2 wk. Alcian Blue (Gangolli *et al.* 1973), light filter Wratten CC32 ×400.

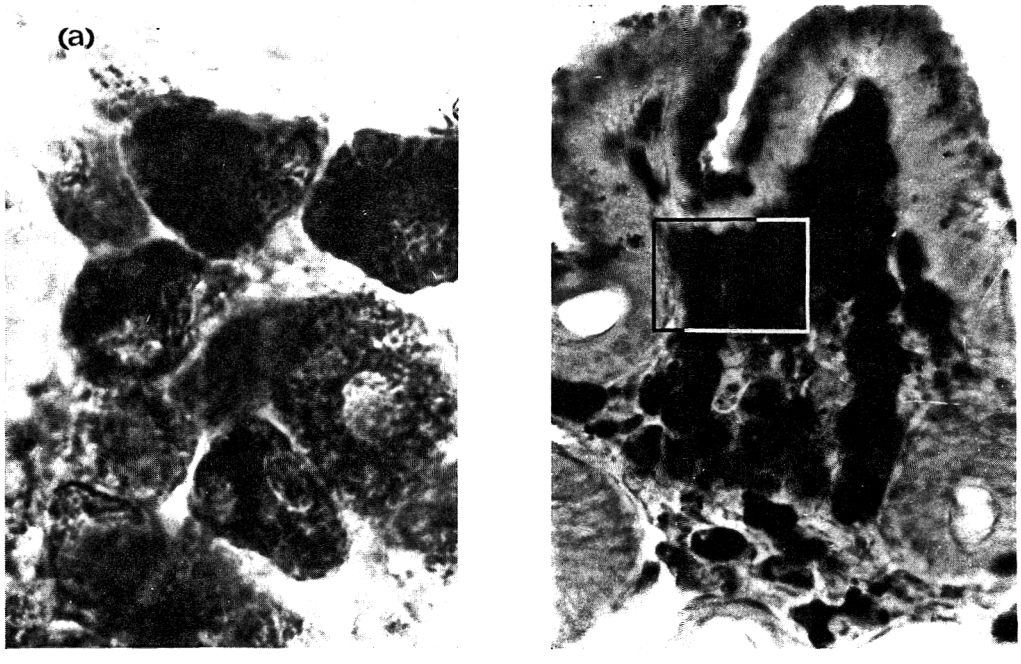

FIG. 6. Acid-phosphatase activity in an early granuloma in the caecum of a guinea-pig given 1% degraded carrageenan in the drinking-water for 2 wk. Gomori acid phosphatase ×250. Higher magnification (×800) demonstrates enzyme activity within macrophages (a).

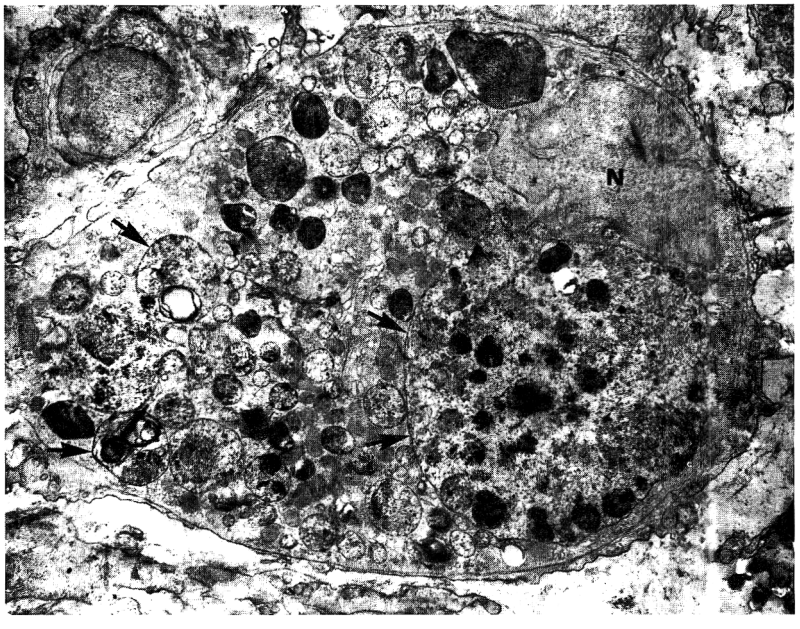

FIG. 7. Ultrastructure of a macrophage from the caecum of a guinea-pig given 1 % degraded carrageenan in the drinking-water for 3 wk, showing numerous phagosomes (arrowed). N—Nucleus. ×7000.

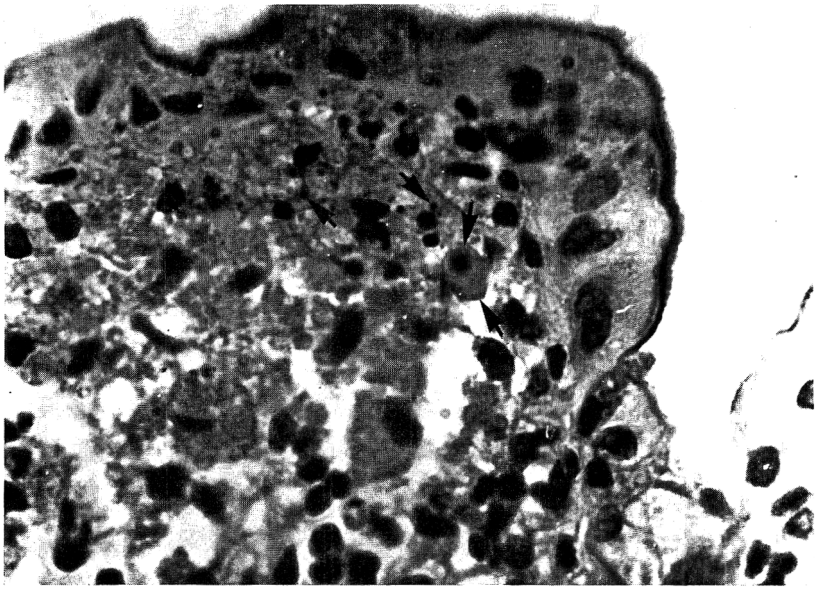

FIG. 8. Macrophage necrosis (arrowed) in a granuloma from the caecum of a guinea-pig given 1 % degraded carrageenan in the drinking-water for 4 wk. Haematoxylin and eosin ×800.

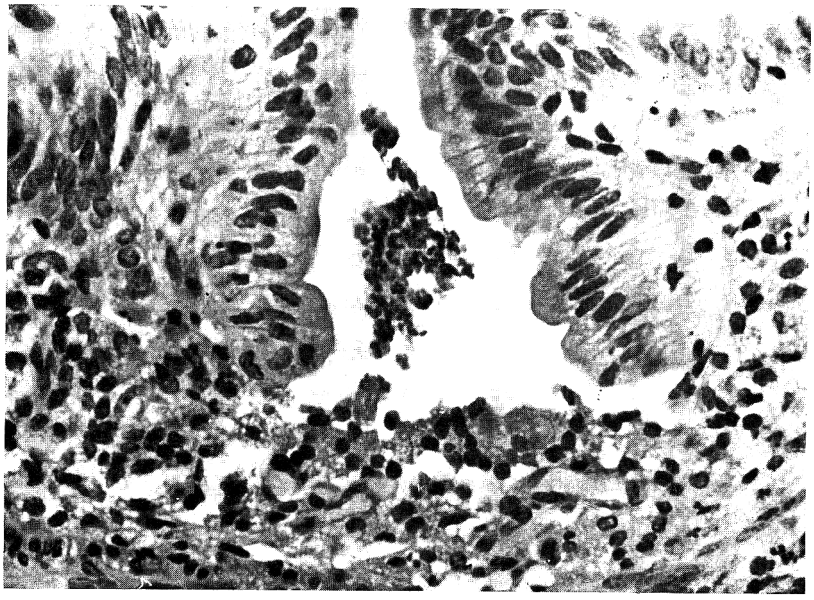

FIG. 9. A small ulcer from the caecum of a guinea-pig given 1% degraded carrageenan in the drinking-water for 4 wk, showing macrophage infiltration. Haematoxylin and eosin ×400.

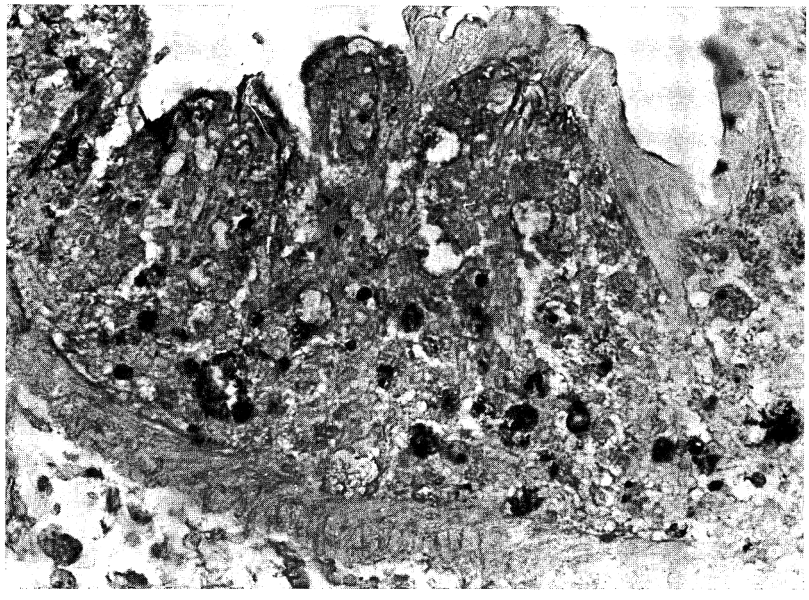

FIG. 10. Carrageenan within macrophages at the base of an ulcer from the caecum of a guinea-pig given 1% degraded carrageenan in the drinking-water for 4 wk. Alcian Blue (Gangolli *et al.* 1973), light filter Wratten CC32 ×400.

gross submucosal oedema, haemorrhage and ulceration (Fig. 2). The cellular infiltrate in the lamina propria consisted mainly of lymphocytes with few macrophages and polymorphonuclear cells.

The caecal and colonic ulcers seen in animals receiving 1% degraded carrageenan in the drinking-water or 5% native carrageenan in the diet were much smaller and developed much later than those observed in guinea-pigs receiving 5% degraded carrageenan in the drinking-water.

The stages leading to ulceration were observed from a study of the animals killed sequentially. Observation at wk 2 showed an increase in the number of macrophages in the lamina propria (Fig. 3). The macrophages often formed dense masses and obliterated the mucosal crypts (granulomas). They varied considerably in size: a few were visible to the naked eye as pale raised areas (Fig. 4). This macrophage infiltration was present in five out of six animals examined.

In sections stained with Alcian Blue at a critical electrolyte concentration of 1 M-$MgCl_2$ after digestion by mucolytic enzymes, most of the macrophages contained carrageenan, visible by this technique as Alcian Blue-positive intracellular inclusions (Fig. 5). Analytical examination of the caecum and colon revealed the presence of carrageenan in amounts ranging from 36 to 400 μg/g tissue (Gangolli *et al.* 1973). The collections of macrophages were strongly acid phosphatase-positive by the Gomori technique (Fig. 6) and ultrastructurally were shown to possess large vacuoles lined by single membranes (Fig. 7). Macrophage necrosis (Fig. 8) was sometimes observed at wk 3 and 4.

Multiple ulcerations were found in seven of the 12 animals examined. They were pinpoint in size when viewed macroscopically and were invariably found microscopically to be accompanied by marked macrophage infiltration. Loss of epithelium occurred only over areas of these pronounced macrophage accumulations (Fig. 9). Carrageenan was demonstrable within the macrophages forming the base of the ulcer (Fig. 10).

Reversibility of carrageenan-induced caecal ulceration in the guinea-pig (Experiment 3)

Three out of four guinea-pigs killed immediately after consuming a 1% solution of degraded carrageenan in the drinking-water for 3 wk were found to have caecal ulcerations. Histologically the lesions were similar to those observed in our earlier studies. No caecal or colonic ulceration was seen in animals killed 1 or 4 wk after the cessation of treatment, but distinct granulomas with demonstrable carrageenan within the macrophages could be identified. In animals killed at a later date, the histology of the caecum and colon was indistinguishable from that in the controls and no carrageenan could be identified in the macrophages within the lamina propria.

Response of ferret, squirrel monkey, rabbit, hamster and rat to carrageenan (Experiment 4)

Faecal softening was the principal effect produced by degraded carrageenan in the ferret, squirrel monkey and rabbit. The rat and hamster developed a frank diarrhoea with watery stools when given 5% degraded carrageenan. Only a slight diarrhoea, marked chiefly by faeces which were semi-solid in consistency, was observed in rats given 1% degraded carrageenan or 5% native carrageenan.

The whole of the gastro-intestinal tract of each species was examined macroscopically for ulceration, which was found only in the rabbit. No accumulation of macrophages was seen in the lamina propria and no carrageenan could be identified histochemically in either the small or large intestine in the non-susceptible species. Granuloma formation was seen

in the caecum and proximal colon of the three rabbits but ulceration was visible macroscopically as small pin-point areas in only one. Histologically, the ulcers resembled closely the ones seen in the guinea-pig (Fig. 9) and histochemically carrageenan was shown to be present within macrophages.

Effect of osmotic cathartics on guinea-pigs (Experiment 5)

All the animals given sodium cyclamate or sodium sulphate developed severe protracted diarrhoea, which was accompanied by loss of blood and considerable loss of weight. Eventually the animals became lethargic, their coats took on a roughened appearance and they looked generally ill. At this stage (between days 6 and 52) they were killed. Some animals became ill within a few days while others maintained a reasonable state of health for several weeks despite the severe diarrhoea. At autopsy, extensive haemorrhagic areas with ulceration were observed in the caecum and colon. Histologically there was gross oedema of the submucosa and muscular layers. In many areas the mucosa appeared to have been stripped off the underlying tissues leaving denuded smooth muscle. In these and nearby areas haemorrhage tended to be severe. This histological picture resembled closely a severe version of the lesion seen in guinea-pigs given 5% degraded carrageenan.

Effect of degraded carrageenan in man

Six patients suffering from malignant disease of the colon were given 5 g degraded carrageenan daily for 10 days before a colectomy was performed. Samples of normal sections of colon obtained at surgery were examined for any signs of ulceration and analysed histochemically and chemically for the presence of degraded carrageenan in the tissue. There were no signs of any ulceration in these samples of gut nor was degraded carrageenan detected by either the histochemical or the analytical method.

DISCUSSION

Our results show that the guinea-pig and rabbit develop ulcers in the large intestine when they receive oral carrageenan, whereas the mouse, ferret, rat, squirrel monkey and hamster do not. Despite the brief period of observation and the smaller doses given in our human studies, the findings suggest that man belongs to the latter group.

The selective action of carrageenan in the guinea-pig and rabbit is in keeping with the results of some other workers (R. S. Anderson, P. Grasso and M. Sharratt, unpublished data, 1970; Maillet *et al.* 1970; Nilson & Wagner, 1959; J. Tournot, personal communication, 1970), but is inconsistent with the published work of Marcus & Watt (1969), who claim that the mouse and the rat are susceptible to the development of colonic and caecal ulceration. In addition, Benitz, Golberg & Coulston (1973) claim that the rhesus monkey is susceptible to the development of caecal ulceration when given degraded carrageenan.

Because degraded carrageenan causes ulceration of the large bowel of the guinea-pig in doses equal to or only slightly above those used therapeutically in man (70 mg/kg body weight) the accepted approach to the assessment of its safety in man could not be used and the significance of the ulcerative lesion could be assessed only by an examination of some of the relevant factors leading to ulceration. In this context, the large ulcers produced by the 5% degraded carrageenan in guinea-pigs do not appear to be relevant since they seem to result from the osmotic effect of the high concentration of low molecular weight carrageenan. They are not considered further in this discussion, which focusses attention on the

pathological lesions leading to the small 'pin-point' ulcers. The accumulation of macrophages in the stages preceding the development of these small ulcers corresponds to the early stage of granuloma formation (Papadimitriou & Spector, 1972; Turk, 1971) and is consistent with the reaction of mammalian tissues in general to particulate macromolecular substances. A similar picture, but on a more extensive scale, has been described as occurring initially in the subcutaneous tissue of the rat after the injection of carrageenan (Williams, 1957).

It appears from our studies that the granuloma formation in the lamina propria of the caecum and colon was a reaction to the carrageenan particles that had traversed the mucosal barrier, since carrageenan could be demonstrated in substantial quantities analytically in the caecum and histochemically within macrophages in this organ (Gangolli *et al.* 1973). It is not clear, however, by what means carrageenan gained access to the lamina propria.

Absorption of macromolecular substances from the small intestine takes place fairly readily during the neonatal period. This process occurs to a greater extent in animals than in man, in whom it appears to be negligible (Balfour & Comline, 1962; Clark & Hardy, 1970; Deutsch & Smith, 1957). There are marked differences between species both in the mechanisms responsible for this absorption (Balfour & Comline, 1962; Hardy, 1969a,b; Morris, 1964; Pierce, 1961) and in the persistence of the process of absorption of macromolecular substances during development (Clarke & Hardy, 1970). It is of some interest, and perhaps also of some relevance to our studies, that cessation of absorption or 'closure' does not occur simultaneously throughout the intestine but commences in the jejunum and gradually progresses down to the terminal ileum (Clarke & Hardy, 1970).

Experimental evidence for the absorption of macromolecular substances after 'closure' has been sought unsuccessfully for a number of years (Clark, 1959). Recently experimental data have accumulated showing fairly clearly that the barrier of the small intestine to the absorption of macromolecules is incomplete. Thus Bockman & Winborn (1966) were able to demonstrate that ferritin administered within the lumen of ligated intestinal loops of the hamster passed into the lamina propria. Volkheimer, Schulz, Aurich, Strauch, Beuthin & Wendlandt (1968) showed a similar passage of starch granules in intact rats, while Warshaw, Walker, Cornell & Isselbacher (1971) demonstrated that horseradish peroxidase could traverse the epithelium of the small intestine of the rat in experiments conducted on isolated loops and in intact animals. In all these experiments, the amounts of ferritin, starch and horseradish peroxidase found in the small intestine were very small and were identified by sensitive histochemical techniques.

The mechanism responsible for the absorption of macromolecular substances in the adult differs considerably from that in the neonate. In the latter, absorption occurs by an intracellular process of pinocytosis (Broughton & Lecce, 1970; Cornell & Padykula, 1969; Kraehenbuhl & Campiche, 1969; Rundell & Lecce, 1972). In the adult this process has not been observed: the epithelial cells at the tips of the intestinal villi absorb macromolecules but do not appear to discharge them into the lamina propria (Warshaw *et al.* 1971). On the other hand, identification of ferritin granules and of particles of starch and horseradish peroxidase between the epithelial cells has led to a suggestion that macromolecular absorption in the adult occurs via the intercellular route (Bockman & Winborn, 1966; Volkheimer *et al.* 1968; Warshaw *et al.* 1971).

There have been no observations on macromolecular absorption by the epithelium of the large intestine in mammals. Our demonstration of carrageenan in the lamina propria of the large intestine in guinea-pigs and rabbits indicates that absorption of some macromolecular substances does occur in these species. The absence of carrageenan within

epithelial cells suggests that such absorption takes place by the intercellular route as in the case of macromolecular absorption by the adult small intestine. It is possible, of course, that carrageenan is ingested by the epithelial cells as well, but our histochemical method is not sufficiently sensitive to detect such small quantities. This may also account for our failure to demonstrate carrageenan between epithelial cells.

It is not certain to what extent the absorption and local accumulation of carrageenan are determined by the physico-chemical characteristics of the compound, such as structure, molecular size, anionic characteristics and configuration, nor is there any data on the biochemical and physiological factors responsible for the species differences observed. Recent reports claiming production of ulcers in the rabbit by sulphated amylopectin (Watt & Marcus, 1972), and the failure to demonstrate polyvinylpyrrolidone in the lamina propria of guinea-pigs treated with 5% of this compound in the diet (M. Sharratt, P. Grasso and F. M. B. Carpanini, unpublished observation 1971) suggest that the physico-chemical characteristics of the compound may be important. On the other hand, physiological factors such as the rate of removal by lymphatic or blood circulation may play an even greater role, and in this context the relatively large size of the caecum and colon in these two herbivorous species, compared with that of the other species studied, may be important. Consideration must also be given to the fact that the large intestine in the guinea-pig and rabbit may be the more important region for the intestinal absorption of macromolecules in the neonatal period, a function which may persist into adolescence and adult life. The observation of a very early 'closure' of macromolecular absorption in the guinea-pig (Clarke & Hardy, 1970) may be important in this respect.

Our results indicate that the ulceration produced by carrageenan is very closely related to the formation of granulomas induced by its accumulation in the lamina propria. No ulceration occurs in species where this pathological process does not develop during the course of carrageenan administration, nor does it occur in the segments of the gastro-intestinal tract of the rabbit and guinea-pig where carrageenan absorption and granuloma cannot be demonstrated. The rapid disappearance of both ulceration and carrageenan from the caecal and colonic mucosa on cessation of carrageenan treatment underlines the importance of the accumulation of carrageenan in the production of the pathological process.

The results of Abraham, Golberg & Coulston (1972) are in contrast to our findings, these authors having reported a very slow rate of disappearance of degraded carrageenan from the fixed macrophages (Kupffer cells) in the liver of the rhesus monkey. This difference may be due to the fact that the type of macrophage in the intestine is different from the Kupffer cells and may possess different properties in relation to the disposal of carrageenan.

Marcus & Watt (1969) suggest that carrageenan ulceration may be of importance to man. Our results indicate that the human response to administration of carrageenan is similar to that of the non-sensitive species, since carrageenan could not be demonstrated by either histochemical or analytical techniques in the human colon after carrageenan administration. Furthermore, long-term therapeutic administration of degraded carrageenan to patients with peptic ulcer has not been reported to be associated with an increased incidence of ulcerative colitis (Bonfils, 1970). Chronic ulcerative colitis in man differs in a number of important respects from the colonic ulceration produced in guinea-pigs by carrageenan. It affects predominantly the rectum and distal colon while the carrageenan ulcers are found predominantly in the caecum and proximal colon (Warren & Sommers, 1949). Histologically, macrophage infiltration is consistently predominant in the carrageenan-induced ulcers,

while this seldom occurs in the human disease (Donnellan & Beal, 1966; Gonzalez-Licea & Yardley, 1966).

The overall observations on carrageenan ulceration of the caecum and colon indicate a high degree of species specificity, which appears to be linked with the accumulation of carrageenan in these organs. All available observations from human studies indicate that man is one of the non-sensitive species, and therefore it is most improbable that the consumption of either native or degraded carrageenan plays any role in the aetiology of chronic ulcerative disease of the gastro-intestinal tract in man and, in particular, of ulcerative colitis.

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Le carragène et les ulcérations du gros intestin chez les mammifères

Résumé—L'administration de carragène à l'état pur ou sous une forme dégradée provoque des ulcérations du gros intestin chez le cobaye et le lapin. Cette ulcération peut être aiguë ou chronique, selon la dose administrée. Les modifications préulcératives consistent principalement en une accumulation de macrophages dans la lamina propria et vont de pair avec la présence de carragène dans les tissus sous-épithéliaux. Aucune de ces modifications n'a été observée chez le rat, le hamster, le sagouin et le furet. Des observations limitées chez l'homme suggèrent que celui-ci est aussi résistant à l'effet décrit ci-dessus et indiquent que le carragène n'intervient probablement pas dans l'étiologie de la colite ulcéreuse chez l'homme.

Untersuchungen über Carrageen und Dickdarmgeschwüre bei Säugetieren

Zusammenfassung—Die Verabreichung von Carrageen in einheimischer oder abgebauter Form verursacht Geschwürbildung im Dickdarm von Meerschweinchen und Kaninchen. Die Ulceration kann je nach der Dosierung akut oder chronisch sein. Präulcerative Veränderungen bestehen hauptsächlich in der Ansammlung von Makrophagen in den Lamina propria, und diese ist mit der Anwesenheit von Carrageen in den subepithelialen Geweben verbunden. Keine der obigen Veränderungen wurde bei Ratte, Hamster, Totenkopfäffchen oder Frettchen beobachtet. Beschränkte Beobachtungen am Menschen lassen annehmen, dass er ebenfalls resistent gegen diese Wirkung ist, und dies lässt darauf schliessen, dass Carrageen wahrscheinlich nichts mit der Ätiologie der ulcerativen Colitis des Menschen zu tun hat.

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Intestinal Effects of Carrageenans in the Rhesus Monkey (*Macaca mulatta*)*

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Abstract—In a study in male and female rhesus monkeys (*Macaca mulatta*), the effects of a native form of carrageenan (HMR), derived from *Chondrus crispus*, have been compared with those of a degraded form (C16) obtained from *Eucheuma spinosum*. Both types were administered in drinking-water for 7–14 wk. Monkeys given 1% HMR (providing an intake of about 1·3 g/kg/day) all gained weight and remained in good condition. Occult blood occurred sporadically in the faeces, as was the case in controls. Only minor changes, none attributable to HMR administration, were found in the intestinal tract at autopsy and on microscopic examination. Similar findings were recorded in animals given 1% HMR for 11 wk and subsequently, after a recovery period of up to 11 wk on tap-water, given escalating daily doses of 50–1250 mg HMR/kg for up to 12 wk. Monkeys given 0·5 or 1·0% C16 solution gained weight, but in those on 2·0% C16 (an intake of approximately 2·9 g/kg/day) weight losses were considerable. All monkeys on C16 lost blood frequently from the intestinal tract and developed some degree of anaemia. Pathological changes seen in the colon ranged from shallow mucosal erosions to ulceration associated with cellular infiltration, granulation tissue in the lamina propria and formation of multiple crypt abscesses. The severity of these effects was dose-dependent, and some reversal was indicated in monkeys given 2% C16 for 14 wk and then allowed to recover on tap-water for 20–24 wk. A marked difference was thus demonstrated between the effects of degraded (C16) and native (HMR) carrageenan in the monkey.

INTRODUCTION

The carrageenan group of edible gums is composed of sulphated polygalactose units of high electronegativity, which react with positively charged polymers such as proteins to form complexes, gels or precipitates (Guiseley, 1968). This property renders such gums extremely valuable to the food industry, in which they are used in the suspension of particles in chocolate milk, in the production of milk puddings and in water-dessert gels. Carrageenan is also an important ingredient of prepared infant formulas. Finally carrageenan is used to thicken soups, sauces and gravies, and as a binder in dentifrices (Klose & Glicksman, 1968).

Carrageenan has also been used as a therapeutic agent. In France, "Coreine", a native carrageenan, has found application in the treatment of colitis since 1911. "Ebimar", a product based on degraded carrageenan, has been employed since 1960 for the treatment of peptic ulcer (Bonfils, 1970; Di Rosa, 1972). For this purpose, only carrageenan derived from *Eucheuma spinosum* has been used.

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Native carrageenan, used as a food additive, is extracted from the so-called "red" seaweeds and in general is made up of a gelling component called κ-carrageenan and a non-gelling component termed λ-carrageenan (Klose & Glicksman, 1968). The ratio of the two fractions varies with the species of seaweed used, with the geographical source and with the time of harvesting (Guiseley, 1968). A third type of carrageenan, designated the iota form, has been extracted from certain carrageenan-bearing seaplants such as *Eucheuma spinosum* and *Eucheuma cottonii* (Guiseley, 1968). This ι-fraction forms an elastic gel with calcium salts.

The experiments described in this report were designed to explore the distinctive biological effects of undegraded (native) and degraded carrageenans on the intestinal tract in a non-human primate species.

EXPERIMENTAL

Materials. Two forms of carrageenan were used, as described by Abraham, Golberg & Coulston (1972). The high molecular, alcohol-precipitated material was provided by Marine Colloids, Inc., Rockland, Maine, as Gelcarin HMR Lot No. 282400, and was composed largely of κ-carrageenan. This undegraded carrageenan will be referred to as HMR. Degraded carrageenan (C16) was supplied by Laboratoires Glaxo, Paris (Batch L4006). The analytical data applicable to this material were: moisture, 5%; ester sulphate, 33·05%; free sulphate, 0·15%; total number of bacteria, 75/g with no coliforms, anaerobic bacteria, yeasts or moulds.

Animals and dosage. Rhesus monkeys (*Macaca mulatta*) were housed individually in wire-bottom cages. Drop pans and cage bottoms were changed and cleaned regularly. Food (Purina Monkey Chow) and drinking-water (or solutions of carrageenan) were freely available at all times. Each form of carrageenan was dissolved in sterile distilled water and the solutions were administered as the sole source of drinking-water. Freshly-prepared solutions were used every day and samples were taken for microbiological examination. No contamination was found during a 24-hr period under use conditions in the temperature-controlled air-conditioned animal quarters. In other experiments, solutions of HMR ranging in concentration from 1–3% were administered daily by stomach tube.

Experimental design and conduct

The overall plan of research and the number of each specific monkey used was presented in Table 1 of an earlier paper (Abraham *et al.* 1972). A total of ten male and ten female monkeys were used in this study. Three males and three females received HMR in the drinking-water as a 1% solution, corresponding to an average daily intake of 1·3 g/kg/day. Two animals were sacrificed after 7 and 11 wk, respectively, the remaining four being put back on plain tap-water and allowed to recover for 11 wk. These animals were then used for a dose-ranging experiment, using escalating daily doses of HMR ranging from 50 to 1250 mg/kg daily for up to 12 wk. The highest dose was given for 8 wk. After this second period of exposure, all four monkeys were killed.

Five males and five females received various concentrations of C16 in the drinking-water up to wk 14. Six animals drank a 2% solution, two a 1% solution and the remaining two a 0·5% solution. From measurements of fluid consumption, the corresponding average daily intakes of C16 were calculated to be 2·9, 1·4 and 0·7 g/kg, respectively. Two monkeys from the 2% group were sacrificed after 7 and 11 wk of exposure. The remaining four animals

from this group were kept on their dosage regimen for 14 wk and were then allowed to recover on tap-water for a period of 20–24 wk, at which time they were killed. The four animals given the other dose levels were killed at the end of the 14-wk treatment period.

Two male and two female monkeys were used as controls. Standard haematological methods were used for the determinations of haemoglobin concentration, haematocrit and total red and white cell counts. For detecting occult blood in faeces, Hematest tablets were used; these tests were performed at least 3 times/wk.

The monkeys were killed by an iv overdose of barbiturate and autopsied immediately. All three body cavities were opened, their contents were inspected *in situ* and the organs were dissected. The gastro-intestinal system was removed *en bloc*. All portions were opened, the contents were gently removed and the inner and outer surfaces were examined using a $\times 10$ magnifying lens and transmitted light. Various representative samples from stomach, duodenum, small intestine, caecum, colon and rectum were placed on cork discs to avoid curling and fixed in 10% buffered formalin. Sometimes adjacent samples were fixed in Zenker's solution. Paraffin sections 6 μm thick were stained with hematoxylin and eosin. The material from the caecum, ileocolic valve, colon and adjacent lymph nodes was sectioned at six adjacent levels and stained with hematoxylin and eosin and by a modified trichrome method (Ladewig & Dessau, 1948). A few sections, including mesenteric lymph nodes, were stained with 0·1% toluidine blue in 30% ethanol for metachromasia (Thompson, 1966).

RESULTS

Native carrageenan (HMR)

All animals treated with HMR remained in good condition and gained weight during the initial 7–11 wk period. Occasionally there was a slight change in the consistency of the stools, which became soft. Positive results for faecal occult blood were found sporadically, but a similar pattern was seen in the control monkeys. Thus no clinical observations suggested any adverse effect from the administration of HMR. Haematological indices after 9 wk of exposure to 1% HMR in drinking-water revealed in all instances a fall in red cell count and in four out of six monkeys, decreased values for haemoglobin and haematocrit. A similar consistent trend was noted in control monkeys drinking tap-water. No cause for these changes was revealed at autopsy of any of these animals.

Of the two animals sacrificed at 7 and 11 wk respectively, the female (no. 1026) had a grossly normal gastro-intestinal tract. Only in the colon a few minute areas of capillary hyperaemia were apparently associated with compacted faecal contents compressed into the intestinal haustrae. Microscopic examination showed focal capillary hyperaemia and sometimes a slight degree of mucosal oedema. All other parts of the gastro-intestinal tract were microscopically normal. The male monkey (no. 1002) had some traces of freshly digested blood in the stomach, in the form of a few brown flakes thought to stem from swallowed blood due to a freshly-broken upper incisor. In the colon, at points 15 and 25 cm distal from the ileocolic valve, two trichobezoars were found, together with stool of pasty consistency. Microscopic examination of all portions of the gastro-intestinal tract revealed no abnormality.

The four remaining monkeys (males 1003 and 1004 and females 1034 and 1035 in Table 1, Abraham *et al.* 1972) were allowed to recover after 7–11 wk of HMR administration. During the 11-wk recovery period, all clinical observations corresponded to those in the

control animals. After this period, the four monkeys received HMR solutions by stomach tube for up to 12 wk in escalating doses of 50–1250 mg/kg/day and were then killed.

The substantial quantities of HMR ingested did not appear to affect the animals adversely. They continued to gain weight and remained in good condition. The stools were usually well formed and normal in consistency and colour throughout the study, except for an occasional soft or watery stool which was pale. Only sporadic positive reactions for occult blood occurred, more or less similar to those in controls. The haematological indices remained unaltered during the course of the experiment.

Gross and microscopic examination of the intestinal tract revealed no change attributable to the administration of HMR. Special attention was given to the caecum, ileocolic valve and colon and their regional lymph nodes. The microscopic appearance of these structures was essentially normal. The mucosa was intact and there were no unusual cellular infiltrations or small crypt abscesses indicative of an inflammatory response. Scattered foci of slight capillary hyperaemia were seen in some colon samples from monkey no. 1004. Parasitic abscesses were found in the regional lymph nodes of two of the four animals. The mesocolonic lymph nodes from the other two animals were normal.

Degraded carrageenan (C16): Short-term treatment

Five of the six monkeys given 2% C16 in the drinking-water did not gain weight. By 10–11 wk, weight losses ranged from 150–440 g. The control monkeys registered weight gains ranging from 560–720 g. Almost immediately after the onset of C16 administration, the consistency of the stools became loose, unformed and watery, and remained in this state as long as the compound was given. In addition, after 2–3 wk, occult blood was found frequently in stool samples, and melaena appeared, sometimes associated with discharge of frank blood and mucus. After 10 wk, two of the six monkeys (nos. 1006 and 1017) became ill and showed signs of dehydration. Male 1006 recovered after receiving antibiotics and electrolytes for 2 days, but the condition of no. 1017 did not improve and the animal had to be killed.

Monkeys given drinking water containing lower levels of C16 (1 or 0·5%) gained weight or maintained their initial body weights and were generally in much better condition than those receiving 2% C16. Faeces were for the most part firm or soft, rarely loose or watery. Occult blood in the faeces of these animals appeared 2–3 wk after the beginning of treatment and thereafter a consistent pattern was observed. The animals receiving 1% C16 appeared to pass blood continuously, while the two monkeys on 0·05% C16 had a lower frequency of positive occult blood in the faeces.

The haematological data (pre-dose and terminal) indicated a moderate degree of secondary anaemia at the end of the exposure period, characterized by consistently decreased haematocrit values and numbers of erythrocytes and a less regular reduction in haemoglobin concentration. As noted above for HMR, the haematological indices obtained for the control monkeys tended to decrease during the experiment. Although all values, both for C16-treated animals and controls were within normal limits for rhesus monkeys (Bushby, 1970; Rollins, Hobbs, Spertzel & McConnell, 1970; Schermer, 1967), the fact remains that, following a recovery period of up to 24 wk, only the C16-treated monkeys showed appreciable increases in the above-mentioned indices.

Two monkeys were killed after receiving 2% C16 for 7 and 11 wk. In female no. 1024 killed at 7 wk, the entire intestinal tract was macroscopically normal and the microscopic

abnormalities were limited to the colon. In one area, a small focal haemorrhage occurred in the tunica propria. The epithelium in this particular area was thinner than usual and covered with mucus containing several erythrocytes. Thinning of the epithelium occurred also in other areas without haemorrhage. Elsewhere in the colon the tunica propria contained a few macrophages, occasionally mixed with leucocytes. The regional lymph nodes were slightly hyperplastic and contained a few foamy macrophages.

In monkey no. 1017, killed at 11 wk, more pronounced changes were seen in the caecum and colon and their regional lymph nodes. The caecum contained liquid stool and the mucosal surface was normal. Microscopically, slight oedema, minor capillary hyperaemia and a slightly increased number of macrophages were seen. The ileocolic valve was grossly normal except for some small mucosal areas with slight hyperaemia. Microscopically, two submucosal abscesses measuring approximately 3·0 and 1·8 mm in diameter were seen under the colonic epithelium of the ileocolic valve. Both abscesses had necrotic centres containing fibrinoid material and cellular debris; one of the abscesses was draining its contents into the intestinal lumen. At the periphery of the necrotic areas, and generally in the immediate vicinity of the abscesses, cross sections of crypts with either hyperplastic or necrotic epithelium were found (Fig. 1). The crypts usually contained eosinophilic, fibrinoid material. Next to these abscesses there was marked cellular infiltration containing macrophages, occasionally multinucleated giant cells and fibroblasts. The rest of the colonic mucosa was oedematous with multiple haemorrhages in the tunica propria and a moderate increase in macrophages.

The colon had multiple fresh mucosal haemorrhages throughout its entire length. The haemorrhagic foci appeared to be covered with clotted red blood that could not be removed from the mucosal surface by rinsing with saline. Microscopically, multiple superficial erosions were seen, sometimes associated with focal shallow ulcerations (Fig. 2). The remaining crypts were surrounded by cellular infiltrations limited to the tunica propria and superficial parts of the submucosa. As a rule, the infiltrations associated with erosions did not penetrate the entire wall but were limited by the muscularis mucosae. However, occasionally the muscularis mucosae was breached and portions were replaced by cellular infiltrates, containing primarily macrophages, lymphocytes and some fibroblasts (Fig. 3).

Crypt abscesses in various stages of development were seen in all parts of the colon, either associated with erosions and ulcerations or away from these lesions. The smallest abscesses comprised crypts with slightly dilated lumina containing a small number of leucocytes mixed with threads of mucus and some cellular debris. As these lesions became larger, dystrophic epithelial changes became noticeable. The cells were flat, atrophic or even necrotic, while the goblet cells became sparse and in more advanced stages were no longer visible. Simultaneously the cellular debris increased in amount. The ultimate fate of these crypt abscesses appeared to be rupture and drainage into the lumen of the colon (Fig. 4). The adjacent parts of the mucosa were usually oedematous, hyperaemic and sometimes haemorrhagic. Occasionally crypt abscesses were seen apparently apart from erosions or ulceration. Abscesses located in the deeper layer of the mucosa were usually associated with focal granuloma formation. Sometimes these macrophagic granulomas penetrated the muscularis mucosae to the extent that it was no longer clearly visible (Fig. 5).

The regional lymph nodes in the mesentery of the colon were visibly slightly enlarged. Microscopically they were somewhat hyperplastic, but the predominant change was a distension of the marginal and especially the central sinus containing reticulum cells with finely granular material. When parallel sections were stained with toluidine blue the fine granules were gamma-metachromatic (Fig. 6). This metachromasia was not stable and was

only seen in minute quantities in fresh preparations. In a few lymph nodes a slight eosinophilic oedema was also present.

The two monkeys that received 1% C16 in the drinking-water for 14 wk had similar lesions in the lower intestinal tract. The caecum from female no. 1020 was grossly normal and microscopically only a few focal areas of accumulated macrophages were seen in the tunica propria. Macroscopically, the ileocolic valve from this animal was slightly hyperaemic. Microscopically, capillary hyperaemia, slight oedema and diffuse or focal accumulations of macrophages were seen in the tunica propria. In addition, one crypt abscess was found, containing moderate amounts of cellular debris. The colon had multiple haemorrhagic areas 30–60 cm distal to the ileocolic valve, their largest diameter being 1–2 mm. Microscopically all sections taken from this portion of the colon showed various degrees of slight oedema, capillary hyperaemia and sometimes haemorrhage in the tunica propria. In addition, several crypt abscesses and shallow mucosal erosions were seen. Both lesions appeared to be less pronounced, but not less frequent, than in female no. 1017 given 2% C16. The lymph nodes in the mesentery were grossly prominent and glassy. The microscopic changes were less pronounced than in the animals from the 2% group, but slight hyperplasia was present, with distension of central sinuses containing enlarged reticulum cells with finely granular cytoplasm.

Abnormal gross findings in male no. 1005 were also limited to the portion of the colon 10–35 cm distal to the ileocolic valve. Slight oedema, focal hyperaemia and minute haemorrhages (largest diameter 1 mm) were seen. Microscopically, the ileocolic valve was also involved. Under a shallow erosion of the mucosa, a cross-section of a mucosal lymph node was seen to contain a necrotic centre, which was presumably an old crypt abscess because in some areas there were remnants of low cuboidal epithelium similar to the flat epithelial lining of the crypt abscesses mentioned above.

In the colon proper, there were slight degrees of oedema, hyperaemia, small focal haemorrhages and macrophage infiltration. About 35 cm from the ileocolic valve, a fresh erosion and some foci of cellular infiltration of the tunica propria were found. Grossly, the regional lymph nodes in the mesentery were glassy-white and enlarged. Microscopic examination again revealed a small degree of hyperplasia and slight distension of the central sinus containing numerous enlarged reticulum cells with finely granular cytoplasm.

The abnormal findings in the two animals that had received 0·5% C16 in the drinking-water for 14 wk were even less conspicuous. Male no. 1008 appeared completely normal, while female no. 1023 had several mucosal haemorrhages (largest diameter 3 mm) in an area 48–52 cm distal to the ileocolic valve. The abnormal microscopic findings in the male were limited to focal areas of capillary hyperaemia and slight oedema of the tunica propria of the colon and the ileocolic valve. The caecum of the female contained a few focal round-cell infiltrations. Three deep crypts with shallow, low cuboidal epithelium containing some epithelial debris were found in the mucosa of the ileocolic valve. Two erosions of the colonic mucosa were seen 20 and 50 cm distal to the ileocolic valve, associated with slight lymphocytic infiltrations containing macrophages. The regional lymph nodes in the colonic mesentery were grossly normal. A minimal degree of hyperplasia, with slight proliferation of reticulum cells in the central sinus, was visible microscopically. These cells were slightly enlarged and contained finely-granular cytoplasm, as described above.

The intestinal tracts of the control monkeys were macroscopically normal. Microscopic examination of tissue samples from the caecum, ileocolic valve, colon (15–20 cm apart) and regional lymph nodes did not reveal any abnormality.

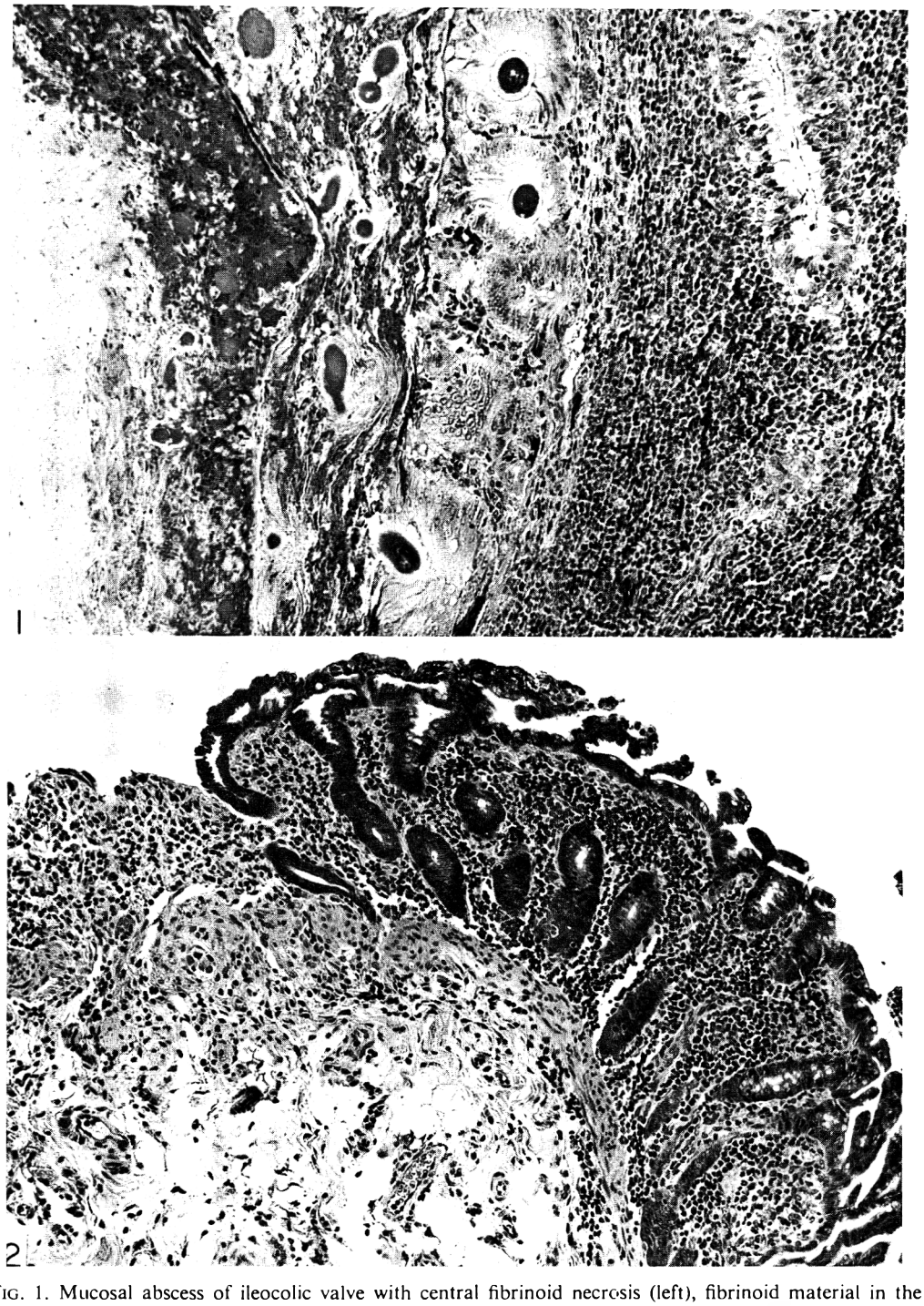

FIG. 1. Mucosal abscess of ileocolic valve with central fibrinoid necrosis (left), fibrinoid material in the necrotic stroma of hyperplastic glands and marked cellular infiltration of the tunica propria (right) from a female monkey (no. 1017) given 2% C16 in the drinking-water for 11 wk. Trichrome stain ×252.

FIG. 2. Margin of erosion in the colon with necrotic epithelium and macrocytic infiltration of the tunica propria limited by the muscularis mucosae in a female monkey (no. 1017) given 2% C16 in the drinking-water for 11 wk. Haematoxylin and eosin ×210.

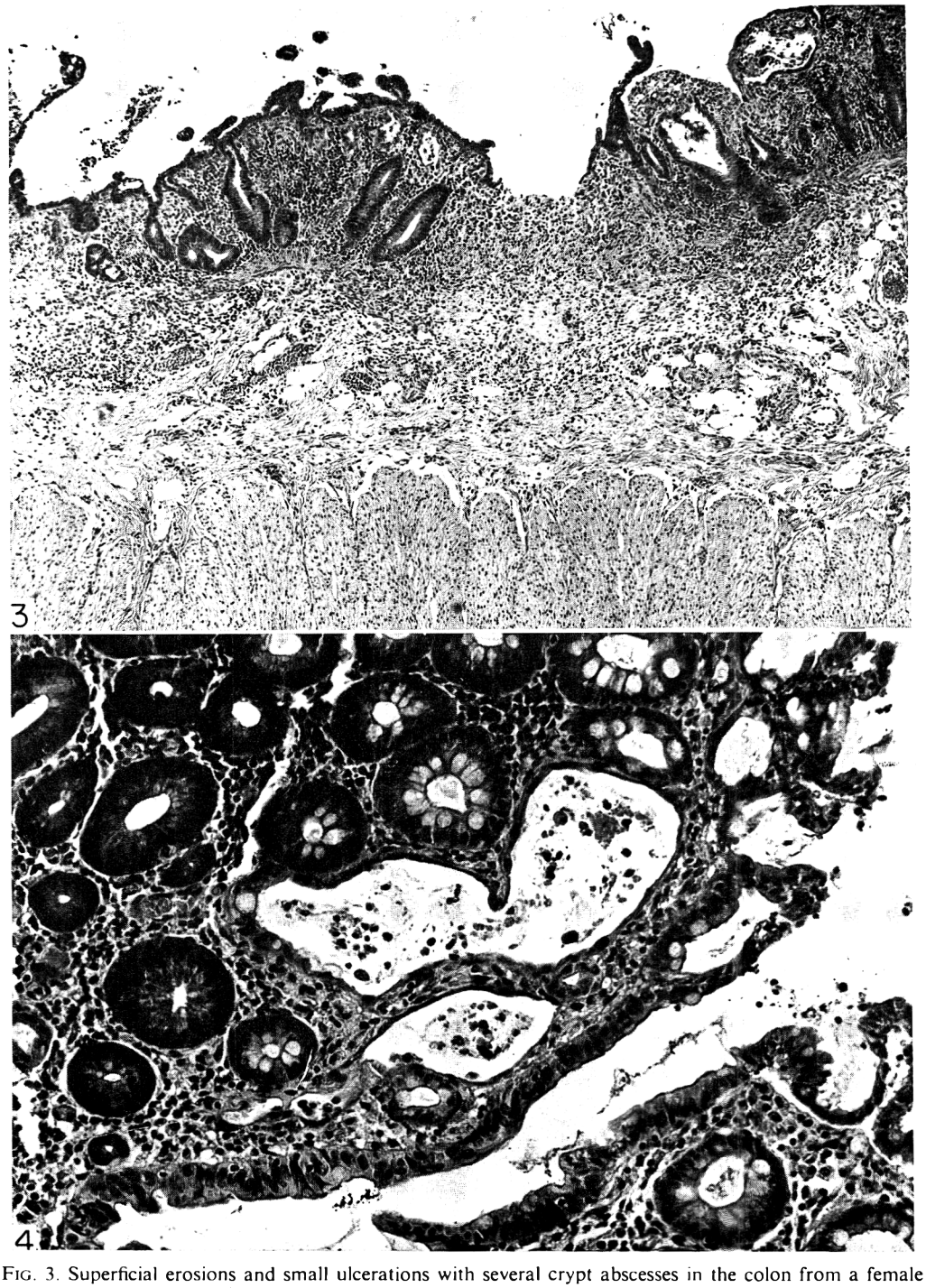

FIG. 3. Superficial erosions and small ulcerations with several crypt abscesses in the colon from a female monkey (no. 1077) given 2% C16 in the drinking-water for 11 wk. The tunica propria shows focal and diffuse macrocytic infiltrations, and at the base of the ulcer there is destruction of the muscularis mucosae. Haematoxylin and eosin × 75.

FIG. 4. Several crypt abscesses in the colonic mucosa, the larger ones with flat, atrophic and partially necrotic epithelium, from a female monkey (no. 1020) given 1% C16 in the drinking-water for 14 wk. Drainage into the lumen can be seen (right). Haematoxylin and eosin × 280.

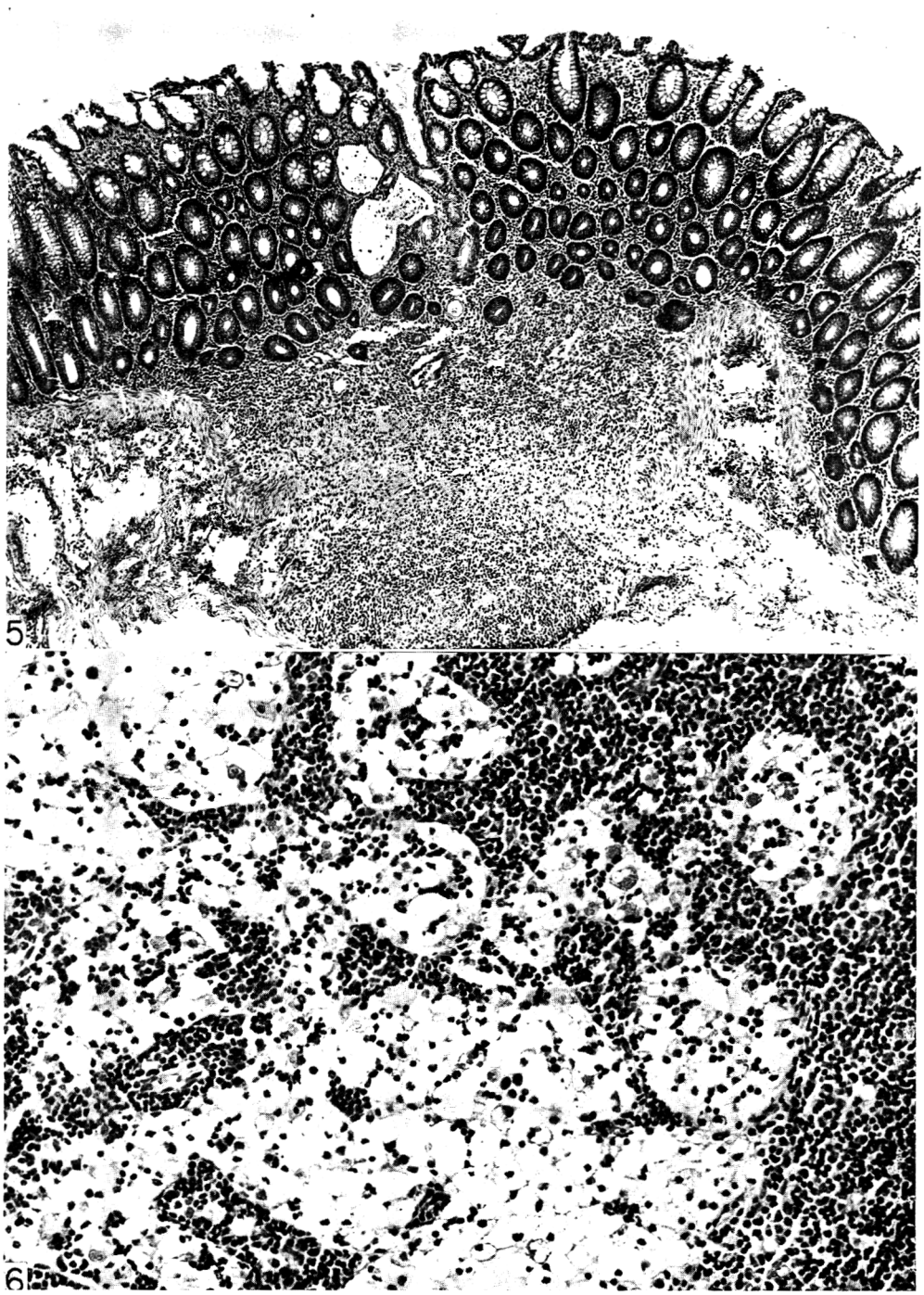

FIG. 5. Two solitary crypt abscesses with focal macrophage granuloma in the submucosa replacing the muscularis mucosae, from a female monkey (no. 1017) given 2% C16 in the drinking-water for 11 wk. Haematoxylin and eosin × 84.

FIG. 6. Enlarged reticulum cells with clear cytoplasm in the central sinus of a mesenteric lymph node next to the ileocolic valve, from a female monkey (no. 1017) given 2% C16 for 11 wk. Toluidine blue × 252.

Degraded carrageenan (C16): Recovery study

Four monkeys that had received 2% C16 in the drinking-water for 10–11 wk were allowed to recover on normal diet and tap-water. Following withdrawal of the carrageenan, the results of tests for faecal occult blood remained consistently positive for 10 wk, after which there was progressive improvement. All four monkeys were killed after 5–6 months of recovery.

At autopsy, the intestinal tract in both males (nos 1006 and 1009) was grossly and microscopically normal. The gastro-intestinal tract of one female (no. 1029) was macroscopically normal, but several large deep crypt abscesses with degenerative changes of the epithelium were found in the colonic part of the ileocolic valve. The colon of the second female (no. 1022) had two large ulcers, one of which was found 5 cm distal to the ileocolic valve, located in the mesenteric portion of the colonic wall. This lesion was oval-shaped, with a maximum diameter of 12 mm and a depth of approximately 0·5–1 mm. It was surrounded by a slightly raised hyperaemic mucosa. The second ulcer was found 5 cm beyond the first; its shape was irregular (diameters 5 × 20 mm) and it was approximately 1 mm deep. Both mucosal defects were microscopically similar. The surrounding mucosa was hyperaemic and contained markedly dilated capillaries. The bed of each ulcer consisted of a dense network of granulation tissue containing many dilated capillaries and numerous leucocytes. There were remnants of small, usually almost obliterated crypts, some of them containing a considerable number of red blood cells.

DISCUSSION

Much of the confusion created by contradictory statements in the literature on carrageenan stems largely from inadequate descriptions of the experiments performed and the results obtained. In addition, uncertainties are compounded by a failure to distinguish clearly between the effects of κ-, λ- and ι-carrageenans made up of native, partially purified but undegraded, and degraded macromolecular forms. The objective of the experiments described in this report was to explore distinctive biological properties of degraded and of alkali-treated alcohol-precipitated undegraded carrageenans with respect to their influence on the intestine of the rhesus monkey. A comparison of the results is given in Table 1. These data indicate distinct differences between the effects of the two types of carrageenan. The undegraded κ-carrageenan (HMR) with a number-average molecular weight of approximately 800,000 (Anderson & Soman, 1966) was well tolerated and did not cause any discernible adverse effect. Administration of the degraded ι-carrageenan (C16) with a number-average molecular weight of about 20,000 (Blakemore & Dewar, 1970) led to intestinal blood loss and anaemia. The morphological abnormalities were seen in the colon, including the colonic portion of the ileocolic valve, and resembled various stages of human ulcerative colitis, an aspect referred to below. Minor degrees of pathological change such as oedema, hyperaemia and an increased number of macrophages were also found in the caecum. All the effects seen in this group were dose-dependent.

These observations differ from the results reported by Sharratt, Grasso, Carpanini & Gangolli (1971a), who administered 1·5 g/kg/day of a similar if not identical degraded carrageenan to squirrel monkeys for 4 wk and found no colonic ulceration. Maillet, Bonfils & Lister (1970) and Sharratt, Grasso, Carpanini & Gangolli (1971b) have postulated that only non-ruminant herbivores like the rabbit and guinea-pig are susceptible to carrageenan-induced ulceration. However, Marcus & Watt (1971) have reported ulceration of the

Table 1. *Comparison of the effects of two forms of carrageenan in rhesus monkeys*

Regimen/Effect	Type of carrageenan			
	HMR (undegraded)	C16 (degraded)		
Concn in drinking-water (%)	1·0	2·0	1·0	0·5
Approx. dose (g/kg/day)	1·3	2·9	1·4	0·7
Duration of treatment (wk)	7–11	7–11	14	14
Clinical signs				
Body-weight loss	Absent	Present	Absent	Absent
Faecal consistency	Soft*	Watery	Loose-soft	Soft-firm
Faecal occult blood	Occasional	Marked	Moderate	Slight
Melaena/frank blood	Absent	Present	Present	Absent
Secondary anaemia	Absent	Moderate	Moderate	Slight
Colon				
Erosions and ulcerations	Absent	Marked	Moderate	Slight
Crypt abscess formation	Absent	Marked	Moderate	Absent
Granulation tissue with macrophages	Absent	Marked	Moderate	Slight
Metachromatic material				
Hyperplastic reticulum cells in regional lymph nodes	Absent	Present	Present	Present
Reticulum cells of spleen†	Absent	Present	Present	Present
Kupffer cells†	Absent	Present	Present	Present

*Occasionally.
†Findings of Abraham *et al.* (1972).

caecum of young rats following oral administration of degraded carrageenan. On the contrary, our experience confirms that of Maillet *et al.* (1970) and Sharratt *et al.* (1970), namely the fact that the caecum and proximal colon of the rat are substantially unaffected under the conditions described. What appears to have been overlooked by other workers, however, is the remarkable sequence of changes in the distal colon of the rat, commencing at the anorectal junction and progressing to squamous metaplasia of the colonic epithelium (Fabian, Abraham, Coulston & Golberg, 1973).

Sharratt *et al.* (1971a) have emphasized the anatomical distribution of the lesions induced by carrageenan in the intestinal tract. The erosions and ulcerations in the rhesus monkey were found not in the caecum but in the first 70 cm of the colon (the entire length of the large intestine, including the rectum, varying from 105 to 125 cm). In the guinea-pig and rabbit the caecum is the primary site of predilection for these lesions. This difference in topographical location is associated with differences in histological detail. Apparently the degraded carrageenans are absorbed in the caeca of guinea-pigs and rabbits. The results from time-sequence studies in guinea-pigs carried out by Sharratt *et al.* (1971b) indicated the initial accumulation of macrophages in the lamina propria and submucosa. These cells appear to take up carrageenan and/or its derivatives, and subsequently the epithelial defects appear. Thus, according to these authors, erosions and ulcerations in the caecum are consequences of this absorption phenomenon.

In the rhesus monkey a different mechanism seems to prevail. The macrophages in the granulation tissue did not appear to have absorbed appreciable amounts of degraded

carrageenan. These cells were normal in appearance and displayed no morphological characteristics suggestive of sulphated polysaccharide uptake; the absence of metachromasia was especially noteworthy. However, there can be no doubt that carrageenan is absorbed by the rhesus monkey, as evidenced by our own studies on tissue storage (Abraham *et al.* 1972) and our unpublished observations on urinary excretion of degraded carrageenan. Analogous quantitative measurements have served to establish that the baboon also absorbs orally-administered degraded carrageenan (Beattie *et al.* 1970). The metachromatic-reaction sites in the enlarged reticulum cells of the regional lymph nodes, observed in animals from the C16 group, indicated the presence of the degraded carrageenan, or of material derived from it, within these cells. This appearance was consistent with the above-mentioned findings reported by Abraham *et al.* (1972) indicating that carrageenan derived from C16 was taken up and stored in Kupffer cells of the liver. The sum total of all these observations provides indisputable evidence that C16 is absorbed from the intestinal tract of the rhesus monkey, whereas in the case of HMR neither a cellular response nor other indication of uptake into the body was forthcoming.

Since carrageenan-containing granulomas do not play a primary role in the formation of mucosal defects in the colon of the rhesus monkey, a different pathogenic mechanism may be postulated. Examination of various segments of colon from monkeys of the C16 group seems to indicate that capillary distension, hyperaemia and subsequent oedema of the mucosa was one of the initiating factors in the formation of mucosal defects. Although the mucosal oedema was usually of moderate degree, this pathological fluid accumulation in the connective tissue would be expected to act as an irritant if it contained absorbed carrageenan. The capillary distension and haemorrhages were usually most marked in the apical portions of the mucosal folds, supporting the concept that the noxious stimulus came from the intestinal lumen. Krauspe (1972) has stressed the importance of such circulatory phenomena in the pathogenesis of the early stages of ulcerative colitis in man.

A second factor in pathogenesis seems to be a primary effect on the crypt epithelium, already postulated by Warren & Sommers (1954). Occasionally, small isolated crypt abscesses and thinning of epithelium were seen without granulomatous reactions. Should it happen that the degraded carrageenan is absorbed through the colonic epithelium, the possibility of direct damage due to the C16 would not be ruled out. Krauspe (1972) has investigated this aspect and confirmed its aetiological role to some extent. The same author mentioned that the oedema and the subsequent cellular infiltrations around the crypts should also be considered as a pathogenic mechanism for epithelial damage and for the formation of crypt abscesses.

Some of the gross and microscopic details of the C16-induced colonic lesions resemble certain morphological changes described as characteristic for ulcerative colitis in man, but it is doubtful whether a chemically-induced reaction to a single stimulus can be regarded as a morphological model disease for human ulcerative colitis, which is characterized by a multifactorial aetiology.

The suggestion has been put forward at various times that colonic ulceration elicited by C16 might be a non-specific manifestation of the response to changes in gut motility and the characteristics of the intestinal contents. Loose stools and frank diarrhoea can result from high doses of C16. Under the conditions of our experiments, however, pathological changes occurred in the colon in the absence of any striking effect on stool consistency (Table 1). Moreover the consequences of administration of an osmotic purgative to rhesus monkeys (sodium cyclamate, in doses as high as 8 g/kg/day) have been observed by Stein,

Serrone & Coulston (1967, and unpublished data). Severe diarrhoea and rectal prolapse resulted, but no intestinal ulceration.

Although the results of the present experiment in rhesus monkeys do little to clarify the ulcerogenic mechanism of C16, the findings do show a definite difference between the effects of degraded C16 carrageenan and native HMR carrageenan. The high molecular, undegraded HMR compound is apparently well tolerated in all species studied so far. The effects of low-molecular degraded C16 on the intestine in guinea-pigs, rabbits, rats and rhesus monkeys are characterized by various degrees of ulceration and granuloma formation. Species differences appear to determine the location of lesions, histological details and presumably differences in the pathogenic mechanism of ulcer formation. It is to the resolution of these problems that further work is now being directed.

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Effets intestinaux de carragènes chez le singe rhésus (*Macaca mulatta*)

Résumé—On a comparé au cours d'essais sur des singes rhésus (*Macaca mulatta*) mâles et femelles les effets d'une forme pure de carragène (HMR) extraite de *Chondrus crispus* avec ceux d'une forme dégradée (C16) extraite d'*Eucheuma spinosum*. Chaque forme a été administrée pendant 7 à 14 semaines dans l'eau de boisson. Tous les singes qui ont reçu 1% de HMR (ce qui représente une consommation d'environ 1,3 g/kg/jour) ont gagné en poids et sont restés en bonne condition physique. Leurs fèces contenaient sporadiquement du sang occulte, mais il en était de même chez les animaux témoins. L'autopsie et l'examen microscopique n'ont révélé que des altérations mineures du tractus intestinal; aucune n'était imputable à la consommation de HMR. Des constatations similaires ont été faites chez les animaux qui avaient reçu 1% de HMR pendant 11 semaines puis, après une période de récupération de 11 semaines, pendant laquelle ils recevaient de l'eau de ville, des doses croissantes de 50 à 1250 mg HMR/kg pendant 12 semaines.

Les singes qui recevaient 0,5 ou 1,0% de solution de C16 ont gagné en poids, mais les pertes de poids ont été considérables chez ceux qui recevaient 2% de C16 (soit une consommation d'environ 2,9 g/kg/j). Tous les singes qui consommaient du C16 ont souffert de fréquentes hémorragies intestinales et contracté un certain degré d'anémie. Les modifications pathologiques du côlon allaient d'érosions peu profondes de la muqueuse à des ulcérations associées à des infiltrations cellulaires, à la présence de tissu de granulation dans la lamina propria et à la formation d'abcès des cryptes. La gravité de ces effets dépendait de la dose et on a observé une certaine récession des effets chez des singes qui avaient été mis au régime de l'eau de ville pendant 20 à 24 semaines après avoir reçu 2% de C16 pendant 14 semaines.

Cette expérience a donc fait apparaître une nette différence entre les effets du carragène dégradé (C16) et ceux du carragène pur (HMR) chez le singe.

Wirkung von Carrageenen im Verdauungstrakt des Rhesusaffen (*Macaca mulatta*)

Zusammenfassung—Bei einem mit männlichen und weiblichen Rhesusaffen (*Macaca mulatta*) durchgeführten Versuch wurde die Wirkung einer einheimischen Form von Carrageen (HMR) aus *Chondrus crispus* verglichen mit der einer abgebauten Form (C16) aus *Eucheuma spinosum*. Beide Typen wurden 7–14 Wochen lang im Trinkwasser verabreicht. Affen, die 1% HMR (entsprechend einer Aufnahme von etwa 1,3 g/kg/Tag) erhielten, nahmen an Gewicht zu und verblieben in gutem Zustand. Okkultes Blut erschien sporadisch in den Faeces wie auch bei den Kontrolltieren. Bei der Sektion wurden im Verdauungstrakt und bei mikroskopischer Untersuchung nur geringfügige Veränderungen festgestellt, von denen keine der HMR-Verabreichung zugeschrieben werden konnte. Ähnliche Befunde wurden bei Tieren verzeichnet, die 11 Wochen lang 1% HMR und nach einer bis 11 Wochen währenden Erholungsperiode mit Leitungswasser bis 12 Wochen lang sich steigernde tägliche Dosen von 50–1250 mg HMR/kg erhalten hatten. Affen, die 0,5 oder 1,0% C16-Lösung erhielten, nahmen an Gewicht zu, aber diejenigen, die 2,0% C16 erhielten (entsprechend einer Aufnahme von etwa 2,9 g/kg/Tag), erlitten beträchtliche Gewichtsverluste. Alle Affen, die C16 erhielten, verloren häufig Blut aus dem Verdauungstrakt und entwickelten einen gewissen Grad von Anämie. Pathologische Veränderungen im Dickdarm reichten von flachen Schleimhauterosionen bis zur mit Zellinfiltration verbundenen Ulceration, Granulationsgewebe in den Lamina propria und multiplen Kryptenabszessen. Die Schwere dieser Wirkungen war dosisabhängig, und eine gewisse Milderung zeigte sich bei Affen, die 2% C16 14 Wochen lang erhalten hatten und sich dann 20–24 Wochen lang mit Leitungswasser erholen konnten. Somit wurde ein deutlicher Unterschied zwischen der Wirkung von abgebautem (C16) und einheimischem (HMR) Carrageen im Affen nachgewiesen.

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Sensitivity of the Guinea-pig to Raw Soya Bean in the Diet

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Abstract—The effects of raw soya bean in the diet were investigated in the guinea-pig. Weanling guinea-pigs fed raw soya bean for 30 days suffered a high mortality rate. The excised pancreases from the test animals were significantly larger than those from control animals fed a casein diet, but the enlargement was not as marked as that reported for rats and chicks fed similar dietary regimens. Pancreatic trypsinogen, chymotrypsinogen and amylase were all increased by soya feeding in the young guinea-pig. Adult guinea-pigs fed the same soya-bean diet suffered no deaths and showed no pancreatic enlargement compared with controls. Pancreatic trypsinogen and amylase activities were higher than those of control animals but chymotrypsinogen was not changed. The response of the guinea-pig to dietary raw soya bean is similar to that previously observed in other rodents.

INTRODUCTION

It is well established that rats (Haines & Lyman, 1961), mice (Nitsan & Bondi, 1965), and chickens (Kakade, Barton, Schaible & Evans, 1967) fed raw soya bean in the diet develop an enlarged pancreas, secrete an abnormally high level of pancreatic enzymes and subsequently exhibit growth depression. These changes appear to be caused primarily by heat-labile trypsin inhibitors present in the soya bean (Khayambashi & Lyman, 1966). More recent investigations have shown that cattle (Gorrill & Thomas, 1967), pigs (Pekas, 1966) and dogs (Patten, Richards & Pope, 1971) appear to be resistant to some of the dietary effects of raw soya bean. In view of this indication of species dependence, we sought to determine whether the response of the guinea-pig to diets containing raw soya bean was similar to that of other rodents.

EXPERIMENTAL

Material. The raw soya bean used was obtained from Central Soya, Chicago, Ill.

Animals. Weanling and adult male English short-haired guinea-pigs, weighing approximately 200 and 600 g, respectively, were purchased from Rich-Glo Rabbitry, El Campo, Texas.

Diets. Animals were fed *ad lib.* either a 35% casein (control) diet or a diet containing 30% raw soya bean and supplemented with casein to balance the nitrogen content with that of the control diet. The nutrient compositions of the diets fed to young and adult animals are listed in Table 1. All animals were given water containing 3 mg ascorbic acid/ml *ad lib.* throughout the experimental period.

Experimental design and conduct. The adult and weanling animals were divided in each case into two groups of approximately the same size. Animals were housed individually

in wire cages throughout the 30-day experimental period. For three consecutive days at 10-day intervals during the experimental period, weanling guinea-pigs were placed in metabolic cages so that food intake could be recorded and urine and faeces separately collected. Body weights were recorded twice weekly for all animals. At the conclusion of the experimental period, the guinea-pigs were fasted for 14 hr and then killed with ether. The

Table 1. *Composition of test and control diets fed to weanling and adult guinea-pigs*

	Composition (%) of diets for			
	Young guinea-pigs		Adult guinea-pigs	
Component	Control	Experimental	Control	Experimental
Casein	35·0	18·0	33·2	16·0
Soya-bean flakes*	—	30·0	—	30·0
Cornstarch	15·0	9·0	13·0	7·2
Alfalfa meal	—	—	10·0	10·0
Cellophane spangles	15·0	15·0	12·0	12·2
Sucrose	10·3	6·3	9·1	5·0
Glucose	7·8	4·8	6·9	3·8
Corn oil	7·3	7·3	7·1	7·1
Salts†	6·0	6·0	6·0	6·0
CH_3COOK	2·5	2·5	1·6	1·6
MgO	0·5	0·5	0·5	0·5
Vitamin mixture‡	0·6	0·6	0·6	0·6

*Raw, defatted, dehulled soya bean flakes.
†Briggs Chick Salt A (Nutritional Biochemicals Co., Cleveland, Ohio).
‡Compounded according to Reid & Briggs (1953).

pancreases were excised, weighed and homogenized at 4°C in 0·15 M-NaCl containing 0·1% Triton X-100. The pancreatic homogenate was centrifuged and the supernatant was assayed for total protein by the method of Waddell (1956). Pancreatic trypsinogen, chymotrypsinogen and amylase activities were determined as described previously (Patten *et al.* 1971). Total faecal nitrogen was assayed by the micro-Kjeldahl procedure of Folin & Farmer (1912) and urea nitrogen was estimated by reaction with diacetyl monoxime as described by Kaplan (1969).

The results were statistically evaluated by Student's *t* test.

RESULTS

During the 30-day experimental period, food and nitrogen intake as well as body weight gain were significantly depressed ($P < 0·01$) in young guinea-pigs fed the 30% raw soya-bean diet, compared with controls receiving the casein diet (Table 2). There appeared to be no significant difference between control and experimental animals with respect to nitrogen loss in the urine. However, guinea-pigs fed raw soya bean excreted significantly more faecal nitrogen daily ($P < 0·01$) than control animals. This difference in faecal nitrogen excretion was observed between the groups after wk 1 of the study and was maintained throughout the remaining experimental period. In contrast to the body-weight changes, the excised pancreases from young animals fed the soya-bean diet were significantly larger ($P < 0·01$)

Table 2. *Effects of 30-day feeding of a diet containing raw soya bean on body weight, food intake and nitrogen excretion in young guinea-pigs*

Parameter	Control group	Experimental group
Number of animals	10	13
Total body-weight gain†, g	173·9 ± 9·1	99·7 ± 9·2*
Food intake‡, g/day	24·1 ± 0·7	18·9 ± 0·7*
N intake‡, g/day	1·16 ± 0·03	0·91 ± 0·03*
Urinary urea N‡, mg/day	14·08 ± 3·25	8·72 ± 0·93
Faecal N‡, mg/day	85·7 ± 3·4	155·0 ± 10·8*

†Over 30-day experimental period.
‡Determined on three consecutive days at 10-day intervals.
Values are means ± SEM and those marked with an asterisk differ significantly (Student's *t* test) from the control value: $*P<0·01$.

than those from casein-fed controls (Table 3). Pancreatic enzyme activities were significantly higher ($P<0·01$) in young guinea-pigs fed raw soya bean than in control animals, but no difference in pancreatic protein concentration was apparent between the two groups. The activities of amylase, trypsinogen and chymotrypsinogen (estimated per gram of pancreas) in soya-fed guinea-pigs were at least double those of animals maintained on the casein diet (Table 3).

In our studies in young guinea-pigs, 20 animals were originally placed on the raw soya-bean diet. After approximately 14 days on the experimental diet, seven of these animals showed progressive signs of B-vitamin deficiency. These symptoms included profuse salivation, loss of hair and degenerating equilibrium. The affected animals died between experimental days 18 and 21 and autopsies of three animals revealed enlarged caeca and occasional cases of Salmonella and bronchopneumonia. No other abnormal pathology was reported. In a subsequent pilot study, supplementation with vitamins of the B complex, either in the diet or parenterally, did not alleviate the detrimental effects of the 30% raw soya-bean diet on young guinea-pigs. Approximately 70% of the total number of animals

Table 3. *Effects of 30-day ingestion of raw soya bean on the pancreas of young guinea-pigs*

Parameter	Control group	Experimental group
Number of animals	10	13
Absolute pancreas weight, g	1·23 ± 0·04	1·42 ± 0·05*
Relative pancreas weight, g/100 g body weight	0·29 ± 0·01	0·42 ± 0·01*
Protein, mg/g pancreas	160·13 ± 6·01	149·29 ± 6·33
Trypsinogen		
K-units/g pancreas	0·78 ± 0·04	1·80 ± 0·13*
Units/mg protein	4·90 ± 0·36	12·36 ± 1·03*
Chymotrypsinogen		
K-units/g pancreas	0·12 ± 0·01	0·27 ± 0·02*
Units/mg protein	0·75 ± 0·05	1·83 ± 0·18*
Amylase		
K-units/g pancreas	7·87 ± 1·24	17·19 ± 1·92*
Units/mg protein	51·11 ± 3·65	121·63 ± 9·63*

Values are means ± SEM and those marked with an asterisk differ significantly (Student's *t* test) from the control value: $*P<0·01$.

fed the raw soya-bean diet with vitamin supplementation died before completion of the 30-day experimental period, and pancreatic enlargement and elevated enzyme levels were still found.

Adult guinea-pigs fed the 30% raw soya-bean diet lost an average of 113 g body weight during the experiment, while the casein-fed control animals gained 126 g during the same time (Table 4). There was no evidence of pancreas enlargement in soya-fed animals when

Table 4. *Effects of 30-day ingestion of raw soya bean on body weight and the pancreas of adult guinea-pigs*

Parameter	Control group	Experimental group
Number of animals	5	5
Change in body weight†, g	+125·6 ± 16·3	−113·2 ± 9·9*
Pancreas weight, g	2·00 ± 0·02	1·95 ± 0·16
Relative weight, g/100 g body weight	0·29 ± 0·01	0·38 ± 0·02*
Protein, mg/g pancreas	117·6 ± 5·4	107·8 ± 9·8
Trypsinogen		
K-units/g pancreas	0·88 ± 0·09	1·55 ± 0·14*
Units/mg protein	7·67 ± 1·08	14·47 ± 0·84*
Chymotrypsinogen		
K-units/g pancreas	0·20 ± 0·02	0·21 ± 0·03
Units/mg protein	1·72 ± 0·20	1·92 ± 0·17
Amylase		
K-units/g pancreas	7·80 ± 0·78	16·03 ± 1·70*
Units/mg protein	67·08 ± 8·38	150·64 ± 14·17*

†Over 30-day experimental period.
Values are means ± SEM and those marked with an asterisk differ significantly (Student's *t* test) from the control value: $*P < 0{\cdot}01$.

the absolute weight of the pancreas was considered. Although pancreas weight relative to body weight was significantly greater ($P < 0{\cdot}01$) for adult guinea-pigs fed raw soya bean than for controls, this difference was considered to reflect changes in body weight rather than in pancreas weight. Pancreatic protein levels did not appear to be affected by the inclusion of raw soya bean into the diet, but some changes were found in pancreatic enzyme activity (Table 4). Twofold increases in pancreatic amylase and trypsinogen activity were found in the soya-fed animals compared with controls. Chymotrypsinogen activity, however, was similar in the experimental and control groups. No adult guinea-pigs fed the raw soya-bean diet died during the experimental period, and none suffered the gross body changes observed in young guinea-pigs.

DISCUSSION

In the present study both young and adult guinea-pigs were adversely affected by dietary raw soya bean, but a high mortality rate occurred only in the young animals, which also showed greater pancreatic changes than did the adult animals. The gross appearance of young soya-fed guinea-pigs prior to death resembled previously reported observations in guinea-pigs fed various diets deficient in B vitamins (Reid, 1958). This suggested to us that the absorption and/or metabolism of essential nutrients might have been interfered with in young guinea-pigs fed raw soya bean, and that this might have lowered the animals' resistance to various infectious agents. Other workers have previously reported that the absorption of vitamin B_{12} (Edelstein & Guggenheim, 1969) and other nutrients (Miller,

Ullrey, Zutant, Haefer & Luecke, 1965) is impaired by the feeding of dietary raw soya bean. Although a pilot study in our laboratory did not indicate B-vitamin deficiency as the direct cause of death in young guinea-pigs, a more thorough absorption study is certainly required. High mortality rates have not been reported in other experimental animals fed raw soya bean.

The lower food intake of young guinea-pigs fed raw soya compared with casein-fed controls was apparently a major factor in depressing the growth response of the soya-fed group and also in increasing their susceptibility to disease. The reduced weight gain of young guinea-pigs may also have been related to factors causing the significantly enhanced faecal nitrogen losses observed in young animals fed raw soya bean. These factors include the enhanced pancreatic secretion of enzymes, as previously observed in studies with rats (Haines & Lyman, 1961), increased sloughing of intestinal epithelial cells (de Muelenaere, 1964) and/or a reduction in the absorption of exogenous and endogenous protein digests (Kwong & Barnes, 1963). This last factor appears to be supported in the present study by the trend towards lower urea-nitrogen values observed in guinea-pigs fed raw soya bean as compared with controls. The adult soya-fed guinea-pigs also lost weight, but these animals did not show the extensive gross body changes subsequently leading to death that were observed in young animals.

Although significant pancreas enlargement did occur in young guinea-pigs fed unheated soya bean, the increase in pancreatic size was not of the magnitude reported by other investigators in studies in chicks (Kakade *et al.* 1967) and young rats (Rackis, 1965). The response of the adult guinea-pig to raw soya bean in the diet appeared to be similar to that of the dog (Patten *et al.* 1971), calf (Gorrill & Thomas, 1967) and pig (Pekas, 1966), showing no pancreatic enlargement. The enzyme analyses of pancreases from both young and adult guinea-pigs fed raw soya bean in this investigation showed some similarities to those observed in other studies with rats (Konijn, Birk & Guggenheim, 1970) and chickens (Ma'Ayani & Kulka, 1968).

Although pancreatic trypsinogen was elevated in both young and adult experimental animals, chymotrypsinogen was increased only in the young. In addition, amylase was elevated by raw soya-bean feeding in both young and adult animals, a finding in opposition to those previously reported in rats (Konijn *et al.* 1970) and chickens (Ma'Ayani & Kulka, 1968).

That these pancreatic changes in size and enzyme activity may be due to trypsin inhibitors present in raw soya beans is supported by studies in rats (Melmed & Bouchier, 1969). Furthermore, it has since been indicated that soya-bean trypsin inhibitor causes the release of a humoral pancreozymin-like substance (Khayambashi & Lyman, 1969). Rothman & Wells (1967) had previously found that repeated administration of pancreozymin to rats caused pancreatic enlargement and increased synthesis of pancreatic trypsinogen, chymotrypsinogen and amylase. Hence, it has been suggested that the direct or indirect release of pancreozymin from the duodenal mucosa by soya-bean constituents may cause the subsequent pancreatic enlargement and increased enzyme concentrations observed in animals fed raw soya bean (Konijn *et al.* 1970; Melmed & Bouchier, 1969). In the present study in guinea-pigs, raw soya bean appeared to elicit a pancreatic response similar to that observed by Rothman & Wells (1967) in rats following pancreozymin administration. The release of pancreozymin from the duodenum and its specificity of action may vary in different animal species and age groups and may, therefore, produce the different responses observed in animals fed raw soya bean.

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Sensibilité du cobaye à un régime de soja cru

Résumé—On a étudié sur le cobaye les effets de la consommation de soja cru, en graines. Le taux de mortalité a été très élevé chez les cobayes sevrés nourris pendant 30 jours avec du soja cru. Les pancréas excisés des animaux d'essai étaient significativement plus grands que ceux des animaux témoins soumis à un régime de caséine, mais l'hypertrophie n'était pas aussi marquée que celle signalée chez des rats et des poussins soumis à des régimes alimentaires similaires. Le régime au soja a fait augmenter le trypsinogène pancréatique, le chymotrypsinogène et l'amylase chez les jeunes cobayes. Le même régime au soja n'a pas provoqué de mortalité ni d'hypertrophie du pancréas chez les cobayes adultes, comparativement aux animaux témoins. Les taux de trypsinogène pancréatique et d'activité de l'amylase étaient plus élevés que chez les animaux témoins, mais le chymotrypsinogène n'était pas modifié. La réaction du cobaye au régime de soja cru est semblable à celle observée précédemment chez d'autres rongeurs.

Empfindlichkeit des Meerschweinchens gegen rohe Sojabohnen im Futter

Zusammenfassung—Die Wirkung roher Sojabohnen im Futter wurde am Meerschweinchen untersucht. Abgesetzte Meerschweinchen, an die 30 Tage lang rohe Sojabohnen verfüttert wurden, zeigten eine hohe Mortalität. Die ausgeschnittenen Bauchspeicheldrüsen der Versuchstiere waren signifikant grösser als die von Kontrolltieren, an die ein Kaseinfutter verfüttert worden war, aber die Vergrösserung war nicht so markant wie die bei in gleicher Weise gefütterten Ratten und Küken. Pankreastrypsinogen, -chymotrypsinogen und -amylase waren im jungen Meerschweinchen sämtlich durch die Sojaverfütterung erhöht. Erwachsene Meerschweinchen, welche das gleiche Sojabohnenfutter erhielten, starben nicht und zeigten im Vergleich mit Kontrolltieren keine Pankreasvergrösserung. Die Pankreastrypsinogen- und -amylaseaktivität war höher als die bei Kontrolltieren, aber die Chymotrypsinogenaktivität war unverändert. Die Reaktion des Meerschweinchens auf rohe Sojabohnen im Futter ist ähnlich der, die schon bei anderen Nagetieren beobachtet wurde.

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Toxic Effects of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin

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Abstract—The toxic compound 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (dioxin) was shown to cause pericardial oedema and death in chickens after a single oral dose of 25–50 μg/kg. The extreme toxicity of dioxin to the guinea-pig was confirmed. Female rats given a single oral dose of 200 μg dioxin/kg had a depressed food intake and lost weight. The mean time to their death was 40·4 days. The animals showed no consistent post-mortem appearance, although gastric haemorrhage and jaundice were common. In the first 3 days after dosing there were significant changes in liver constitution, although there were no alterations in plasma components indicative of liver malfunction. The changes in blood cell counts, haematocrit, haemoglobin and plasma-protein levels 1 wk after dosing were most probably associated with the reduction in food intake. The liver showed pathological changes at later periods, in particular the formation of multinucleate parenchymal cells.

INTRODUCTION

Since the 1930s, chloracne has been recognized as an occupational hazard associated with the production or use of chlorinated aromatic compounds (Key & Birmingham, 1966). In particular, it has been linked with the manufacture of 2,4,5-trichlorophenol, a compound used in the preparation of the herbicide 2,4,5-trichlorophenoxyacetic acid and of the bactericide hexachlorophene (Poland, Smith, Metter & Possick, 1971). In groups of cases studied in Germany in the 1950s (Bauer, Schulz & Spiegelberg, 1961; Hofmann, 1957; Kimmig & Schulz, 1957; Schulz, 1968), the chloracne was associated with other disorders and the causative agent was identified as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (referred to henceforth as dioxin). Besides inducing the hyperkeratosis of chloracne, dioxin was found to be highly toxic to various animal species. In rabbits the LD_{50}, administered as a single oral dose, was 10 μg/kg (Schulz, 1968).

The formation of dioxin has been shown to occur when an alkali metal salt of 2,4,5-trichlorophenol is heated at elevated temperatures (Milnes, 1971). It is readily produced in 2,4,5-trichlorophenol manufacture, but if the process is carefully controlled dioxin contamination of the trichlorophenol is less than 1 ppm.

In an incident in the United States in 1957, some millions of chickens died, the most characteristic symptom being pericardial oedema. This condition was traced to the inclusion in the diet of a fat from which the toxic factors could be isolated in yields of approximately 100 μg/kg of fat (Harman, Davis, Ott, Brink & Kuehl, 1960; Wootton, Artman & Alexander, 1962; Yartzoff, Firestone, Banes, Horwitz, Friedman & Nesheim, 1961). One of these compounds was eventually identified by crystallography as 1,2,3,7,8,9-hexachlorodibenzo-*p*-dioxin (Cantrell, Webb & Mabis, 1969). Dioxin itself has been found to be very toxic in the chick oedema bioassay (Higginbotham, Huang, Firestone, Verrett, Ress & Campbell,

1968). Structurally similar compounds, tetra- and pentachlorodibenzofurans, are contaminants of polychlorinated biphenyls that cause hydropericardium in chicks and hyperkeratosis of the rabbit's ear (Vos & Beems, 1971; Vos & Koeman, 1970; Vos, Koeman, van der Maas, ten Noever de Brauw & de Vos, 1970). Other studies have shown that dioxin is a potent teratogen and foeticidal agent for rats and mice (Courtney & Moore, 1971; Neubert & Dillmann, 1972; Sparschu, Dunn & Rowe, 1971).

In the work now described, some effects of dioxin on various species were studied in an attempt to elucidate its mode of action.

EXPERIMENTAL

Preparation of dioxin. Dibenzo-*p*-dioxin was prepared by the method of Gilman & Dietrich (1957) save that the reaction was carried out in refluxing diethylene glycol dimethyl ether for 5–8 hr. Prior to crystallization, the dibenzodioxin was purified by steam-distillation, since this procedure removed a minor contaminant which otherwise conferred a pale-blue fluorescence and a yellow phosphorescence on the product. Dibenzodioxin was dissolved in glacial acetic acid and chlorine gas was bubbled through the solution under reflux for 4–8 hr (cf. Tomita, Ueda & Narisada, 1959). The solvent was evaporated and the residue was washed with ether. The resultant solid was crystallized several times from benzene to give material which was shown by gas–liquid chromatography (GLC) to contain 8·2% trichlorodibenzodioxin. (GLC was carried out on a Pye Series 104 chromatograph fitted with an electron-capture flame ionization detector head, using a 7 ft column of 3% E 30 on 100–120 mesh Diatomite CQ at 200°C and a carrier gas (nitrogen) flow rate of 67 ml/min.) It was assumed that the flame ionization detector response to the dioxins was proportional to their molecular weight; any decreased sensitivity to dioxin itself due to the extra chlorine atom would imply a lower degree of contamination than that indicated above. Dioxin was administered to animals either as a 100 μg/ml solution in dimethylsulphoxide (DMSO) or arachis oil or, for higher doses, as a suspension in arachis oil.

Animals and treatment. Male and female albino rats of the Porton strain, bred in this laboratory, were given Diet 41B and water *ad lib.* White Leghorn chickens (4–6 wk old) from Southern Biological Ltd., Carshalton, were given Layers Pellets and water *ad lib.* Adult albino guinea-pigs of the Porton strain had free access to Diet IGP and water supplemented with ascorbic acid. All the test animals were given a single orally intubated dose of dioxin in solution or suspension while controls received an equivalent volume of the appropriate solvent. For all three species, body weights were recorded at intervals throughout the observation period and autopsies were performed on all animals. In addition some of the treated rats given single doses up to 500 μg dioxin/kg were used for more extensive studies, covering food-consumption, haematology and liver weight and histology, in addition to growth and mortality. Details of the doses given and the periods of observation are reported in the appropriate parts of the 'Results' section. Six weanling SPF and two adult germ-free rats were also treated with dioxin in a small comparative mortality study.

Blood assays. For the assay of plasma components, blood was taken on days 1, 3, 9 and 21 after treatment in heparinized syringes from the heart of female rats, following the opening of the thorax under ether anaesthesia. The blood was centrifuged at 5°C as soon as possible after collection and the plasma was separated and stored frozen. Blood samples for cell counts and haemoglobin assays were taken weekly from the tail. Portions of these samples were diluted in Isoton® containing 30% bovine serum albumin solution (100:1,

®Trade name of Coulter Electronics Ltd., Dunstable, Beds.

v/v). The albumin stabilized the erythrocytes against haemolysis. Blood cell counts were performed on a Coulter counter. Total plasma bilirubin was assayed by the method of Ferro & Ham (1967) save that all operations were performed in a dimly-lit room, since it was found that bilirubin solutions, the colour reagent and mixtures of the two were all photolabile. Plasma protein was estimated with the biuret reagent. Kits for the assay of plasma alkaline phosphatase, glutamic–oxalacetic transaminase (GOT) and glutamic–pyruvic transaminase (GPT) were obtained from Sigma Chemical Co., Ltd., London, and a kit for the assay of plasma cholesterol by the Liebermann–Burchard reaction was obtained from Roche Products Ltd., London.

Liver studies. The weight and water content of liver were determined 24 and 72 hr after dioxin treatment on samples removed as rapidly as possible following decapitation and dried to constant weight at 110 ± 5°C. The livers were from male and female animals which had been used for the determination of hexobarbitone sleeping times (Greig, 1972).

Histology. Female rats were killed 2–60 days after oral doses of dioxin (50–400 μg/kg body weight). Samples were taken from all the major organs except the brain and fixed in formol alcohol. Paraffin sections were prepared in the usual way and stained with Harris' haematoxylin and eosin.

Statistical methods. The significance of the results was assessed by Student's *t* test, unless otherwise indicated.

RESULTS

Toxicity of dioxin to chickens

The toxicity of dioxin towards chickens was confirmed, a dose of 25–50 μg/kg given orally killing them 12–21 days later. The only immediate effect of the dioxin on the birds was that they gained weight less rapidly than controls. Prior to death some birds lost weight and were in poor condition, with laboured breathing, the beak agape and feathers ruffled. The most common post-mortem finding was some accumulation of serous fluid in the pericardial sac, as has been described for the chick oedema syndrome (Allen & Carstens, 1966; Schmittle, Edwards & Morris, 1958). It thus appears that a single dose of dioxin is as effective as prolonged feeding in causing pericardial oedema (Allen & Carstens, 1966).

Toxicity of dioxin to guinea-pigs

The effect of dioxin (2, 4 or 10 μg/kg) on groups of three male and three female adult guinea-pigs was a 15–30% loss of body weight within 8–24 days. Two animals (a female given 10 μg/kg and a male given 4 μg/kg) died within this period and the others were killed when in poor condition. Autopsy revealed no obvious abnormalities of the major viscera, except that there was little or no food in the gastro-intestinal tract and the stomachs were distended with gas. This confirmed the extreme susceptibility of the guinea-pig to dioxin (Sparschu *et al.* 1971).

Effects of dioxin in rats

Growth, mortality and post-mortem studies. The oesophageal intubation of female rats (8–11 wk old, body weight 180–200 g) with solutions of 200 μg dioxin/kg in DMSO led to an immediate and prolonged reduction in food intake, with a loss of body weight (Fig. 1). There was a wide variation in the response of the animals to dioxin, as is shown by the greater standard errors of body-weight measurements on dosed animals compared to their

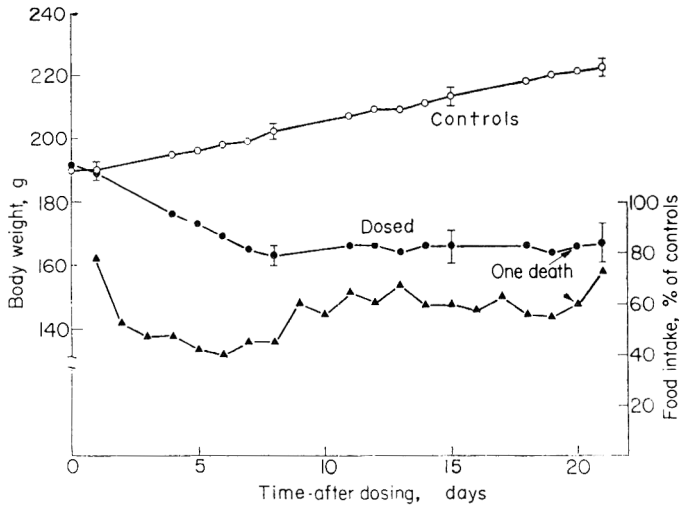

FIG. 1. Mean body weights (±SEM where indicated) of female control rats (○) and of female rats given a single oral dose of 200 μg dioxin/kg (●), and food intake of the treated rats (▲) expressed as a percentage of the intake in the control group. The dosed and control groups each comprised 18 animals. Immediately after dosing, the pellet diet was replaced with powdered diet and the food consumption of groups of six animals was measured daily.

controls. A few animals maintained a nearly constant weight for 3–7 wk after dosing and then underwent a rapid loss of weight. The effect on weanling female rats (4–5 wk old, 60–70 g) of single, oral doses of 25–200 μg/kg was a lower or zero growth rate compared with controls.

Attempts to estimate an LD_{50} value in female rats given a single oral dose of dioxin failed because of the irregular distribution of deaths in the treated groups. The mortality figures from two experiments are set out in Table 1. The mean time to death in these experiments was 40·4 ± 4·5 (SEM) days.

Table 1. *90-Day mortality of female rats given a single oral dose of 0–300 μg dioxin/kg*

Experiment 1*		Experiment 2†	
Dosage (μg/kg)	Deaths/no. in group	Dosage (μg/kg)	Deaths/no. in group
0	0/12	0	0/6
30	1/6	126	3/6‡
48	1/6	199	1/6
75	1/6	315	1/6
120	1/6	500	4/6
190	3/6		
300	3/5		

*Rats (170–200 g, 8–9 wk old) received dioxin in dimethylsulphoxide, of which controls received 3 ml/kg.
†Rats (170–200 g, 9–10 wk old) received dioxin in arachis oil, of which controls received 5 ml/kg.
‡Excluding one animal killed at 42 days with severe swelling of the limbs and urine staining in the genital area.

In rats dying after dioxin treatment, no consistent changes were observed at autopsy. A common finding was gross haemorrhage, usually originating from the mucosa of the glandular stomach but on occasions involving the ileum or the retroperitoneal tissue. Some rats were jaundiced and this was sometimes associated with distension of the bile duct as far as the duodenum. No physical obstruction other than abnormal amounts of mucus in the duodenum could be found in these rats. Changes in the lungs were common and ranged from petechial haemorrhage or perivascular oedema to massive infection. Six weanling SPF Grade IV and two adult germ-free rats dosed with dioxin and maintained in a sterile isolator showed no signs of infection at autopsy, but deaths still occurred (two SPF rats by 24 days and one of the germ-free adults by 21 days). One of these rats had bile-duct enlargement and another had duodenal haemorrhage associated with jaundice.

A tenfold increase in the oral dose of dioxin (1·25–5·0 mg/kg suspended in arachis oil) administered to six female rats (170–190 g) did not accelerate the appearance of toxic effects. The weight loss of these animals after 7 and 14 days (7·1 ± 3·8 and 19·7 ± 3·4%, respectively) did not differ significantly from that in rats given 200 μg/kg (19·8 ± 2·8 and 18·9 ± 3·1%). Two of the animals on the high doses died at 16 and 21 days, but the post-mortem appearance of the four survivors, which were killed at 23 days, was similar to that of the animals given a lower dose and killed after 3–4 wk, save that excessive peritoneal fluid was present in three of the animals.

Haematology. A single oral dose of dioxin (200 μg/kg) administered to female rats (180–200 g) raised their red cell count by the end of 1 wk (Table 2). This increase persisted for 2 and 3 wk after dosing and was associated with a rise in the haematocrit (Table 3) and haemoglobin content of the blood. Leucocyte counts in both dosed and control groups fluctuated considerably but were significantly elevated in the dosed group at 2 and 3 wk.

Liver function and weight. The jaundice seen in some rats at autopsy suggested that the liver might be a target organ. Therefore the levels of various plasma components indicative of liver function were measured (Table 3). None of these were altered to any large extent either 1 or 3 days after the administration of an oral dose of dioxin; later the level of plasma bilirubin was raised and that of plasma protein was lowered.

Although measures of liver function were not affected by dioxin at 1 and 3 days, there were significant alterations in liver components (Table 4). The wet and dry weights of the liver, expressed as a percentage of body weight, were increased in male and female rats on days 1 and 3 after treatment, respectively. In both sexes the water content was significantly increased on days 1 and 3. This effect was not due to the altered food intake of dioxin-treated rats, since when food was withheld immediately after treatment from both dosed and control male rats, similar increases were observed in the dosed group 24 hr later.

Histology. Scattered areas of necrosis were present in the centrilobular zone of the rat liver 3 wk after dioxin treatment. This loss of parenchymal cells resulted in apparent dilatation of surrounding sinusoids. The scattered necrosis adjacent to the central veins continued throughout the period studied and was associated with a prominent infiltration of mononuclear cells. By day 60 the walls of the central veins were grossly thickened by connective tissue and mononuclear cells and the sinusoidal dilatation persisted (Fig. 2). The arrangement of liver trabeculae was distorted. The parenchymal cells varied in size and shape, with many multinucleate forms containing 4–30 nuclei (Fig. 3). These were found in the centrilobular area. Large hepatocytes with between four and six nuclei appeared as early as day 21. Within the multinucleate cells an occasional pyknotic nucleus was observed. No mitotic figures were seen in the multinucleate cells. The continuing necrotic lesion was associated

Table 2. *Effect of a single oral dose of 200 μg dioxin/kg on the haematology of female rats*

Time after treatment (days)	Treatment†	Body weight (g)	Erythrocytes (10^6/mm^3)	Leucocytes (10^3/mm^3)	Haemoglobin (g/100 ml)
0	Control	209·6 ± 2·1	7·75 ± 0·15	8·58 ± 0·89	16·0 ± 0·3
	Dioxin	202·8 ± 3·6	8·00 ± 0·22	10·22 ± 1·60	16·5 ± 0·5
7	Control	217·2 ± 3·9	7·36 ± 0·17	4·20 ± 0·60	15·1 ± 0·4
	Dioxin	178·7 ± 6·2***	9·40 ± 0·31***	6·72 ± 1·25	19·0 ± 0·6***
14	Control	224·4 ± 4·7	7·56 ± 0·15	3·16 ± 0·38	16·1 ± 0·2
	Dioxin	160·3 ± 6·9***	10·43 ± 0·43***	7·15 ± 1·72*	20·2 ± 0·5***
21	Control	231·8 ± 5·7	8·24 ± 0·30	5·90 ± 0·73	16·5 ± 0·5
	Dioxin	156·8 ± 9·7***	10·65 ± 0·85*	14·27 ± 1·70**	19·7 ± 1·7

†Dioxin (200 μg/kg) was dissolved in dimethylsulphoxide, while controls received an equivalent volume of the vehicle alone. Values are means ± SEM for five control or six treated rats and those marked with asterisks differ significantly from the controls: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table 3. *Effect of a single oral dose of 200 μg dioxin/kg on blood constituents of female rats*

Time after treatment (days)	Treatment†	Haematocrit (%)	Plasma levels of: Protein (mg/ml)	Cholesterol (mg/100 ml)	Bilirubin (mg/100 ml)	Alkaline phosphatase (Sigma units/ml)	GOT (Karmen units/ml)	GPT (Karmen units/ml)
1	Control	45·6 ± 1·1	76·9 ± 0·9	73·3 ± 6·8	0·50 ± 0·1	3·02 ± 0·63	181·0 ± 63·0‡	30·2 ± 2·3
	Dioxin	47·0 ± 0·8	76·5 ± 0·2	74·2 ± 4·4	0·55 ± 0·1	1·73 ± 0·07	75·3 ± 5·3§	26·1 ± 1·3§
3	Control	45·9 ± 0·7	68·7 ± 1·2	65·3 ± 5·3	0·25 ± 0·04	2·3 ± 0·2	81·2 ± 3·3	26·5 ± 1·6
	Dioxin	48·0 ± 0·8	69·6 ± 2·5	73·9 ± 5·0	0·49 ± 0·11	2·1 ± 0·2	98·9 ± 4·5*	24·2 ± 3·4
9	Control	43·3 ± 0·7	68·7 ± 1·2	—	—	—	—	—
	Dioxin	48·4 ± 1·1**	62·3 ± 2·5*	—	—	—	—	—
21	Control	42·1 ± 0·7	—	—	0·33 ± 0·13	—	—	—
	Dioxin	47·7 ± 1·6*	—	—	10·97 ± 4·63§‖	—	—	—

GOT = Glutamic–oxalacetic transaminase GPT = Glutamic–pyruvic transaminase

†Dioxin (200 μg/kg) was dissolved in dimethylsulphoxide, while controls received an equivalent volume of the vehicle alone.

‡Haemolysis affected results.

§One sample lost.

‖Value differs significantly (ranking test) from control: $P = 0.008$.

Values are means ± SEM for groups of six rats (or five in the case of controls at 1 and 21 days). Those marked with asterisks differ significantly from the controls: $*P < 0.05$; $**P < 0.01$.

Table 4. *Weight and water content of the liver in rats given an oral dose of 200 μg dioxin/kg*

Time after treatment (hr)	Sex	Treatment†	Liver weight (g/100 g body weight)		Water content of liver (%)
			Wet weight	Dry weight	
24	M	Control	4·74 ± 0·10	1·42 ± 0·03	70·08 ± 0·11
		Dioxin	5·28 ± 0·10**	1·54 ± 0·03*	70·86 ± 0·12***
	F	Control	4·20 ± 0·06	1·24 ± 0·02	70·45 ± 0·19
		Dioxin	4·64 ± 0·17*	1·33 ± 0·04	71·38 ± 0·28*
72	M	Control	4·71 ± 0·17	1·39 ± 0·04	70·61 ± 0·12
		Dioxin	4·95 ± 0·22	1·40 ± 0·06	71·63 ± 0·06***
	F	Control	3·81 ± 0·06	1·13 ± 0·02	70·44 ± 0·14
		Dioxin	4·61 ± 0·09***	1·32 ± 0·02***	71·41 ± 0·28**
24‡	M	Control	3·61 ± 0·09	1·05 ± 0·02	70·77 ± 0·11
		Dioxin	4·02 ± 0·08**	1·15 ± 0·02**	71·37 ± 0·19*

†Dioxin (200 μg/kg) was dissolved in dimethylsulphoxide, while controls received an equivalent volume of the vehicle alone.
‡Food was withheld from both groups immediately after dosing. The vehicle was arachis oil.
Values are means ± SEM for groups of six rats (or 12 in the case of treated and control females at 72 hr). Those marked with asterisks differ significantly from controls: $*P < 0·05$; $**P < 0·01$; $***P < 0·001$.

with an increased incidence of parenchymal cell mitoses throughout the liver lobule. Identical histological changes were produced at each dose level studied and there was no evidence that higher doses increased the parenchymal cell loss.

No lesions attributable to dioxin were observed in the rat lungs, but many animals that died following dioxin dosage had severe chronic and acute inflammatory lung lesions. Several animals had pronounced congestion of the mucosa and submucosa of the stomach and duodenum. No definite ulcers were seen in the sections examined. No significant abnormality was seen in the spleen and lymph nodes.

DISCUSSION

The findings of this study were generally in accord with others already reported. Thus a depression in growth rate or a loss of weight has been reported to follow a dose of 1 μg–10 mg dioxin/kg given ip or orally (Buu-Hoï, Pham-Huu Chanh, Sesqué, Azum-Gelade & Saint-Ruf, 1972a; Cunningham & Williams, 1972). An increase in plasma bilirubin and reduction in plasma protein was also described by Buu-Hoï, Pham-Huu Chanh, Sesqué, Azum-Gelade & Saint-Ruf (1972b) following massive (10 mg/kg) doses of dioxin given ip. It is possible that the prolonged reduction in food intake may have contributed to the depression in plasma-protein levels, and also the rises seen in the red and white blood cell counts, haematocrit and haemoglobin levels. Similar changes associated with inanition have been reported by Weimer (1961). Norback & Allen (1972) observed moderate hypertrophy of the liver in male rats receiving a diet containing 0·002% "chlorinated diphenyl-*p*-dioxin". In the present study, the increase in dry liver weight did not correlate with any changes in the protein or DNA content of the organ but may have been due to an increase in liver lipids (Cunningham & Williams, 1972).

The delayed deaths of rats and guinea-pigs after a single small dose of dioxin cannot be explained by any consistent pathological change in the dead or dying animals. Although

all rats had liver damage, the extent of this was such that it was difficult to attribute the death of the animals to a gross hepatic failure. The histological appearance of the liver was unusual in that there was a progressive lesion with the formation of multinucleate cells and some fibrosis. These changes developed in the absence of extensive focal or zonal necrosis when the livers were examined by conventional histological methods. The multinucleate cells may have been formed by fusion of parenchymal cells and the mechanism of this is under investigation. Liver lesions similar to those seen in this study have been reported in *Macaca mulatta* monkeys fed toxic fat (Allen & Carstens, 1967) and in male Wistar rats given 10 mg dioxin/kg body weight by ip injection (Buu-Hoï *et al.* 1972a). Both of these groups described multinucleate cells and a centrilobular lesion, although the abnormal cells were less bizarre than those described in this study.

The deaths and similar morbidity in germ-free and SPF rats given dioxin indicated that the terminal pulmonary infection in conventional animals was not the primary cause of death.

Our interpretation of the findings is that dioxin interferes with the capacity of the liver cells to maintain their correct organization; in some cells this leads to death and in others to disorganization of structure. This is not inconsistent with a mode of action 'of a cascade-type involving a series of biochemical lesions' (Buu-Hoï *et al.* 1972b). We have confirmed that the liver microsomal mixed-function oxidase system of rats given dioxin is seriously disturbed (Greig, 1972; J. B. Greig, unpublished results 1972). The possibility that the mode of action of dioxin is linked with this prolonged disturbance is being studied.

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Effets toxiques de la 2,3,7,8-tétrachlorodibenzo-*p*-dioxine

Résumé—On a démontré qu'une seule dose orale de 25 à 50 μg/kg du composé toxique 2,3,7,8-tétrachlorodibenzo-*p*-dioxine (dioxine) provoque de l'oedème du péricarde et la mort chez le poulet. L'extrême toxicité de la dioxine chez le cobaye est confirmée. Une dose orale unique de 200 μg de dioxine/kg a fait diminuer la consommation de nourriture et le poids chez des rats femelles, qui n'ont plus survécu, en moyenne, que de 40,4 jours. Quoique des hémorragies gastriques et des jaunisses fussent courantes, le tableau d'autopsie de ces animaux n'était pas uniforme. La constitution du foie s'était modifiée significativement dans les 3 jours suivant l'administration, sans toutefois que les composants du plasma présentassent des altérations révélatrices d'une mauvaise fonction du foie. Les modifications de la numération globulaire, de l'hématocrite, de l'hémoglobine et des taux de protéines du plasma observées une semaine après l'administration étaient très probablement associées à la diminution de la consommation de nourriture. Des modifications pathologiques du foie se sont manifestées plus tard; il s'agissait plus particulièrement de formations de cellules parenchymales multinucléées.

Toxische Wirkungen von 2,3,7,8-Tetrachlordibenzo-*p*-dioxin

Zusammenfassung—Die toxische Verbindung 2,3,7,8-Tetrachlordibenzo-*p*-dioxin (Dioxin) verursacht, wie gezeigt wurde, pericardiales Ödem und den Tod von Küken nach einer einzelnen oralen Dosis von 25–50 μg/kg. Die extreme Toxizität von Dioxin beim Meerschweinchen wurde bestätigt. Bei weiblichen Ratten, die eine einzelne orale Dosis von 200 μg Dioxin/kg erhalten hatten, kam es zu herabgesetztem Futterverbrauch und zu Gewichtsverlust. Die mittlere Zeit bis zu ihrem Tod betrug 40,4 Tage. Die Tiere zeigten keine gleichmässige Postmortem-Erscheinung, wenn auch Magenblutung und Gelbsucht häufig waren. In den ersten 3 Tagen nach der Verabreichung traten signifikante Veränderungen in der Leberkonstitution ein, obwohl es keine Änderungen bei den Plasmabestandteilen gab, die auf eine gestörte Leberfunktion deuteten. Die Änderungen der Blutkörperchenzahl, des Hämatokritwertes, des Hämoglobins und der Plasmaproteinkonzentrationen 1 Woche nach der Verabreichung waren höchstwahrscheinlich mit der Reduzierung des Futterverbrauchs verbunden. Die Leber zeigte zu späteren Zeiten pathologische Veränderungen, besonders die Bildung mehrkerniger Parenchymzellen.

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Is Oestrogenic Activity Present in Hops?

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Abstract—During the last 20 yr, several claims have been made for the presence of large quantities of oestrogens in hops and it has been alleged that this hormonal activity appears in beer. The oestrogenic activities reported were very high in comparison with those in other plants. A number of purified hop components, including the essential-oil fraction and the α- and β-bitter acids obtained from hop resins, have now been examined and various organic-solvent extracts of hops grown in the USA and in Europe have been prepared and tested for oestrogenic activity, both directly and following saponification in base. Commercial hop extracts were also tested for oestrogenic activity. Wide ranges of dilutions of all preparations were examined by the uterine-weight assay in the immature female mouse. No oestrogenic activity was detectable in any of these preparations.

INTRODUCTION

The presence of significant quantities of steroidal and nonsteroidal oestrogens in plants has been documented repeatedly. The original demonstration of the occurrence of oestrone in date palm with a structure and activity identical to those of the normal animal hormone (Butenandt & Jacobi, 1933) has been followed by the identification of several other steroidal oestrogens from plant sources. A dramatic instance of the ill-effects of plant oestrogens on animals was the demonstration that the interference with the reproductive processes of grazing animals feeding on subterranean clover could be ascribed to the presence of a new class of oestrogenic compounds, the isoflavones (Bradbury & White, 1954). The coumoestrol family of compounds represents yet another type of structure with oestrogenic activity and is reported to occur in a number of legumes (Bickoff, Booth, Lyman, Livingston, Thompson & DeEds, 1957).

There has been recent public concern over reports that hops used in the brewing industry contain sizeable quantities of oestrogens which may even enter beer. This concern appears to be based on three published scientific reports.

In the first of these, a brief note totally lacking in experimental detail, Koch & Heim (1953) of the Veterinary Institute of the University of Munich reported that oestrogenic activity was present in hops and beer. The attention of these investigators was directed toward a search for oestrogenic activity in hops by the legend in lay circles that when women who normally lived at a distance from hop gardens assembled for the harvesting of hops, they began regularly to menstruate 2 days after commencing to pick the hops. Although hops were used medicinally as sedatives at that time, no studies on their hormonal content appeared to have been reported. The oestrogen content of other plants had been documented, however.

Koch & Heim (1953) used the Allen–Doisy test (vaginal cornification in castrated rats) and claimed that the oestrogenic activity of hops was 20,000–300,000 IU/100 g, which would correspond to the presence of the equivalent of 20–300 μg 17β-oestradiol/g. This is in fact an extraordinarily large amount in relation to other plants and is comparable to, or higher than, the daily intake of oestrogens by women taking certain oral contraceptive preparations. According to Koch & Heim (1953) only a small fraction of the solid material of hops enters the beer. The usual ratio of hops to final beer is 2–2·5 g hops/litre beer. On analysing beer, Koch & Heim (1953) found 10–360 IU/litre in different samples, i.e. 1–36 μg 17β-oestradiol equivalent/litre, and they claimed that the oestrogen content of "heavily hopped" beer was higher. It would be concluded on the basis of these figures that only 2·5–5% of the oestrogen content of the hops found its way eventually into the beer.

The next contribution on this subject appears to have been that of Zenisek & Bednar (1960), who reported on the oestrogenic hormonal activity associated with the β-bitter acid fractions (Stevens, 1967) but could not account for the entire oestrogenic activity of hops on the basis of the hormonal activity of the β-bitter acids. Using the Allen–Doisy test and expressing oestrogenic activity in μg 17β-oestradiol equivalents/g, these authors found that the activity of an extract prepared by mixing a water extract of hops (tannins) with a lipophilic-solvent extract (bitter acids and resins) was 1000 and that of an extract prepared with a lipophilic solvent (petroleum ether) only and containing more bitter acids was 1250, while the oestrogenic activities of β-bitter acid and of hops were 1500 and 30–300 respectively.

Subsequently Churý (1960) claimed the presence of oestrogenic activity in a 95% ethanol extract of hops that had been subjected to base saponification. In this work, hops, red clover, peas and cabbage were extracted three times with boiling 96% ethanol which was partially evaporated. This was followed successively by acidification with oxalic acid to precipitate phytin and chlorophyll, filtration, alkalinization with 1 N-NaOH, heating at 100°C for 10 min, acidification with 1 N-HCl and extraction with ether. The ether extract was dried with Na_2SO_4, evaporated to dryness and dissolved in olive oil. The extracts were assayed by the uterine-weight assay in immature mice (sc and oral administration) and the vaginal smear test in castrated rats (intravaginal administration) and the oestrogenic activities found were: in hops 1–2, in red clover 0·006–0·009, in peas 0·004–0·006 and in cabbage 0·024 μg 17β-oestradiol equivalents/g.

The medicinal use of hops has a long and romantic history. Zenisek & Bednar (1960) reviewed some of the popular legends and commented on the rejuvenating effects of cold brewery-sludge baths. The cold sludge baths contained 30% hop extracts. According to legend, King Wenceslas IV in 1406 permitted the incorporation of the hop cone into the coat of arms of the brewers in recognition of the rejuvenating effects of cold brewery-sludge baths. It is said that, even quite recently, hop-sludge baths have been used in Germany for the treatment of a variety of gynaecological disorders. The case of a railroad worker who noted the regular appearance of acne after extensive beer consumption (3–4 litres) and the reputed frequency of acne among brewers, led Amann (1965) to suggest a causal connexion between this condition and the presence of oestrogens in hops.

Apart from the obvious potential hazard of the ingestion or absorption of large quantities of oestrogens from beer and hops by the human population, our own interest was directed to the possible presence of oestrogens in hops for two other reasons. If the report of Koch & Heim (1953) could be confirmed, the oestrogen content of hops would be extraordinarily high in comparison with that of other plants. Furthermore, such high activity might be

associated with oestrogenic compounds of novel structure. In fact, all our experiments on hops, hop extracts and hop derivatives failed to detect any oestrogenic activity.

EXPERIMENTAL

Oestrogen bioassays. The assays were carried out on 21-day-old female Carworth Farm (CF1) mice, according to the method of Evans, Varney & Koch (1941). Hop extracts dissolved or suspended in sesame oil or in mixtures of sesame oil and 95% ethanol were injected sc twice daily (at *c.* 09.00 and 17.00 hr) for 3 days. The volume of each injection was 0·05 ml. The mice were killed on the morning of day 4 by ether and their uteri were dissected, blotted rapidly on filter paper and weighed on a torsion balance. In a typical calibration experiment, uteri from sesame oil-treated control animals weighed 9·0 ± 0·8 mg (mean ± SD) while uteri from animals receiving a total dose of 0·18 μg 17β-oestradiol weighed 52·4 ± 10·5 mg (mean ± SD). The mice were housed in plastics cages in groups of between five and ten and were maintained at 24°C with constant light and dark cycles of 12 hr each. Rockland mouse pellets and water were supplied. Between five and ten animals were assigned to each dosage group. Each preparation was assayed at three or more levels over a 10,000-fold range of concentrations.

Materials

Purified hop components. Various purified hop components, including crystalline α- and β-acids as well as essential hop oils, were all tested for oestrogenic activity over a wide range of concentrations (Table 1). Although the detailed descriptions of these materials are given elsewhere (Stevens, 1967), general lack of familiarity with hop chemistry merits the following brief description. Lupulin is the name given to the bright yellow waxy grains, which are

Table 1. *Results of assays of the oestrogenic activity of hop components*

Material*	Total amount injected/mouse†	No. of animals/group	Mean uterine weight (mg ± SD)
Essential hop oil	3	5	12·5 ± 2·9
	0·3	6	11·1 ± 1·8
	0·03	6	10·8 ± 2·0
	0·003	6	12·4 ± 2·9
α-Bitter acids	3	6	9·2 ± 1·5
	0·3	6	9·4 ± 0·8
	0·03	6	10·2 ± 1·9
	0·003	5	11·0 ± 2·4
β-Bitter acids	3	6	11·3 ± 2·9
	0·3	5	10·8 ± 1·9
	0·03	6	11·3 ± 2·6
	0·003	6	13·0 ± 2·3
17β-Oestradiol	0·00018	6	52·4 ± 10·5
— (Controls)	—	6	9·0 ± 0·8

*The chemical nature of the fractions has been described by Ashurst (1967) and by Stevens (1967).
†Expressed in μl for the essential hop oil, and otherwise in mg.

shed by the hop plants (*Humulus lupulus*) and contain hop oil as well as the α- and β-acids. If hops or lupulin grains are subjected to steam distillation, the clear essential hop oil is obtained. The bittering value of hops is not associated with essential oil, but originates from the resin content of the lupulin grains. The hop resins are obtained by extraction with methanol. The α-acids are those precipitable from the methanol extracts as lead salts and comprise the humulones, a series of cyclic terpenoids. These undergo structural isomerizations on heating in the presence of malt to isomeric α-acids, which account for the principal bitter component of beer. The β-acids, which do not precipitate with lead, are known as the lupulones. These also undergo isomerization on heating (to hulupones), but are responsible in only a minor capacity for the bitter taste of beer.

Preparation of hop extracts. Our first experiments were carried out on a sample of partially dried and pressed hops (177 g) obtained from the National Brewing Co., Baltimore. This was extracted exhaustively with ether in a Soxhlet-type apparatus. The extract was evaporated and the residue was mixed with cold methanol to give an insoluble wax and soluble total resins. Evaporation of the resin fraction to dryness gave a residue of 24·8 g and this was mixed with sesame oil to give an emulsion which was injected into the test animals (Table 2). An aliquot (5 g) of the total resin was heated with 0·5 N-NaOH at 100°C

Table 2. *Results of assays of oestrogenic activity of hydrolysed and unhydrolysed hop components*

Material	Total amount injected (mg/mouse)	No. of animals/group	Mean uterine weight (mg ± SD)
Total hop resins*	30	8	8·0 ± 1·8
	3	10	8·3 ± 2·1
	0·3	10	7·4 ± 2·2
	0·03	10	9·4 ± 2·7
	0·003	10	8·8 ± 3·5
17β-Oestradiol	0·00018	9	50·0 ± 5·6
— (Controls)	—	8	8·1 ± 2·0
Saponified total hop resins			
Basic and neutral fraction	51	6	8·1 ± 1·1
	5·1	6	12·0 ± 2·8
	0·5	6	11·1 ± 3·0
	0·05	6	10·2 ± 2·0
	0·005	6	12·5 ± 3·6
Acidic fraction	33	6	13·8 ± 1·9
	3·3	6	11·5 ± 3·3
	0·33	6	9·9 ± 2·1
	0·03	6	11·7 ± 2·6
	0·003	6	14·7 ± 4·2
17β-Oestradiol	0·00018	5	53·1 ± 4·0
— (Controls)	—	5	11·7 ± 1·9

*The weight of material injected is based on the total methanol-soluble resin fraction.

for 10 min to give a brown solution which was extracted with ether. The evaporated ether extract (1·2 g) represented the basic and neutral fractions (Table 2). The aqueous phase was

acidified with 2 N-HCl and again extracted with ether. Evaporation gave the acidic fraction (2·1 g). Both basic and acidic fractions were dissolved in sesame oil and subjected to bioassay.

A number of European and American hops of known pedigree, as well as commercially prepared brewers extracts, were also subjected to bioassay for oestrogenic activity (Table 3).

Table 3. *Results of assays of oestrogenic activity of ethanolic extracts of hops saponified according to Chuý (1960) and of commercially available hop extracts*

Material	Total amount injected (mg/mouse)	Nc. of animals/group	Mean uterine weight (mg ± SD)
Saponified ethanolic extracts of			
Yakima hops	26	5	12·8 ± 2·3
	0·3	5	9·6 ± 4·6
	0·003	5	8·9 ± 5·4
Hallertau hops	24	4	9·8 ± 0·6
	0·3	5	9·3 ± 2·7
	0·003	5	8·9 ± 2·9
Zatec hops	25	5	12·8 ± 2·4
	0·3	4	7·3 ± 2·4
	0·003	5	8·2 ± 1·7
Commercial hop extracts*			
"Simple" extract†	30	5	9·3 ± 1·4
	0·3	4	9·6 ± 2·2
	0·003	5	9·6 ± 3·1
"Diluted" extract‡	30	5	9·1 ± 3·2
	0·3	5	10·9 ± 6·5
	0·003	4	10·3 ± 2·8
"Preisomerized" extract§	30	5	8·7 ± 1·0
	0·3	5	9·2 ± 1·9
	0·003	5	13·5 ± 5·4

*Values given are for unsaponified samples, but in each case the saponified material was similarly free of hormonal activity.
†Methanol extract (1 g of preparation containing extract from 10 g hops).
‡Methylene chloride or 2-propanol extract (1 g of preparation containing extract from 3·9 g hops).
§Sodium borohydride-treated hexane extract (1 g of preparation containing extract from 15 g hops).

Following the procedure of Chuý (1960), described on p. 598, partially dried Yakima, Hallertau and Zatec hops were extracted overnight in a Soxhlet extractor with 95% ethanol. The solvent was evaporated and the residue was heated under nitrogen on a steam bath with 1 N-NaOH for 10 min. Acidification with 1 N-HCl was followed by extraction with ether and the ethereal solutions were dried over Na_2SO_4, evaporated and administered as solutions or suspensions in sesame oil. Domestic commercial extracts of hops, prepared by extraction with methanol, with either methylene chloride or isopropanol (the precise method was unknown) or by treatment of a hexane extract with sodium borohydride in an alkaline aqueous medium (as specified by the US Food and Drug Administration in

Sec. 121.1082 (b,1) of the Code of Federal Regulations) were tested for oestrogenic activity without further treatment and also after saponification, as described above.

RESULTS AND DISCUSSION

No oestrogenic activity was detected in any of the purified hop components (Table 1), in the hydrolysed or unhydrolysed hop resins (Table 2) or in hop extracts prepared commercially or in the laboratory (Table 3).

The discrepancy between our findings and those of earlier work is difficult to reconcile. Thus, we could not detect any oestrogenic activity in the β-bitter acid fraction, whereas Zenisek & Bednar (1960) found the equivalent of 1·5 mg 17β-oestradiol/g. Our experiments would have measured the equivalent of 0·03 μg 17β-oestradiol (Adams, Jacobson, Levy & Talalay, 1965) and we injected as much as 3·0 mg β-bitter acids (Table 1). Thus our experimental design would have permitted detection of a level of oestrogenic activity 0·7% of that reported by Zenisek & Bednar (1960).

There is an extraordinary lack of experimental detail in the reports of Koch & Heim (1953) and Zenisek & Bednar (1960). Neither of these pairs of authors saponified their hop extracts. Chuŕý (1960) provided considerably more detail on his extraction and saponification procedures. However, the extensive tests reported in this paper on various US and European hops, extracted in a variety of ways and tested directly or after saponification, gave uniformly negative results for oestrogenic activity. In our hands, lupulin and the various more refined chemical fractions of hops were also devoid of oestrogenic activity. We conclude, therefore, that contemporary preparations of hops of this type are free of oestrogens extractable by classical procedures.

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Le houblon a-t-il une activité oestrogénique?

Résumé—Plusieurs auteurs ont prétendu au cours des 20 dernières années que le houblon contient de grandes quantités d'oestrogènes et que cette activité hormonale se manifeste dans la bière. Les activités oestrogéniques signalées ici sont très fortes comparativement à celles observées dans d'autres plantes. On a examiné ici un certain nombre de composants épurés du houblon, dont la fraction d'huiles essentielles et les acides amers α et β obtenus de résines de houblon. On a aussi préparé différents extraits aux solvants organiques de houblons cultivés aux Etats-Unis et en Europe et étudié leur activité oestrogénique, aussi bien directement qu'après saponification par des bases. L'activité oestrogénique d'extraits de houblon commerciaux a également été étudiée. De larges séries de dilutions de toutes les préparations ont été examinées par détermination du poids de l'utérus chez la souris femelle immature. Aucune activité oestrogénique n'a été décelée dans aucune de ces préparations.

Ist im Hopfen Östrogenaktivität vorhanden?

Zusammenfassung—Während der letzten 20 Jahre wurde verschiedentlich die Behauptung erhoben, im Hopfen seien grosse Mengen Östrogene enthalten, und es wurde unterstellt, dass diese Hormonaktivität im Bier erscheine. Es wurde über Östrogenaktivitäten berichtet, die sehr hoch im Vergleich mit denen in anderen Pflanzen waren. Eine Anzahl gereinigter Hopfenbestandteile einschliesslich der Fraktion essentieller Öle und der α- und β-Bittersäuren aus den Hopfenharzen wurden jetzt untersucht und verschiedene Extrakte mit organischen Lösungsmitteln aus Hopfen, der in den USA und in Europa angebaut wird, wurden hergestellt und auf Östrogenaktivität direkt und nach der Verseifung der Base geprüft. Hopfenextrakte des Handels wurden ebenfalls auf Östrogenaktivität geprüft. Weite Bereiche von Verdünnungen aller Präparate wurden mittels Uterusgewichtsuntersuchung an der geschlechtsunreifen weiblichen Maus geprüft. In keinem dieser Präparate war Östrogenaktivität festzustellen.

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Excretion and Metabolism of Orally Administered Aflatoxin B_1 by Rhesus Monkeys

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Abstract—The metabolic fate of [^{14}C]aflatoxin B_1 administered orally in solid form was determined in two groups (H and L) of three male rhesus monkeys. The animals of group H were each given a single dose of 0·4 mg/kg, while the animals of group L received a dose of 0·015 mg/kg. Within 7 days, 40% of the dose was excreted in the urine and 42% in the faeces, excretion being independent of the dose level. The urine and blood still contained detectable radioactivity 5 wk after administration, and the liver retained higher amounts. The liver of one monkey dosed with 0·015 mg/kg retained 1% of the dose at that time. The rate of disappearance of liver radioactivity was slower in the animals treated with the lower dose of the toxin.

Of the urinary metabolites excreted during day 1, 74% were chloroform-soluble and represented 24% of the administered dose. The percentage of the extractable urinary radioactivity declined sharply thereafter. Five radioactive urinary metabolites were separated from the chloroform-soluble fraction. Aflatoxin M_1 was the major metabolite, approximately 20 and 18% of the oral dose being excreted as aflatoxin M_1 in the urine of the animals of group H and L, respectively, during days 1–4. Unmetabolized aflatoxin B_1 was excreted only during day 1 and represented 0·2% (group H) and 0·05% (group L) of the dose. Of the unidentified metabolites, X_1 and X_2 (each representing 0·04–0·07% of the dose) were less polar than aflatoxin M_1 and appeared during days 3 and 2, respectively. The other two metabolites, X_3 and X_4, were more polar than M_1, were excreted during days 1–4 and accounted for 1 and 0·5% of the dose, respectively. Aflatoxin P_1 in urine represented approximately 5% of the administered dose (3·3% as glucuronide and 1·2% as sulphate conjugate).

Only 15% of the excreted faecal radioactivity was solvent-extractable. Aflatoxins M_1 and B_1 in the faeces accounted for 2·6 and 2·2% of the dose, respectively. The solvent-extractable faecal radioactivity declined with time of excretion, reaching the low level of 1% of the faecal radioactivity excreted during day 4.

INTRODUCTION

It is important to acquire a detailed knowledge of the metabolic fate of aflatoxin B_1 in experimental animals for several reasons. This information is essential to an understanding of the biochemical mechanisms underlying the toxicity and carcinogenicity of the compound, and may ultimately provide an explanation for the diversity of response found among experimental animal species. Furthermore, suitable application of this approach to

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human tissues and to excreta of exposed populations will provide an indication of the manner in which the toxin is metabolized by man. This information in turn would facilitate evaluation of the health significance of human exposure.

In order to provide information for these purposes, we have been investigating the metabolism of aflatoxin B_1 by rhesus monkeys. By utilizing ^{14}C-labelled toxin, it has been possible to determine quantitatively the general distribution, metabolic conversion and excretion of metabolic derivatives with a higher degree of recovery and accuracy than is possible using fluorescence techniques. In earlier studies (Dalezios & Wogan, 1972; Dalezios, Wogan & Weinreb, 1971), we described the fate of ip administered toxin. The experiments reported here were planned to determine the fate of toxin administered orally under conditions more nearly approximating those encountered with naturally-contaminated diets, and also to investigate the metabolism over a longer period of time than in the previous study. To simulate actual toxin exposure, which is normally by ingestion, aflatoxin B_1 was given orally in solid form to rhesus monkeys. Two dose levels were used so that the metabolism of aflatoxin B_1 administered orally in a low or high dose could be compared with that of the ip injected toxin in our earlier study.

EXPERIMENTAL

Aflatoxins. Radioactive aflatoxin B_1, labelled in the ring carbons, was produced in this laboratory by isolation from *Aspergillus parasiticus* cultures supplemented with [1-^{14}C]acetate according to the method of Hsieh & Mateles (1971). The purity of the [^{14}C]aflatoxin B_1 preparation, as determined by autoradiography of thin-layer chromatographs and scintillation counting, was found to be approximately 98% aflatoxin B_1. Aflatoxin B_1 was purchased from Calbiochem, La Jolla, Cal., and aflatoxin B_{2a} was prepared from aflatoxin B_1 according to the method of Pohland, Cushmac & Andrellos (1968). Aflatoxin M_1 was isolated from *Aspergillus parasiticus* cultures. Aflatoxin P_1 was isolated from rhesus monkey urine (Dalezios *et al.* 1971).

Materials. Ketodase (β-glucuronidase, 5000 units/ml), was purchased from Warner-Chilcott Co., Morris Plains, N.J., aryl sulphatase (type III), from Sigma Co., St. Louis, Mo., DEAE-Sephadex A-25 from Pharmacia Fine Chemicals, Inc., Piscataway, N.J., adsorbosil-1 from Applied Science Labs, State College, Pa., Amberlites XAD-2 and XAD-7 from Rohm and Haas Co., Philadelphia, Pa., and casein from Nutritional Biochemical Corp., Cleveland, Ohio. All chemicals and solvents used were of analytical-reagent grade.

Animals. Male rhesus monkeys (*Macaca mulatta*), weighing 3–4 kg, were housed in the National Center for Primate Biology, Davis, Cal., and maintained throughout the experiment on a diet consisting of commercial primate ration (Purina monkey chow, Ralston-Purina Co., St. Louis, Mo.) supplemented with fresh fruit. Water was available continuously. During the experiment, the monkeys were housed individually in stainless-steel metabolism cages.

Experimental procedures

Six animals were randomly assigned to two groups (H and L) and each animal received a single oral dose of a mixture of radioactive and non-radioactive aflatoxin B_1. The appropriate amount of aflatoxin B_1 was dissolved in acetone, mixed with casein, dried in air and packed in hard gelatin capsules, and the capsule was then delivered by stomach-tube according to the technique of Conzelman (1970). The three monkeys (A, B and C) of

group H were each given [^{14}C]aflatoxin B_1 (specific activity 1·12 μCi/μmole) at a dose level of 0·4 mg/kg body weight. The other three monkeys (D, E and F) of group L each received [^{14}C]aflatoxin B_1 (specific activity 29·7 μCi/μmole) in a dose of 0·015 mg/kg body weight. Time of dosing was 09.00 hr in both experiments.

Urine was collected in glass bottles submerged in ice and protected from light, and faecal samples were collected in screw-cap bottles. Samples were collected every 6 hr during days 1 and 2 and every 12 hr for the rest of the experiment; all samples were stored at −25°C until they were analysed. Venous blood samples of 5 ml were obtained at intervals of 1 and 6 hr, 1, 2, 3, 6 and 9 days and 2, 3 and 5 wk after dosing.

Monkeys were killed by iv barbiturate overdosing at the following times after dosing: in group H, monkey A at 4 days, B at 1 wk and C at 3 wk; in group L, monkey D at 1 wk, E at 2 wk and F at 5 wk. Livers were perfused *in situ* with cold saline, excised, washed and frozen at −25°C.

Determination of radioactivity

Radioactive measurements were made in triplicate in a Packard Tri-Carb Model 2425 Liquid Scintillation spectrometer with [^{14}C]toluene as the internal standard. Specific radioactivity of each urine sample was determined by liquid scintillation counting (Bruno & Christian, 1961).

Blood aliquots of 0·4 ml were pipetted directly on to a piece of cotton held in low-ash paper, dried under an infra-red lamp at 70°C, compressed into a pellet and then combusted in a model 305 Tri-Carb Sample Oxidizer (Packard Instrument Co., Downers Grove, Ill.). Liver was minced with scissors and homogenized in saline and aliquots equivalent to 0·3 g wet liver were processed and combusted as described for blood. Faeces were dried in a desiccator for 3 days and triturated in a marble mortar, after which samples of approximately 0·2 g were similarly analysed.

Thin-layer chromatography

Thin-layer chromatography (TLC) plates of 250 μ thickness were prepared from adsorbosil-1 silica gel. The plates were developed with the alternative solvent systems, chloroform–acetone–water (88:12:1·5, by vol.) (Stubblefield, Shannon & Shotwell, 1969) or chloroform–acetone–hexane (85:15:20, by vol.) (Teng & Hanzas, 1969). The fluorescent spots were visualized under long-wave (366 nm) ultraviolet light, and autoradiograms were made on X-ray films. Radioactivity in fluorescent spots was determined by transferring the silica gel into a counting vial and then adding 15 ml of toluene–gel scintillation solution (Snyder, 1964).

Urine-extraction procedure

Urine samples were pooled according to the time of excretion to produce fractions I (0–12 hr), II (13–24 hr), III (25–48 hr), IV (49–72 hr), V (73–96 hr), VI (97–120 hr), VII (121–144 hr), VIII (145–168 hr), IX (8–9 days) and X (10–14 days). Each fraction was then extracted according to the procedure described in detail by Dalezios & Wogan (1972).

Chloroform-insoluble aflatoxin conjugates were isolated by recycling the urinary aqueous phase through an Amberlite XAD-2 or Amberlite XAD-7 column, and the conjugates were then separated by column chromatography on DEAE-Sephadex A-25 (Dalezios & Wogan, 1972). The aflatoxin conjugates were identified and quantified by selective enzymatic (β-glucuronidase and aryl-sulphatase) hydrolysis procedures (Dalezios *et al.* 1971).

Analysis of faeces

The faecal samples of monkeys B and C, and those of D and E were pooled and divided according to the day of excretion into five fractions, dried in a desiccator for 3 days and triturated in a marble mortar. To 5 g aliquots of the combined daily faeces in 250 ml Erlenmeyer flasks were added 50 ml (10 vols) acetone–chloroform–water (38:58:4, by vol.) (Purchase & Steyn, 1969). The flasks were shaken overnight in a rotary shaker at room temperature. The solids were removed by filtration and washed three times with 10 ml of 10% (v/v) methanol in chloroform and finally with 10 ml acetone. Portions of the solids were combusted to determine the retained (non-extractable) radioactivity.

The filtrates were evaporated to near dryness and the residues were dissolved in methanol. To remove the interfering pigments, each sample was processed according to the method of Stoloff, Dantzman & Armbrecht (1971) for the isolation of the aflatoxins excreted in the faeces of wethers. Aflatoxins and metabolites present in the residues were separated by TLC, identified by comparison with standard aflatoxins and quantitated by scintillation counting.

RESULTS

Excretion pattern

The daily excretion of radioactivity in the urine, expressed as a percentage of the dose, is summarized in Table 1. During days 1–7 the monkeys excreted approximately 40% of the dose, mainly during the first day. Excretion was not affected by the dose, and excretion rates for all monkeys reached a maximum about 12 hr after dosing. Urine still contained detectable radioactivity 5 wk after dosing.

Table 1. *Urinary excretion of radioactivity following a single oral dose of [^{14}C]aflatoxin B_1*

Time after dosing (days)	Urinary excretion of radioactivity (% of dose) in					
	Group H monkeys			Group L monkeys		
	A	B	C	D	E	F
1	33·7	35·6	33·0	31·1	28·2	34·5
2	1·5	3·4	3·8	5·7	4·8	1·5
3	1·1	1·3	1·6	1·3	1·3	1·7
4	1·0	1·1	1·1	0·9	1·5	0·4
5	—	1·0	0·6	0·3	1·1	0·3
6	—	0·6	0·5	0·2	0·3	0·2
7	—	0·6	0·5	0·1	0·1	0·1
8	—	—	0·1	—	0·1	0·1
9	—	—	0·09	—	0·01	0·2
10	—	—	0·07	—	0·08	0·2
11	—	—	0·06	—	0·07	—
12	—	—	0·05	—	0·06	—
13	—	—	DR	—	DR	0·03
14	—	—	DR	—	DR	0·05
21	—	—	DR	—	—	0·02
35	—	—	—	—	—	DR

DR = Detectable radioactivity
Group H received a dose of 0·4 mg/kg and group L a dose of 0·015 mg/kg.

On days 1–4, the monkeys excreted approximately 41% of the administered dose in the faeces (Fig. 1). Excretion rates for the four monkeys studied were very similar, reaching a maximum during day 3 when 25% of the dose was excreted and then falling rapidly, approaching 3% of the dose during day 4. Faeces collected on day 7 contained about 0·3% of the administered dose.

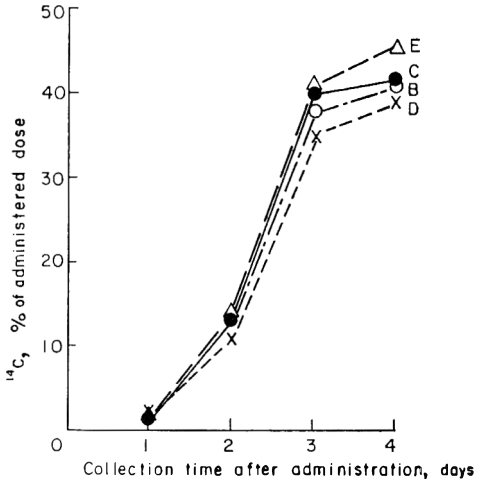

FIG. 1. Cumulative faecal excretion of ^{14}C from four male rhesus monkeys given [^{14}C]aflatoxin B_1 in a single oral dose of 0·4 mg/kg (animals B and C: ○—·—○; ●——●) or 0·015 mg/kg (animals D and E: ×— —×; △— —△).

Radioactivity in blood

One hour after the oral administration of [^{14}C]aflatoxin B_1, the blood of the treated monkeys contained detectable radioactivity. The highest level of radioactivity in the blood (equivalent to 6·3 μg aflatoxin B_1/ml) was attained 1 day after dosing, but this had declined again by 50% by day 3 (Fig. 2). At wk 5 after dosing, the concentration of radioactivity in the blood was still about 20% of the maximum concentration obtained on day 1.

Retention of radioactivity by liver

Liver was found to contain appreciable amounts of radioactivity. The liver of monkey B (dose, 0·4 mg/kg) retained 5·1% of the administered radioactivity 1 wk after intubation, while the liver of monkey D (dose, 0·015 mg/kg) retained 3·4%. The data in Fig. 3 show that the specific activity of liver declined slowly and almost linearly with time. The rate of disappearance of hepatic radioactivity was slower in the animals treated with the lower dose of aflatoxin B_1 than in those treated with the higher dose. The liver of monkey F (dose, 0·015 mg/kg) retained 1% of the dose 5 wk after dosing. Histological examination revealed no pathological abnormalities in the liver samples taken on the day the animals were killed.

Urinary metabolites

Chloroform-soluble metabolites. The proportion of urinary metabolites extractable into chloroform varied with time. Approximately 74% of the radioactivity excreted in urine on day 1 was chloroform-soluble; this represented 24·1% of the administered dose. Table 2

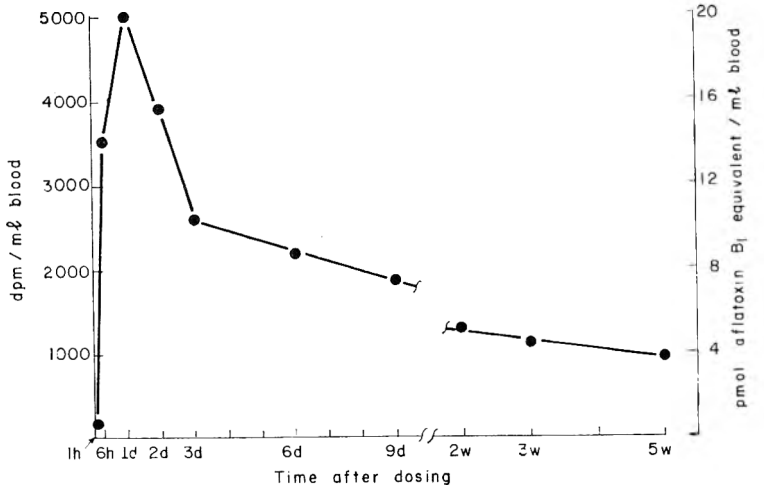

FIG. 2. Blood level of ^{14}C in one male rhesus monkey (F) at various times (h = hours; d = days; w = weeks) following a single oral dose of 0·015 mg [^{14}C]aflatoxin B_1/kg.

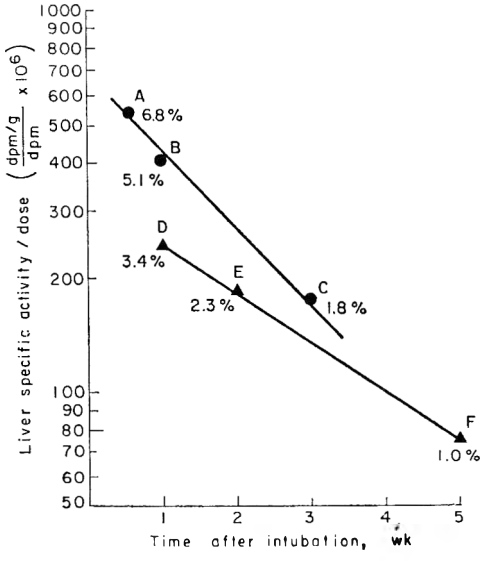

FIG. 3. Retention of ^{14}C by the liver of male rhesus monkeys given [^{14}C]aflatoxin B_1 in a single oral dose of 0·4 mg/kg (●; animals A, B and C) or 0·015 mg/kg (▲; animals D, E and F). Percentages at each point indicate the proportion of the dose retained.

shows that the proportion of chloroform-soluble metabolites declined sharply thereafter.

TLC of the chloroform-soluble material showed that aflatoxin M_1 was the major chloroform-soluble urinary metabolite present, representing approximately 80% of the chloroform-soluble radioactivity excreted by the monkeys of group H during day 1. This accounted for 18·8% of the administered dose (Table 3). The corresponding values for group L were 70 and 16·9%, respectively.

Table 2. *Extraction of urinary aflatoxin metabolites with chloroform*

Time after dosing (days)	Chloroform-soluble metabolites as percentage of	
	Excreted radioactivity	Dose
1	74 (73–76)	24·1
2	32 (23–39)	1·1
3	11 (10–13)	0·2
4	6 (5–6)	0·06
5	4 (3–10)	0·01
6	4 (3–5)	>0·01
7	3 (2–5)	>0·01
		Total... 25·5

Ranges of values are given in parentheses.

TLC separated the chloroform-soluble metabolites into six fractions (plus material remaining at the origin), and these are arranged in Table 3 in order of decreasing R_F value. The identity of aflatoxins B_1 and M_1 was established by co-chromatography with authentic aflatoxins B_1 and M_1. The data in Table 3 show the number of individual compounds and the amounts, as percentages of the dose, excreted during days 1–4 after dosing. Unmetabolized aflatoxin B_1 was excreted only on day 1 and represented 0·24 and 0·05% of the dose for groups H and L, respectively.

Table 3. *Radioactivity of solvent-extractable urinary and faecal metabolites of [^{14}C]aflatoxin B_1 separated by TLC*

Route of excretion	Time after dosing (days)		Aflatoxin B_1 metabolites (% of dose)						
			X_1	B_1	X_2	M_1	X_3	X_4	Origin
			Group H (0·4 mg/kg)						
Urine	1		—	0·24	—	18·80	0·58	0·37	2·37
	2		—	—	0·05	0·53	0·45	0·03	0·16
	3		0·03	—	0·01	0·05	0·03	0·01	0·02
	4		0·01	—	0·01	t	t	t	0·02
		Total...	0·04	0·24	0·07	19·38	1·06	0·41	2·93
Faeces	1		—	0·03	—	0·01	t	t	t
	2		—	2·05	—	1·03	0·19	0·11	0·42
	3		—	0·09	—	1·60	0·34	0·19	0·29
	4		—	—	—	—	—	—	—
		Total...	—	2·17	—	2·64	0·53	0·30	0·71
			Group L (0·015 mg/kg)						
Urine	1		—	0·05	—	16·90	0·98	0·45	1·86
	2		—	—	0·03	0·68	0·09	0·03	0·13
	3		0·04	—	0·01	0·05	0·02	0·02	0·03
	4		0·01	—	t	t	t	t	0·02
		Total...	0·05	0·05	0·04	17·63	1·09	0·50	2·04

t = Trace

Each value is the mean of three analyses of each extract of urine samples from three monkeys or the mean of three analyses of the extract of the combined daily excreted faeces of monkeys B and C.

Quantitatively, aflatoxin M_1 was by far the major urinary metabolite in both groups of animals, appearing mainly during days 1 and 2. A new unknown metabolite, X_1, of very low polarity (R_F 0·95), appeared in the urine during day 3, and on day 4 this became the major urinary metabolite, representing 40% of the urinary radioactivity and 0·01% of the dose. The urine excreted during day 2 contained another unknown metabolite, designated X_2 and accounting for 0·05 and 0·03% of the dose for groups H and L, respectively. Finally, Table 3 shows that two other unknown chloroform-soluble aflatoxins X_3 and X_4, more polar than aflatoxin M_1, were also excreted in the urine. Co-chromatography with standard aflatoxin B_{2a} failed to identify either of these compounds as aflatoxin B_{2a}. All radioactive compounds were fluorescent, but not all fluorescent compounds were radioactive in the chromatograms of the chloroform extracts of urine collected after the first 48-hr period. These non-radioactive fluorescent compounds were urinary pigments which became detectable after the concentration of the chloroform-extracts to a very small volume, a step required for the detection of the minor metabolites of aflatoxin B_1.

Water-soluble metabolites. The Amberlite XAD-2 column retained approximately 90% of the water-soluble metabolites of aflatoxin B_1 excreted in the urine during day 1 after dosing (Table 4). The retained metabolites were eluted with methanol and concentrated.

Table 4. *Retention of water-soluble urinary radioactivity on Amberlite XAD-2 column and enzymatic hydrolysis by β-glucuronidase of the retained radioactive conjugated metabolites of aflatoxin B_1 given to monkeys in a dose of 0·015 mg/kg*

	Water-soluble metabolites retained on XAD-2 column			
		Hydrolysis of retained metabolites		
		Hydrolysed		Unhydrolysed (% of total retained radioactivity)
Time after dosing (days)	^{14}C retained (% of applied radioactivity)	% of total retained radioactivity	% of oral dose	
1	91	34	2·6	64
2	80	31	0·6	76
3	70	10	0·1	79
4 + 5	64	8	*c.*0·0	82
6 + 7	70	3	*c.*0·0	72

The residues were dissolved in 0·2 M-acetate buffer (pH 5·0) and hydrolysed with β-glucuronidase. Data in Table 4 show that the radioactive water-soluble metabolites excreted during days 1 and 2 were hydrolysed by this treatment, suggesting that approximately 30% of the water-soluble urinary metabolites excreted during the first 48-hr period were glucuronides, accounting for 3·2% of the administered dose. The proportion of such metabolites declined thereafter and reached a minimum on day 7. Amberlite XAD-7 was found to be a better material than the XAD-2 for the removal of the aflatoxin conjugates from aqueous solutions.

The unhydrolysed aqueous phase remaining after the extraction of the incubation mixture of the water-soluble metabolites with β-glucuronidase was reconstituted to its original volume with acetate buffer. Sulphatase was added, and the mixture was again

incubated at 37°C for 48 hr. The extract contained approximately 14% of the radioactivity, suggesting that 1·2% of the administered aflatoxin was excreted as sulphate conjugate. A DEAE-Sephadex A-25 column was used to separate the water-soluble radioactivity of the total urine excreted during days 1–7 after dosing. Five subfractions were isolated, the last eluted subfraction containing 20% of the radioactivity. Enzymatic hydrolysis with β-glucuronidase suggested that most of the material in this subfraction was a glucuronide.

Faecal metabolites of aflatoxin

The amounts of the solvent-extractable radioactivity excreted in the daily combined faeces of monkeys B and C decreased from a maximum of 29·5% of faecal radioactivity (3·8% of dose) on day 2 to zero on day 5 of collection (Table 5). TLC of the faecal extracts revealed the presence of four fluorescent radioactive compounds (Table 3). Aflatoxin B_1

Table 5. *Solvent-extraction of faecal metabolites of aflatoxin B_1 given to monkeys in a dose of 0·4 mg/kg*

	Solvent-extractable radioactivity in faeces	
Time after dosing (days)	% of total faecal radioactivity	% of oral dose
1	53·6	0·05
2	29·5	3·8
3	9·5	2·4
4	1·1	0·002
5–7	0	0

was the major solvent-soluble compound excreted in faeces during day 1, accounting for 67% of the extracted radioactivity (0·03% of dose). Aflatoxin M_1 also appeared in the faeces during day 1, accounting for only 14% of the extracted radioactivity (approximately 0·01% of the dose), although the amount increased with time. Thus on day 3 of excretion, aflatoxin M_1 was the major component of the aflatoxins extractable from the faeces, representing 61% of extractable radioactivity or 1·6% of the administered dose.

DISCUSSION

Aflatoxin B_1 administered orally in solid form was rapidly absorbed from the gastrointestinal tract, as indicated by the presence of radioactivity in the blood within 1 hr of intubation. Thereafter, the compound and its derivatives were excreted in urine and faeces. The pattern of urinary excretion was found to be similar to that in monkeys injected ip with aflatoxin B_1 dissolved in dimethylsulphoxide (DMSO) (Dalezios & Wogan, 1972). Approximately 30% of the single orally administered dose was excreted in the urine in the first 24-hr period, and the urinary excretion rate reached a peak approximately 12 hr after dosing. During days 1–7 after dosing, the animals treated with the higher dose (0·4 mg/kg) excreted a total of about 42% of the dose, while those treated with the lower dose (0·015 mg/kg) excreted 38% of the dose in the urine.

Faeces represented an equally important excretion route. Approximately 42% of the orally given dose was excreted in faeces during days 1–4. Total excretion of [^{14}C]aflatoxin

B_1 during days 1–4 therefore amounted to 80–85% of the administered dose. No significant differences were observed in the excretion of radioactivity between the animals treated with the higher and lower doses of [^{14}C]aflatoxin B_1.

Our earlier studies of the metabolism of aflatoxin B_1 in rhesus monkeys (Dalezios & Wogan, 1972) indicated that, when the toxin was administered ip, the urinary metabolites were present mainly in water-soluble form. Approximately 85% of the radioactivity excreted in urine was water-soluble. Aflatoxin P_1 represented the principal urinary aflatoxin derivative, about 50% being present as the glucuronide, 10% as the sulphate and 3% as unconjugated phenol. Together these represented more than 20% of the injected [^{14}C]aflatoxin B_1. Aflatoxin M_1 accounted for only 2·3% of the administered dose and unmetabolized aflatoxin B_1 for about 0·05% of the dose.

The present studies showed that when aflatoxin B_1 was adsorbed on casein and administered orally to male rhesus monkeys at equivalent dose levels, aflatoxin M_1 became the principal urinary metabolite, approximately 19% of the oral dose being excreted in this form. Unmetabolized aflatoxin B_1 accounted for 0·2% of the dose. Enzymatic hydrolysis suggested that only 3·3% of the dose was excreted as aflatoxin P_1 glucuronide and 1·2% as sulphate conjugate. No unconjugated aflatoxin P_1 was detectable by TLC of the chloroform-extractable fraction. The urine contained four additional unidentified chloroform-extractable metabolites. Two of them, more polar than M_1, were excreted during days 1–4. The other two, X_1 and X_2 (less polar than M_1), appeared during days 3 and 2, respectively. On day 4, these two metabolites, X_1 and X_2, were the most prominent in the chloroform-soluble fraction. Their general distribution patterns were independent of aflatoxin dosage. All metabolites separated chromatographically were both radioactive and fluorescent, suggesting that the chromophore moiety of the toxin molecule remained essentially intact.

One possible explanation for the difference in metabolism following injection and oral dosing of aflatoxin B_1 is that the DMSO used as an injection vehicle grossly alters general and intracellular distribution kinetics and thereby alters metabolic fate or induces the activity of the *O*-demethylase.

Retention of radioactivity by the liver of animals in the present experiment was in close agreement with that found in injected monkeys (Dalezios & Wogan, 1972). The liver of monkey A which was given an oral dose of [^{14}C]aflatoxin B_1 identical to that given in the injection experiment (0·4 mg/kg) retained 6·8% of the dose 4 days after dosing. This is comparable to 5·6% of the dose retained by the liver in the injection experiment in the same period of time. The liver of monkey F (given the lower amount of the toxin, 0·015 mg/kg) still retained about 1% of the dose 5 wk after administration. Rat liver was also found to retain appreciable amounts of aflatoxin B_1 for long periods of time (Lijinsky, Lee & Gallagher, 1970; Wogan, Edwards & Shank, 1967). These results show that the monkey and rat livers exhibit a similar pattern of retention of [^{14}C]aflatoxin B_1. In both species the retained radioactivity is comparable in amount and similar in persistence. In addition, most of the retained radioactivity is associated with the total liver protein (Dalezios & Wogan, 1972; Lijinsky *et al.* 1970). Patterson & Roberts (1972) found that aflatoxin hemiacetal, B_{2a} (an *in vitro* metabolic product of aflatoxin B_1), binds to mixed plasma proteins about four times more strongly than does aflatoxin B_1.

The higher rate of disappearance of the radioactivity from the liver of the monkeys treated with the higher dose of the toxin possibly reflects greater binding between the aflatoxin metabolite(s) and liver macromolecules. This could be a direct result of a higher concentration of [^{14}C]aflatoxin B_1 in the liver of these animals. Consequently, during

catabolism of the liver macromolecules (i.e. proteins), more molecules of aflatoxin metabolite(s) will be eliminated from the liver for each molecule of protein catabolized in the animals treated with the higher amount of [^{14}C]aflatoxin than in those treated with the lower dose.

Results of studies on faeces indicated that only 15% of the excreted faecal radioactivity was solvent-soluble. This suggests that the majority of the aflatoxins excreted in faeces are conjugates (not solvent extractable) or aflatoxins which are strongly bound to faecal components. The finding that the proportion of solvent-soluble aflatoxins declined with time of excretion suggests that the non-extractable aflatoxins are metabolic products of aflatoxin B_1 and that the composition of the mixture of the metabolites changes with time. Of the identified faecal aflatoxins, aflatoxin M_1 represented only 3% and aflatoxin B_1 about 2% of the administered dose.

On the basis of the data presented, it is concluded that when aflatoxin B_1 adsorbed on casein is given orally to male rhesus monkeys, aflatoxin M_1 is the major urinary metabolite. Thus, a male rhesus monkey of 3 kg body weight fed 45 μg aflatoxin B_1 excreted in the urine approximately 8 μg aflatoxin M_1 during the first 24-hr period.

If man metabolizes ingested aflatoxin B_1 in a pattern similar to that of the monkey, urinary aflatoxin M_1 is an appropriate index for estimating the toxin intake.

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Excrétion et métabolisme de l'aflatoxine B_1 administrés par voie orale au singe rhésus

Résumé—On a déterminé chez deux groupes (H et L) de trois singes rhésus mâles le destin métabolique de l'aflatoxine B_1 marquée au ^{14}C, administrée sous forme solide et par voie orale. Les animaux du groupe H ont reçu chacun une dose unique de 0,4 mg/kg et ceux du groupe L une dose de 0,015 mg/kg. 40% de la dose ont été excrétés dans l'urine et 42% dans les fèces en l'espace de 7 jours, l'excrétion étant indépendante du niveau de dosage. L'urine et le sang manifestaient une radioactivité détectable encore 5 semaines après l'administration. Le foie retenait des quantités plus importantes: celui d'un singe auquel on avait injecté 0,015 mg/kg retenait à ce moment 1% de la dose. La radioactivité du foie disparaissait plus lentement chez les animaux qui avaient reçu la plus faible dose de toxine.

74% des métabolites urinaires excrétés le premier jour etaient solubles dans le chloroforme; ils représentaient 24% de la dose administrée. Le pourcentage de radioactivité urinaire extractible diminuait ensuite de manière abrupte. Cinq métabolites urinaires radioactifs ont été séparés de la fraction soluble dans le chloroforme. Le principal était l'aflatoxine M_1: les animaux des groupes H et L ont excrété sous cette forme respectivement 20 et 18% de la dose orale dans leur urine pendant les 4 premiers jours. De l'aflatoxine B_1 non métabolisée n'a été excrétée que le premier jour; elle représentait 0,2% (groupe H) et 0,05% (groupe L) de la dose. Parmi les métabolites non identifiés, X_1 et X_2 (chacun représentant 0,04 à 0,7% de la dose) étaient moins polaires que l'aflatoxine M_1 et apparaissaient l'un le 2e et l'autre le 3e jour. Les deux autres métabolites, X_3 et X_4, plus polaires que M_1, ont été excrétés pendant les 4 premiers jours et représentaient respectivement 1,0 et 0,5% de la dose. L'aflatoxine P_1 trouvée dans l'urine représentait environ 5% de la dose administrée (3,3% sous forme de glycuronide et 1,2% sous forme de sulfate conjugué).

Seulement 15% de la radioactivité excrétée dans les fèces étaient extractibles par un solvant. Les aflatoxines M_1 et B_1 présentes dans les fèces représentaient l'une 2,6 et l'autre 2,2% de la dose. La radioactivité fécale extractible par un solvant diminuait tout au long de l'excrétion et descendait le 4e jour au faible niveau de 1% de la radioactivité fécale excrétée.

Ausscheidung und Stoffwechsel von oral verabreichtem Aflatoxin B_1 durch Rhesusaffen

Zusammenfassung—Das Stoffwechselschicksal von in fester Form oral angewendetem [^{14}C]-Aflatoxin B_1 wurde mit zwei Gruppen (H und L) von drei männlichen Rhesusaffen bestimmt. Die Tiere der Gruppe H erhielten je eine Einzeldosis von 0,4 mg/kg, während die Tiere der Gruppe L eine Dosis von 0,015 mg/kg erhielten. Innerhalb von 7 Tagen wurden 40% der Dosis im Urin und 42% in den Faeces ausgeschieden, wobei die Ausscheidung unabhängig von der Grösse der Dosis war. Urin und Blut enthielten noch 5 Wochen nach der Verabreichung feststellbare Radioaktivität, und die Leber hielt noch grössere Mengen zurück. Die Leber eines Affen, der 0,015 mg/kg erhalten hatte, enthielt in diesem Zeitpunkt 1% der Dosis. Die Abbaugeschwindigkeit der Radioaktivität der Leber war langsamer bei den Tieren, welche die niedrigere Dosis des Toxins erhalten hatten.

Von den Stoffwechselprodukten, die am ersten Tag im Urin ausgeschieden wurden, waren 74% chloroformlöslich und stellten 24% der verabreichten Dosis dar. Der Prozentsatz der extrahierbaren Radioaktivität des Urins sank danach steil ab. Aus der chloroformlöslichen Fraktion wurden fünf radioaktive mit dem Urin ausgeschiedene Stoffwechselprodukte getrennt. Der Hauptmetabolit war Aflatoxin M_1; etwa 20 und 18% der oralen Dosis wurden während des 1. bis 4. Tages im Urin der Tiere der Gruppe H bzw. L als Aflatoxin M_1 ausgeschieden. Unmetabolisiertes Aflatoxin B_1 wurde nur während des ersten Tages ausgeschieden and stellte 0,2% (Gruppe H) bzw. 0,05% (Gruppe L) der Dosis dar. Von den unidentifizierten Metaboliten waren X_1 und X_2 (jeder stellte 0,04–0,7% der Dosis dar) weniger polar als Aflatoxin M_1 und erschienen während des 2. bzw. 3. Tages. Die anderen zwei Metaboliten, X_3 und X_4, waren polarer als M_1, wurden während des 1. bis 4. Tages ausgeschieden und stellten 1 bzw. 0,5% der Dosis dar. Aflatoxin P_1 im Urin stellte etwa 5% der angewendeten Dosis dar (3,3% als Glucuronid und 1,2% als Sulfatkonjugat).

Nur 15% der ausgeschiedenen faecalen Radioaktivität waren mit Lösungsmittel extrahierbar. Aflatoxin M_1 und B_1 in den Faeces stellten 2,6 bzw. 2,2% der Dosis dar. Die lösungsmittelextrahierbare faecale Radioaktivität nahm mit der Zeit der Ausscheidung ab und erreichte die niedrige Zahl von 1% der ausgeschiedenen faecalen Radioaktivität während des 4. Tages.

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Inhibition of Alcohol and Lactic Dehydrogenases by Patulin and Penicillic Acid *In Vitro*

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Abstract—The inhibition of yeast alcohol dehydrogenase (ADH) and rabbit-muscle lactic dehydrogenase (LDH) by the mycotoxins patulin and penicillic acid has been studied *in vitro*. Both mycotoxins were non-competitive inhibitors of ADH, but were competitive with regard to LDH. The inhibition constants (K_I) of patulin and penicillic acid, respectively, were found to be $5{\cdot}0 \times 10^{-5}$ M and $1{\cdot}1 \times 10^{-4}$ M for ADH and $6{\cdot}2 \times 10^{-6}$ M and $7{\cdot}2 \times 10^{-5}$ M for LDH. Cysteine reversed the inhibitory effect of both mycotoxins on LDH but not on ADH. The interaction of the thiol groups at the active site of the dehydrogenases with both mycotoxins is discussed.

INTRODUCTION

Patulin and penicillic acid are fungal toxins produced by several species of Aspergillus and Penicillium (Abraham & Florey, 1949) and containing the α,β-unsaturated lactone structure. Both toxins have antibiotic activity and are toxic to animals (Ciegler, Mintzlaff, Weisleder & Leistner, 1972; Hooper, Anderson, Skell & Carter, 1944; Norstadt & McCalla, 1968; Oxford, 1942; Waksman, Horning & Spencer, 1943). They have been shown to have a carcinogenic effect in rats and mice (Dickens & Jones, 1961 & 1965) and their presence in foods and feeds for human and animal consumption has therefore been considered hazardous. Nevertheless, both toxins are readily inactivated by thiols (Atkinson & Stanley, 1943; Dickens & Cooke, 1965; Dickens & Jones, 1961; Geiger & Conn, 1945). It has been suggested that the SH group of the thiol reacts with the double bond conjugated to the lactone carbonyl group through Michael addition (Jones & Young, 1968). More recently, Ciegler *et al.* (1972) has reported that the terminal unconjugated double bond of penicillic acid could also be a site of interaction with thiols. The possibility of an addition reaction between an amino group and penicillic acid has also been suggested (Ciegler *et al.* 1972). Although early studies suggested that the antibiotic activity of both toxins might be due to their reaction with SH groups of bacterial enzymes (Atkinson & Stanley, 1943; Geiger & Conn, 1945), no reports have been found in the literature to substantiate this hypothesis. This paper presents an account of the inhibitory effect of patulin and penicillic acid on two thiol enzymes, alcohol dehydrogenase (ADH) and lactic dehydrogenase (LDH).

EXPERIMENTAL

Preparation of toxins. Penicillic acid was produced in rice by *Penicillium cyclopium* NRRL 1888 and was purified according to the method of Bentley & Keil (1962). Patulin was kindly supplied by Dr. T. M. McCalla, of the University of Nebraska, and was further recrystallized from ethyl ether (Norstadt & McCalla, 1968). The purity of both toxins was verified

by thin-layer chromatography, melting point and spectrophotometric and mass-spectroscopic analyses. Freshly prepared aqueous solutions of appropriate concentration were used for the inhibition studies. Molar absorptivity values of $1{\cdot}4 \times 10^4$ at 222 nm for penicillic acid and of $2{\cdot}0 \times 10^4$ at 276 nm for patulin were used to calculate toxin concentration in certain cases.

Enzyme assays. The enzymes, ADH derived from yeast and LDH from rabbit muscle, and the chemicals required for their assay were purchased from Sigma Company, St. Louis, Mo. ADH was assayed according to the method of Vallee & Hoch (1955), its concentration being determined using a molar absorptivity of $1{\cdot}89 \times 10^5$ (Hayes & Velick, 1954) and a molecular weight of 151,000 (Kagi & Vallee, 1960). LDH was assayed according to the method of Pesce, McKay, Stolzenbach, Cahn & Kaplan (1964). Its concentration was determined using a molar absorptivity of $2{\cdot}0 \times 10^5$ and a molecular weight of 140,000 (Pesce *et al.* 1964).

Inhibition studies. The inhibitory effect of the toxins was determined by adding an appropriate amount of toxin to the reaction mixture and comparing the initial velocity in the presence of the toxin to that in its absence under similar conditions. The data were then treated by the methods of Lineweaver & Burk (1934) and Hunter & Downs (1945) for the determination of the type of inhibition, the Michaelis constant (K_m), and the inhibition constant (K_i). The reversibility of the inhibited reaction was determined by adding freshly prepared cysteine solution to the reaction mixture at zero time and after the reaction had proceeded for 2 min in an amount to give a final concentration of $6{\cdot}3 \times 10^{-4}$ M.

RESULTS

Preliminary observations. The inhibitory effect of patulin and penicillic acid on ADH and LDH activities is illustrated in Figs 1 and 2. It is readily seen that both toxins inhibited the action of these enzymes.

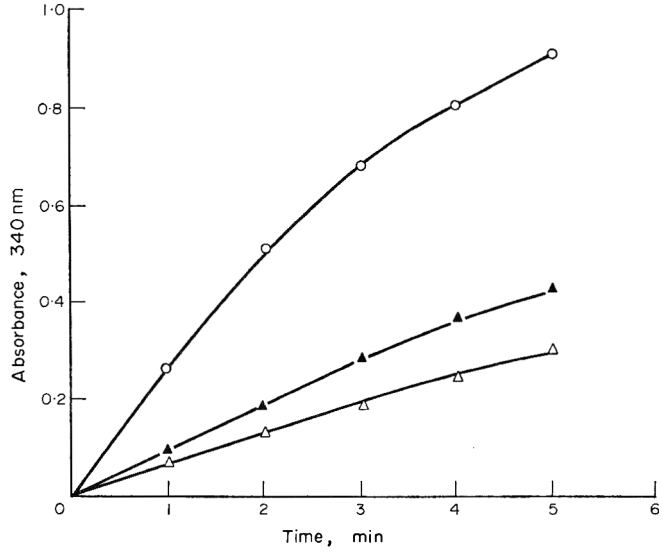

FIG. 1. Effect of penicillic acid and patulin on the enzyme activity of ADH. The reaction was carried out in 0·016 M-pyrophosphate buffer (pH 8·8) at 25°C in the absence of mycotoxins (○——○), in the presence of $8{\cdot}0 \times 10^{-5}$ M-penicillic acid (▲——▲) and in the presence of $8{\cdot}6 \times 10^{-5}$ M-patulin (△——△). The concentrations of enzyme, ethanol and NAD were $2{\cdot}0 \times 10^{-5}$ M, $3{\cdot}4 \times 10^{-1}$ M and $8{\cdot}3 \times 10^{-3}$ M, respectively.

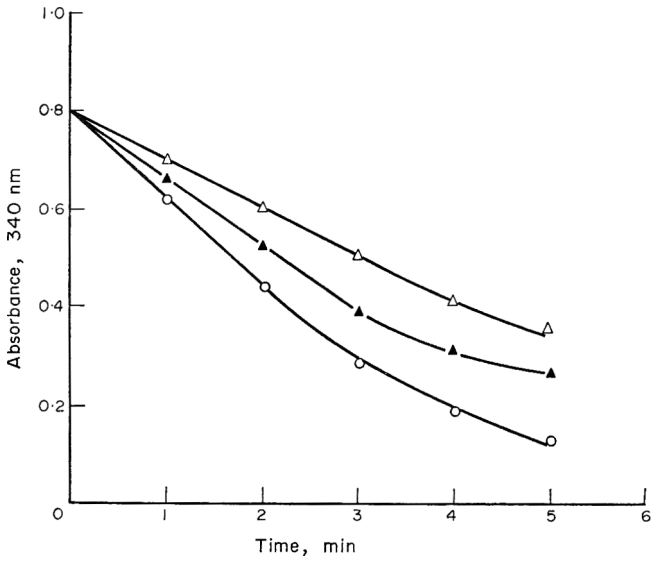

FIG. 2. Effect of penicillic acid and patulin on the enzyme activity of LDH. The reaction was carried out in 0·029 M-phosphate buffer (pH 7·4) at 25°C in the absence of mycotoxins (○——○), in the presence of $8·0 \times 10^{-5}$ M-penicillic acid (▲——▲) and in the presence of $1·7 \times 10^{-5}$ M-patulin (△——△). The concentrations of enzyme, pyruvate and NADH were $1·5 \times 10^{-8}$ M, $3·3 \times 10^{-4}$ M and $6·7 \times 10^{-5}$ M, respectively.

Effect of mycotoxin concentration on enzyme activity. The effect of varied amounts of patulin and penicillic acid on the activity of the enzymes at a constant concentration of substrate was examined. The data, presented in Fig. 3, show that patulin appears to be more effective than penicillic acid as an inhibitor for ADH and LDH. The concentrations of

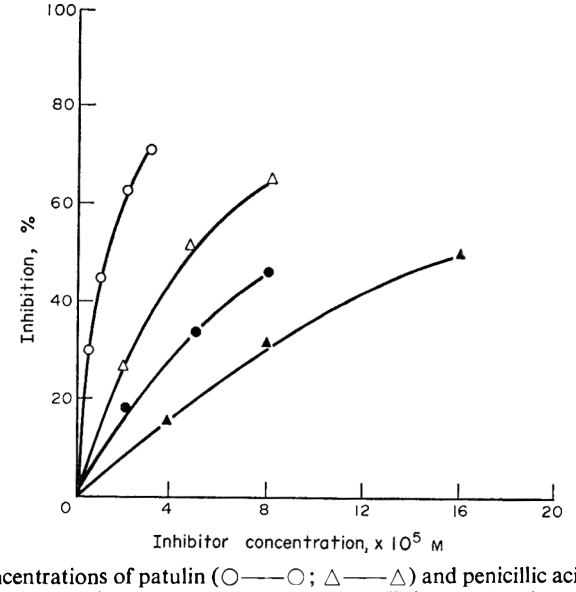

FIG. 3. Effect of the concentrations of patulin (○——○; △——△) and penicillic acid (●——●; ▲——▲) on the enzyme activity of ADH (△——△; ●——●) and LDH (○——○; ▲——▲). The reaction conditions were the same as indicated in Figs 1 and 2.

patulin required for 50% inhibition of ADH and LDH were $4{\cdot}8 \times 10^{-5}$ M and $1{\cdot}2 \times 10^{-5}$ M, respectively, compared with $9{\cdot}0 \times 10^{-5}$ M and 16×10^{-5} M, respectively, for 50% inhibition of the same enzymes by penicillic acid. The molar ratios of patulin to ADH and LDH required to cause 50% inhibition were found to be 2·4 and 800, respectively, while the molar ratios of penicillic acid to ADH and LDH to cause the same percentage of inhibition were 4·5 and 10666, respectively.

Effect of substrate concentration on enzyme-inhibiting activity. In order to determine the type of enzyme inhibition induced by patulin and penicillic acid, the substrate concentration was varied in the absence and presence of constant amounts of either mycotoxin. The results, plotted according to the method of Lineweaver & Burk (1934) are shown in Figs 4 and 5. The plots indicate that patulin and penicillic acid inhibit ADH non-competitively, whereas LDH was competitively inhibited by both mycotoxins. The inhibition constants (K_i)

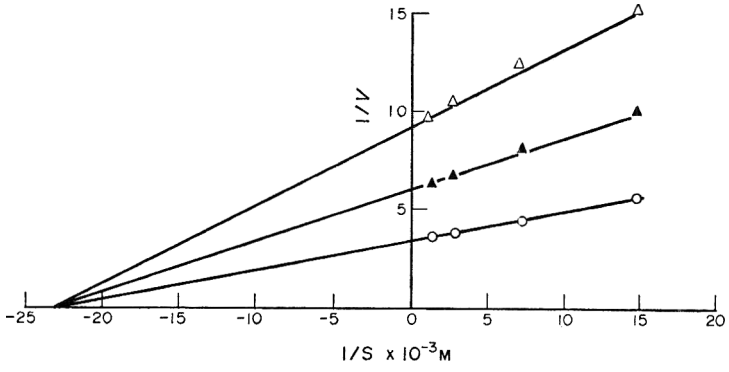

FIG. 4. Lineweaver–Burk plots for ADH: control reaction (○——○); reaction inhibited by penicillic acid (▲——▲); reaction inhibited by patulin (△——△). The reaction conditions were the same as those indicated in Fig. 1, except that ethanol concentration ranged from $6{\cdot}7 \times 10^{-2}$ M to $6{\cdot}7 \times 10^{-1}$ M.

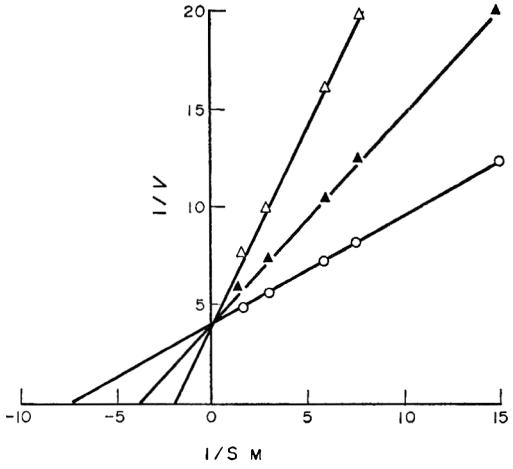

FIG. 5. Lineweaver–Burk plots for LDH: control reaction (○——○); reaction inhibited by penicillic acid (▲——▲); reaction inhibited by patulin (△——△). The reaction conditions were the same as those indicated in Fig. 2, except that pyruvate concentration ranged from $6\,7 \times 10^{-5}$ M to $6{\cdot}7 \times 10^{-4}$ M.

obtained by the Lineweaver–Burk and the Hunter–Downs methods were in good agreement, and the average values, along with the K_m values for the enzymes, are given in Table 1.

Effect of cysteine on enzyme inhibition. The results following addition of cysteine to the reaction mixtures at zero time and after the reaction had proceeded for 2 min are presented in Table 2. The inhibitory effect of patulin and penicillic acid on ADH was not greatly influenced by cysteine, which was added in a concentration 7–8 times higher than that of either mycotoxin. Addition of cysteine at zero time resulted in a slight protective effect. The results indicate that the inhibition of ADH by patulin or penicillic acid was irreversible. No inhibition of LDH was observed when cysteine was added to the reaction system in the presence of either mycotoxin.

Table 1. *Michaelis constants* (K_m) *and inhibition constants* (K_i) *for the action of patulin and penicillic acid on the enzymes ADH and LDH*

Enzyme	K_m (M)	K_i (M) for inhibition by Patulin	K_i (M) for inhibition by Penicillic acid	Type of inhibition
ADH	$4{\cdot}4 \times 10^{-2}$	$5{\cdot}0 \times 10^{-5}$	$1{\cdot}1 \times 10^{-4}$	Non-competitive
LDH	$1{\cdot}4 \times 10^{-4}$	$6{\cdot}2 \times 10^{-6}$	$7{\cdot}2 \times 10^{-5}$	Competitive

TABLE 2. *Effect of cysteine on the inhibitory effect of patulin and penicillic acid on the enzymes ADH and LDH*

	Enzyme activity (% of control value) in presence of mycotoxin					
	No cysteine added		Cysteine* added			
			At zero time		After 2 min	
Enzyme	PT	PA	PT	PA	PT	PA
ADH	35	56	53	64	40	57
LDH	56	80	100	100	100	100

PT = Patulin PA = Penicillic acid

*Freshly prepared solution added in a final concentration of $6{\cdot}3 \times 10^{-4}$ M.

DISCUSSION

The interaction of α,β-unsaturated ketones and lactones, including patulin and penicillic acid, with thiol compounds has been well documented in the literature (Gavins & Friedman, 1968; Jones & Young, 1968; Scott & Somers, 1968). The fact that patulin and penicillic acid are readily inactivated by thiols (Ciegler *et al.* 1972; Dickens & Jones, 1965; Geiger & Conn, 1945) has led to the hypothesis that the mode of action of these mycotoxins is due to their interaction with the SH residues in enzymes. Our data support this hypothesis by showing that both the mycotoxins inhibited ADH and LDH, which are thiol enzymes (DiSabato & Kaplan, 1963; Fondy, Everse, Driscoll, Castillo, Stolzenbach & Kaplan, 1965; Snodgrass,

Vallee & Hoch, 1960). Since both mycotoxins have a higher affinity for the enzymes than do their respective substrates, i.e. the K_i values are lower than the K_m values (Table 1), they should be considered effective inhibitors of these enzymes.

Patulin has a higher affinity for the dehydrogenases (a substantially lower K_i) than does penicillic acid. Furthermore, less patulin than penicillic acid was required to cause enzyme inhibition (Fig. 3). These results parallel the *in vivo* pattern of biological effects in mice and rats, in which the carcinogenic or lethal dose of patulin is much lower than that of penicillic acid (Dickens & Jones, 1961). Patulin has only one thiol addition site but there are two such sites in penicillic acid.

On a molar basis (mycotoxin to enzyme), the amount of mycotoxin needed to inhibit ADH was much smaller than that needed to inhibit LDH (Fig. 3). The inhibitory effect of both mycotoxins on ADH was non-competitive and irreversible, whereas the opposite was true for LDH. These observations are comparable to the effect of mercurials on the thiol enzymes. Snodgrass *et al.* (1960) reported that *p*-chloromercuribenzoate inhibited ADH instantaneously by combining with thiol groups of the enzyme and thus causing dissociation of the enzyme into subunits. The inhibition was partially reversible on the addition of excess amounts of glutathione. On the other hand, the inhibition of LDH by *p*-mercuribenzoate was completely reversed on addition of excess cysteine (Di Sabato & Kaplan, 1963; Fondy *et al.* 1965). Both oxidized and reduced co-enzymes protected the thiol groups in the active site against binding with *p*-mercuribenzoate. Such protection was less effective for thiol groups other than those at the active site. The same authors suggested that *p*-mercuribenzoate inhibits LDH by binding with the thiol groups near the active site of the enzyme. It is ponsible that patulin and penicillic acid resemble mercurials in reacting with the SH grou ps is the active centre of ADH. Further experiments involving, for example, the isolation of labelled peptides and the study of their amino acid composition, as well as the peptide sequence, should substantiate this theory.

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Inhibition *in vitro* de l'alcool-déshydrogénase et de la lacticodéshydrogénase par la patuline et par l'acide pénicillique

Résumé—On a étudié *in vitro* l'inhibition par deux mycotoxines, la patuline et l'acide pénicillique, de la déshydrogénase de l'alcool de levure (ADH) et de la lacticodéshydrogénase du muscle de lapin. Les deux mycotoxines se sont comportées en inhibiteurs non compétitifs de l'ADH, mais en inhibiteurs compétitifs de la LDH. Les constantes d'inhibition (K_I) déterminées pour la patuline et pour l'acide pénicillique sont respectivement de $5{,}0 \times 10^{-5}$ M et de $1{,}1 \times 10^{-4}$ M pour l'ADH et de $6{,}2 \times 10^{-6}$ M et de $7{,}2 \times 10^{-5}$ M pour la LDH. La cystéine a annihilé l'effet inhibiteur des deux mycotoxines sur la LDH, mais non sur l'ADH. L'article examine l'interaction des radicaux thiols et des deux mycotoxines au site actif des déshydrogénases.

Inhibition von Alkohol- und Milchsäuredehydrogenase durch Patulin und Penicillinsäure *in vitro*

Zusammenfassung—Die Inhibition von Hefe-Alkoholdehydrogenase (ADH) und Kaninchenmuskel-Milchsäuredehydrogenase (LDH) durch die Mycotoxine Patulin und Penicillinsäure wurde *in vitro* untersucht. Beide Mycotoxine waren nichtkonkurrierende Inhibitoren von

ADH, konkurrierten aber hinsichtlich LDH. Die Inhibitionskonstanten (K_i) von Patulin und Penicillinsäure waren $5{,}0 \times 10^{-5}$ M und $1{,}1 \times 10^{-4}$ M für ADH und $6{,}2 \times 10^{-6}$ M und $7{,}2 \times 10^{-5}$ M für LDH. Cystein kehrte den Inhibitionseffekt beider Mycotoxine auf LDH, nicht aber auf ADH um. Die Reaktionsbeteiligung der Thiolgruppen an der aktiven Stelle der Dehydrogenasen mit beiden Mycotoxinen wird besprochen.

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Hexachlorophene Concentrations in Blood Associated with the Use of Products Containing Hexachlorophene*

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Abstract—Hexachlorophene (HCP) concentrations in the blood of human adults were measured by gas–liquid chromatography to establish a baseline value and to determine the effects of using products containing from 0·5 to 3·0% HCP. The mean baseline concentration in 30 control subjects was 0·02 μg HCP/ml of blood. Values ranged from 0·38 μg/ml in an individual using a 3% HCP cleanser for whole-body washing to less than 0·005 μg/ml (limit of detection) in some individuals using 1 or 3% HCP cleansers for hand-washing only. HCP concentrations resulting from prolonged use of a soap containing 0·75% HCP ranged from 0·02 to 0·14 μg/ml while those resulting from the use of a mouthwash containing 0·5% HCP for 3 wk ranged from 0·02 to 0·12 μg/ml. The mean concentrations in human adipose tissue obtained from the neck and abdomen were 0·01 and 0·04 μg/g of tissue, respectively.

INTRODUCTION

Hexachlorophene (2,2′-methylenebis-(3,4,6-trichlorophenol); HCP) is a well-known antimicrobial agent first synthesized in 1939 (Gump, 1941). It has been used in soaps at concentrations up to 3% as a germicide and in cosmetics at concentrations up to 0·2% as a preservative. Its effectiveness in use is primarily limited to Gram-positive organisms (Gucklhorn, 1969 & 1970; Mirecki, 1954).

The toxicology of HCP has been reviewed by Kimbrough (1971) and by Gump (1969), both of whom reported cases of human poisoning following accidental ingestion of HCP or application of products containing HCP to burned skin or even to intact skin when the product was not washed off. Signs of poisoning were noted in these individuals and included diarrhoea, vomiting, anorexia, coma, convulsion, weakness of the lower extremities, twitching of the extremities and ocular abnormalities, some of these signs appearing in all patients. In those cases where death did not occur, the toxic signs eventually disappeared when the use of HCP was discontinued.

Kimbrough & Gaines (1971) reported the development of brain changes suggestive of oedema in rats following the feeding of HCP at a dietary level of 500 ppm for 12–14 wk. Microscopic examination of these brains revealed a diffuse spongiosis of the white matter. These effects were reversible, at least in part, on removal of HCP from the diet. Rats

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maintained on a diet containing 100 ppm HCP for 98 days had marginal brain changes (Kimbrough & Gaines, 1971). The mean HCP concentration in the blood of rats fed a similar diet containing 100 ppm HCP for 258 days was 1·2 μg/ml (Curley & Hawk, 1971).

Curley & Hawk (1971) also reported a mean HCP concentration of 0·028 μg/ml of blood in 14 volunteers whose use of HCP products was unknown. The range was from <0·001 to 0·089 μg/ml. Curley, Hawk, Kimbrough, Nathenson & Finberg (1971a) determined the HCP concentrations in the blood of newborn human infants exposed to one or two daily whole-body washing(s) with pHisoHex® (3% HCP) for 1–11 days, finding levels in these infants when discharged from the hospital ranging from 0·009 to 0·646 μg/ml, with a mean of 0·109 μg/ml.

This paper is concerned with concentrations of HCP in blood in relation to the use of HCP products. To obtain a baseline value, HCP concentrations were measured in blood samples from 30 volunteers selected from Food and Drug Administration office and technical workers. The HCP concentration was also measured in fat obtained from surgical patients or at autopsy.

EXPERIMENTAL

Materials. The HCP used as a standard for the gas–liquid chromatography (GLC) determinations was obtained from Sindar Division, Givaudan Corp., Clifton, N.J., under the trade name G-11. The products containing HCP were obtained in the commercial market.

Sampling procedures. In each of the experiments involving the drawing of blood (5–10 ml) for the determination of HCP, the site of venepuncture (antecubital vein) was carefully wiped with 70–90% ethanol to remove HCP from the skin surface. The actual experimental regimen for each test is described in the Results section. For determinations of HCP in fat, samples of adipose tissue were removed from the neck at surgery or from the abdominal midline at autopsy. These samples were frozen at −20°C until assayed.

Analyses. HCP was determined in the blood or fat as previously described (Ulsamer, 1972). The blood was extracted twice with ether–ethanol (18:7, v/v) and the acetyl derivative of HCP was prepared from the dried extract. The amount of HCP was then estimated by GLC, using a 3% OC-101 column in a Nuclear-Chicago Model 5000 gas chromatograph with a ^{63}Ni electron-capture detector. HCP in fat was measured similarly, except that chloroform–methanol (1:1, v/v) was employed as the extracting solvent. This extract was washed with 0·2 N-NaOH, acidified with HCl and then extracted with chloroform. The acetyl derivative of HCP was prepared from the dried chloroform extract. The limit of detection was about 0·005 μg HCP/ml.

RESULTS

Experimental checks and baseline data

The results of an experiment to determine whether HCP absorbed on to the skin surface could lead to erroneously high estimations of HCP levels in blood are shown in Table 1. For this purpose a 3% HCP commercial cleanser was applied for 2–3 min daily for 5 days to the area of the right arm from which blood was to be drawn and the area was then rinsed with warm tap-water. Before blood was drawn from the HCP-treated right arm and the untreated left arm, the site of venepuncture was wiped carefully with 90% ethanol, except in the case of subject MAR who was dabbed once with a 70% ethanol-saturated gauze

®Trade name of Sterling-Winthrop Corp.

Table 1. *HCP concentrations in blood drawn from HCP-treated and untreated arms*

Arm	HCP (μg/ml) in blood from subject			
	PAL	YOD	MAR	BRO
Untreated	0·04	0·02	0·02	0·05
Treated*	0·03	0·02	0·04	0·05

*HCP was applied to the site of venepuncture once daily for 5 days.

sponge. The HCP concentrations in blood from the treated and untreated arms were essentially the same for the three individuals whose skin was thoroughly wiped with 90% ethanol. MAR had a somewhat higher concentration of HCP in blood from the HCP-treated arm, which may have reflected the inadequate skin-wiping of this individual although the significance of an increase of only 0·02 μg/ml is doubtful. All other blood samples were drawn from arms that had been thoroughly wiped.

HCP concentrations were determined in the blood of 30 volunteers from Food and Drug Administration personnel to establish a baseline value. Fifteen subjects had no detectable HCP in the blood (i.e. <0·005 μg/ml), eleven had levels between 0·01 and 0·03 μg/ml, three had levels between 0·05 and 0·08 μg/ml and one individual had 0·20 μg/ml. The average value for the group was 0·02 μg/ml. Excluding the highest value, the average was approximately 0·01 μg/ml.

Total-body washing

Table 2 gives the HCP concentrations in blood resulting from the long-term use of a soap containing 0·75% HCP. Blood samples were obtained from 12 individuals who had washed daily with the soap for 0·3–16 yr from 1 to 6 times each day over the whole body. The HCP concentrations in blood ranged from 0·02 to 0·14 μg/ml with a mean of 0·07 μg/ml. There appeared to be no definite correlation between the blood concentration of

Table 2. *HCP concentrations in human blood following long-term whole-body washing with a soap containing 0·75% HCP*

Subject	Use of HCP preparation		Concn of HCP (μg/ml)
	Duration (yr)	Frequency (times/day)	
STO	0·3	1	0·06
McV	4·5	2	0·05
SUT	5	2	0·05
CUN	8	3	0·10
MAR	9	1	0·02
COW	10	1	0·13
DAR	10	6	0·06
CAM	13	1	0·04
GRA	15	1	0·08
BRE	16	1	0·02
HES	16	2	0·09
McK	16	5	0·14
			Mean... 0·07 ± 0·04

HCP and either the duration or frequency of use. The mean concentration of 0·07 μg/ml was 3·5 times higher than the mean of the baseline samples. The possibility that these individuals were exposed to HCP from other sources cannot, of course, be excluded.

Seven of the individuals whose blood concentration of HCP did not exceed 0·01 μg/ml were selected from the control group used to establish baseline values. They were asked to use a 3% commercial cleanser for whole-body washing once daily in the shower for 3–6 wk. The resulting blood levels are shown in Table 3. The mean HCP concentration in this

Table 3. *HCP concentrations in human blood following 3–6 wk of daily whole-body washing with a commercial cleanser containing 3% HCP*

Subject	Duration of use (days)	Concn of HCP (μg/ml)
YOD	21	0·27
PAL	23	0·25
MEU	23	0·28
EVA	23	0·11
DAV	23	0·38
NAC	23	0·27
MAR	41	0·10
		Mean . . . 0·24 ± 0·08
WEG	5 (yr)	0·37

group was 0·24 μg/ml with a range of 0·10–0·38 μg/ml. This represented an average increase over the baseline value of 0·24 μg/ml in 3 wk. A similar HCP concentration was found in the blood of a physician (WEG) who had been following this regimen for 5 yr (Table 3).

Hand washing

Table 4 lists the HCP concentrations in blood samples from eight dentists who washed their hands with a commercial cleanser containing 1% HCP 3–40 times/day for 6–11 yr. The mean concentration was 0·02 μg/ml, which is similar to the baseline value. It is not known whether any other products containing HCP were used by these individuals.

Table 5 presents the results of blood analyses on 29 hospital-nursery workers who washed their hands 4–34 times each day for 1–136 months with a commercial cleanser containing

Table 4. *HCP concentrations in the blood of dentists following use of a hand cleanser containing 1% HCP*

	Use of HCP preparation		
Subject	Duration (yr)	Frequency (times/day)	Concn of HCP (μg/ml)
SEN	5	40	0·03
VER	5	10	0·01
NIS	6	40	0·02
PER	6	20–30	0·03
SAV	7	20–30	0·03
FOX	10	—	0·00
HIC	10	30	0·05
ABR	11	3	0·01
			Mean . . . 0·02 ± 0·02

Table 5. *HCP concentrations in the blood of hospital-nursery personnel using a commercial hand cleanser containing 3% HCP*

Parameter	Mean*	Range*	No. of subjects	HCP concn (μg/ml) Mean	HCP concn (μg/ml) Range
Duration of exposure (months)					
Up to 12	5	1–10	15	0·04	0·00–0·14
Over 12	61	15–136	14	0·03	0·00–0·06
Frequency of exposure (washes/day)					
Up to 15	9	4–14	10	0·04	0·00–0·09
Over 15	26	17–34	10	0·04	0·00–0·14
Other HCP products					
Used			12	0·04	0·00–0·07
Not used			8	0·03	0·00–0·14

*Values are the means and ranges of the parameter and are expressed in the same units.

3% HCP. The overall mean value was 0·03 μg/ml with a range of 0·00–0·14 μg/ml, which is similar to the baseline value. The HCP concentration appeared to be somewhat higher in the blood of individuals exposed for less than 12 months (0·035 μg/ml) than in individuals using the HCP cleanser for longer than 12 months (0·025 μg/ml), but this difference was probably not significant. The frequency of daily exposure did not appear to affect the HCP concentration; the mean value was 0·031 μg/ml for those individuals averaging 15 washes/day. The concomitant use of other products containing HCP, such as soaps, deodorants and handcreams with a low HCP content, did not appear to influence the results, but the HCP content of these products and the frequency of their use was unknown. Thus in those cases where no other products were used the mean value was 0·033 μg/ml while in those instances where they were knowingly used the mean was 0·035 μg/ml.

Table 6. *HCP concentrations in human blood following daily use of a mouthwash containing 0·5% HCP for approximately 3 wk*

Subject	Duration of use (days)	Concn of HCP (μg/ml)
SHI	17	0·12
WRI	20	0·05
STR*	21	0·05
THO	21	0·06
BRO	23	0·05
ENG	23	0·05
GAL	23	0·02
HIL	23	0·08
NEW	23	0·05
WEN	23	0·04
		Mean . . . 0·06 ± 0·02

*Subject used product twice daily.

Use of mouthwash containing 0·5% HCP

Ten volunteers with HCP concentrations in the blood of less than 0·01 μg/ml were asked to gargle once daily for approximately 1 min with a supplied mouthwash (0·5% HCP) over a period of about 3 wk. At the end of this time, the mean HCP concentration in the blood was 0·06 μg/ml with a range of 0·02–0·12 μg/ml (Table 6). This represents a mean increase of approximately six times the initial value.

Adipose tissue study

Human adipose tissue obtained during routine neck surgery (nine samples) or removed from the abdomen at routine autopsy (seven samples) were analysed for HCP. The mean concentration for neck fat was 0·01 μg/g of tissue with a range of 0·00–0·05 μg/g, and the mean abdominal fat was 0·04 μg/g with a range of 0·02–0·05 μg/g.

DISCUSSION

Penetration of HCP through intact skin has been demonstrated in animals (Carroll, Salak, Howard & Pairent, 1967; Curley, Hawk & Linder, 1971b) and in man (Curley *et al.* 1971a; Feldmann & Maibach, 1970). The degree of this penetration in man was reported by Feldmann & Maibach (1970) to be 3·1% of a topically applied dose of radio-labelled HCP through intact skin in 5 days. Larson (1968) reported that penetration of HCP (a 3% commercial cleanser) through burned skin in man resulted in blood concentrations up to 17 μg/ml, whereas the highest blood concentration of HCP obtained in the present work (Table 3) with whole-body washing using a 3% cleanser was 0·38 μg/ml. HCP is also retained by the skin and this retention appears to be related to the concentration of HCP in the soap, the frequency of washing, the duration of each application and the total exposure (Manowitz & Johnston, 1967). Other investigators have reported that HCP accumulated on the skin for the first three or four washes and then the amount remained more or less constant (Shemano & Nickerson, 1954), and that the duration of skin contact during each application made no difference after the initial 5 or 10 min (Compeau, 1960). From the data on whole-body washing with preparations containing 0·75 and 3·0% HCP (Tables 2 & 3, respectively), it appears that the amount of HCP in the blood is related to the concentration of HCP in the product applied to the skin surface. The mean concentration after whole-body washing with the soap containing 0·75% HCP was 0·07 μg/ml, whereas that after whole-body washing with the 3% commercial cleanser was about 3 times higher at 0·24 μg/ml, even though the soap containing 0·75% HCP was used for a longer period of time and with greater frequency. A comparison of the data in Table 3 with that in Table 5, relating to use of a cleanser of the same strength (3% HCP) for hand-washing only, shows that the concentration of HCP in the blood is also related to the area of skin application, even though the hand-washing was more frequent and of longer duration. Thus for whole-body washing the average was 0·24 μg/ml, whereas for hand washing the average value was 0·03 μg/ml.

Although the data in Table 2 (on long-term whole-body washing with a soap containing 0·75% HCP) and Table 5 (on hand-washing with a commercial cleanser containing 3% HCP) are limited, it appears that there is no definite increase in the blood concentration of HCP either with an increase in duration of total exposure or with an increase in the number of times the product is used daily. Analysis of the data in Table 2 by a multiple regression technique revealed no evidence that duration and frequency of use were related to HCP

concentrations in the blood (regression, F 2,9 = 0·91; NS) Furthermore, the coefficient of multiple determination (the percentage of the variance in HCP accounted for by the relationship of duration and frequency of use with HCP in the blood) was 17%. This indicated that most of the variation in HCP concentration must have been due to variables not included in regression. Such variables could include the use of other products and/or individual variation. It is also of interest that the HCP concentration in the blood of subject WEG (Table 3), who used a commercial cleanser containing 3% HCP for 5 yr for whole-body daily washing, was no higher than the highest value found in those who used it for 3 wk. Thus HCP may not accumulate in the blood with continued use once equilibrium is reached. The mean concentration of HCP in human fat (0·025 μg/g tissue) was similar to our mean baseline value (0·02 μg/ml). HCP, therefore, does not appear to accumulate preferentially in the fat as do some of the chlorinated pesticides, such as DDT (Hoffman, Fishbein & Andelman, 1964). The difference between values for fat from the neck and abdomen was significant ($P < 0·01$) but the tissues were obtained from completely different populations.

The highest blood level of HCP obtained with the commercial cleanser containing 3% HCP (Table 3) was 0·38 μg/ml, approximately 33% of the toxic level that caused brain damage in rats in a long-term feeding study (Curley & Hawk, 1971). All the mean blood levels determined so far after uses of HCP preparations other than whole-body washing with the 3% HCP cleanser were less than 10% of the toxic level produced in these rats (Curley & Hawk, 1971). The variations seen in blood concentrations of HCP in relation to product use appear to be a reflection of differences in individual skin permeability and metabolism. This is best seen in the experiment carried out under controlled conditions (Table 3), in contrast to other soap and cleanser experiments in which the possibility of use of other products complicated the results.

There is no reason to believe that blood becomes contaminated with HCP as a result of venepuncture through contaminated skin, provided the skin is thoroughly swabbed with 70–90% ethanol (Table 1). Furthermore, the drawing of large samples (5–10 ml) of blood minimized the effects of any possible contamination.

The concentration of HCP in the blood samples used to determine a baseline value and the data from Tables 4 and 5 suggest that the average HCP level in the blood of the adult population at large is likely to be low. Only two of the 67 individuals in these groups had HCP concentrations that were more than 10% of the level reported by Curley & Hawk (1971) to be toxic in rats. However, there is a possibility that toxic blood levels could be reached in an individual who has very permeable skin and uses a cleanser containing 3% HCP for daily whole-body washing(s) and who is also exposed to a variety of other products containing HCP. Organ toxicity would, of course, depend on the length of time that potentially dangerous HCP concentrations were maintained in the blood. In long-term percutaneous absorption studies in monkeys, the blood levels of HCP showed only slight daily fluctuations, in contrast to parenteral and intestinal absorption, where peaking occurred (A. G. Ulsamer, P. Yoder & F. N. Marzulli, unpublished data, 1972).

As toxicity results from the continuous entry of HCP into vulnerable tissues of the central nervous system, high concentrations of short duration may be of less consequence than a low concentration that is relatively steady.

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Concentrations d'hexachlorophène dans le sang en corrélation avec l'emploi de produits contenant de l'hexachlorophène

Résumé—On a mesuré par chromatographie gazeuse et liquide les concentrations d'hexachlorophène (HCP) dans le sang d'hommes adultes afin d'établir une valeur de référence et de déterminer les effets que peut avoir l'emploi de produits contenant de 0,5 à 3,0% d'HCP. Le taux sanguin moyen de référence de 30 sujets témoins était de 0,02 μg HCP/ml. Les valeurs relevées s'étalaient entre 0,38 μg/ml, chez un individu qui se servait d'un nettoyant à 3% d'HCP pour se laver tout le corps, et moins de 0,005 μg/ml (limite de détection) chez quelques individus qui employaient des produits à 1 ou à 3% d'HCP, uniquement pour se laver les mains. Les taux d'HCP résultant de l'usage prolongé d'un savon à 0,75% d'HCP se situaient entre 0,02 et 0,14 μg/ml; tandis que ceux résultant de l'emploi pendant 3 semaines d'un bain de bouche à 0,5% d'HCP se situaient entre 0,02 et 0,12 μg/ml. Les concentrations moyennes dans le tissu adipeux prélevé au cou et à l'abdomen atteignaient respectivement 0,01 et 0,04 μg/g de tissu.

Hexachlorophenkonzentrationen im Blut verbunden mit der Verwendung von hexachlorophenhaltigen Produkten

Zusammenfassung—Hexachlorophen (HCP)-Konzentrationen im Blut erwachsener Menschen wurden durch Gas-Flüssigkeitschromatographie bestimmt, um einen Ausgangswert zu erhalten und die Wirkung von Produkten messen zu können, die 0,5 bis 3,0% HCP enthielten. Die mittlere Grundkonzentration betrug bei 30 Kontrollpersonen 0,02 μg HCP/ml Blut. Die Werte reichten von 0,38 μg/ml bei einer Person, die ein Reinigungsmittel mit 3% HCP für Ganzkörperwaschungen benutzte, bis zu weniger als 0,005 μg/ml (Grenze der Feststellbarkeit) bei manchen Personen, die Reinigungsmittel mit 1 oder 3% HCP nur zum Händewaschen benutzten. HCP-Konzentrationen, die vom längeren Gebrauch einer Seife mit 0,75% HCP herrührten, reichten von 0,02 bis 0,14 μg/ml, während die durch den Gebrauch eines Mundwassers mit 0,5% HCP auf die Dauer von 3 Wochen von 0,02 bis 0,12 μg/ml reichten. Die mittleren Konzentrationen im Fettgewebe des Menschen aus dem Hals und vom Bauch betrugen 0,01 bzw. 0,04 μg/g Gewebe.

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Effect of Hexachlorophene on the Rat Brain During Ontogenesis

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Abstract—The acute oral toxicity (LD_{50}) of hexachlorophene (HCP) in the 10-day-old rat was 9 ± 2 mg/kg. After this, toxicity decreased as a function of age and the LD_{50} at 32 days of age was 111 ± 12 mg/kg. In adult rats, however, the LD_{50} was only about half that in a 32-day-old rat. HCP began to have a toxic effect on the brain when the rats were between the ages of 8 and 14 days. This was demonstrated by an increase in the weight and water-content of the brain, by the appearance of vacuoles in the white matter and by interference in the functioning of the central nervous system.

INTRODUCTION

Hexachlorophene (HCP) has been used extensively as an antibacterial agent for the past 20 yr. During this period several cases of poisoning have occurred, the chief cause being the accidental drinking of solutions containing HCP (Plueckhahn & Banks, 1972). It has also been observed that HCP is absorbed not only through burns (Larson, 1968) but also through healthy skin (Curley, Hawk, Kimbrough, Nathenson & Finberg, 1971; Herter, 1959) to such an extent that it can bring about toxic symptoms. Experiments performed on adult rats have shown that HCP causes lesions in the white matter of the central nervous system (Kimbrough & Gaines, 1971). The brain of a newly-born rat may be regarded in many respects as an embryonic organ, which only some weeks after birth attains the structure and function seen in the adult animal. We were interested to determine whether the toxicological properties of HCP in the rat differed at the various stages of brain development.

EXPERIMENTAL

Animals. Altogether 320 Sprague–Dawley rats of both sexes and of various ages were used in the experiments.

Acute oral toxicity. In the first experiment the acute oral toxicity of HCP was studied as a function of age, by determining the LD_{50} values in rats 10, 20, 25, 32, 50, 70 and 300 days old. Each age-group contained about the same number of males and females. HCP was administered by stomach tube in 1% carboxymethylcellulose (CMC) and the animals were observed for 10 days. Standard errors were calculated according to Miller & Tainter (1944).

Toxicity to the developing brain. In the second experiment, the toxicity of HCP to the developing brain was examined in rats of both sexes aged 6, 12, 15, 22 and 30 days as well

as in adult female rats. On two successive days, the test animals were given a 1% CMC-suspension of HCP by stomach tube in doses equivalent to approximately 80% of the assumed LD_{50} while the control animals were given only CMC. On day 3 the control animals were killed by decapitation, as were any animals in an obvious state of HCP poisoning, as indicated by distinct signs of effect on the central nervous system and loss of weight. The brains were weighed and then cut longitudinally into two identical halves. One half was used for determinations of water content, the tissue being dried at 110°C for 24 hr, and the other half was fixed in neutral formalin and embedded in paraffin. Sections prepared from both the cerebrum and cerebellum were stained with haematoxylin and eosin and with Luxol fast blue for staining myelin. The statistical significance of variations in the weight and water content of the brains was calculated using Student's *t* test.

RESULTS

Acute toxicity of HCP

The acute oral toxicity of HCP in the rat as a function of age is presented in Fig. 1. The toxicity in 10-day-old rats was high, the LD_{50} being only 9 ± 2 mg/kg. Beyond this age, toxicity decreased almost linearly, with the LD_{50} reaching a maximum of 111 ± 12 mg/kg in the 32-day-old animals. However, the LD_{50} of HCP in the adults was only slightly over half that in the 32-day-old rats.

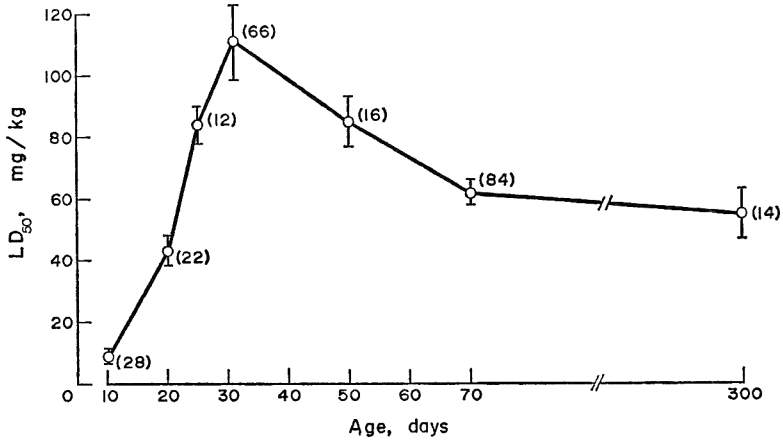

FIG. 1. The acute toxicity of HCP in young and adult rats expressed as a function of age. Results are expressed as the LD_{50} ± SE, with the number of animals in parentheses.

Effects of HCP on the brain

Figure 2 shows the effect of HCP poisoning on the weight of the brain during postnatal development. Toxic doses of HCP administered on two successive days had no effect whatsoever on the weight of the brain of 8- and 14-day-old rats, but in the 17-day-old rats exposed to HCP, the mean weight of the brain was significantly higher ($P < 0{\cdot}001$) than that recorded for the comparable control group. A significant rise in brain weight ($P < 0{\cdot}001$) also occurred in the older age groups under the effect of HCP.

The effect of toxic doses of HCP on the water-content of the brain is shown in Fig. 3.

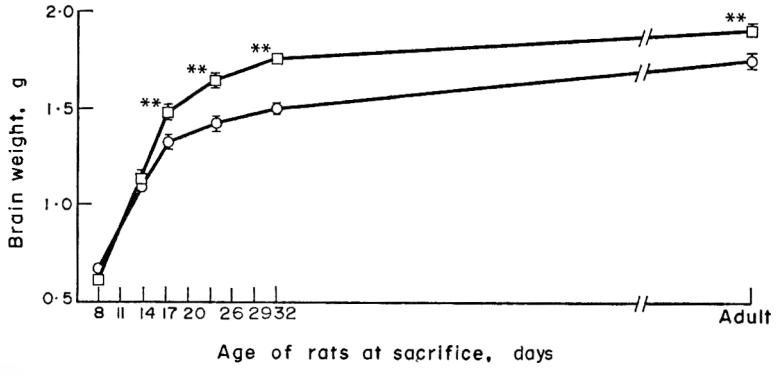

FIG. 2. The effect of HCP poisoning on the weight of the rat brain at different stages of postnatal development. Results for control animals (○) and for animals treated with a toxic dose of HCP for 2 days immediately before sacrifice (□) are expressed as the means ± SEM for groups of 5–11 rats. Values differing significantly (Student's *t* test) from the corresponding controls are marked with asterisks: $^{**}P < 0{\cdot}001$.

It can be seen that in the 8-day-old rats the water-content was lower ($P < 0{\cdot}05$) in the HCP-treated animals than in the controls, but at 14 days of age HCP increased the water-content of the brain ($P < 0{\cdot}05$). In the older age groups there was a more marked rise ($P < 0{\cdot}001$) in the water-content of the brain in rats treated with HCP.

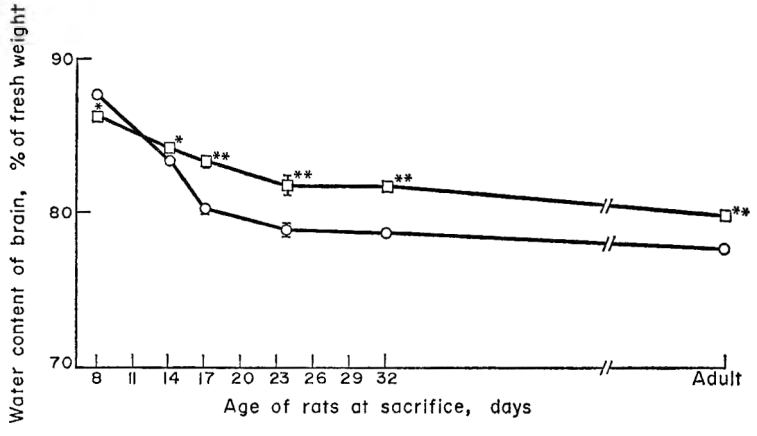

FIG. 3. The effect of HCP poisoning on the water content of the rat brain at different stages of postnatal development. Results for control animals (○) and for animals treated with a toxic dose of HCP for 2 days prior to sacrifice (□) are expressed as the means ± SEM for groups of 5–11 rats. Values differing significantly (Student's *t* test) from the corresponding controls are marked with asterisks: $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}001$.

Rats given HCP on days 6 and 7 after birth showed a marked decrease in body weight but no central nervous system disturbances, such as paralysis of the fore and hind legs. In rats over 12 days old, distinct signs that the HCP was affecting the central nervous system were observed. In the rats killed at 14 and 17 days, it was observed that HCP first depressed the action of the fore limbs, while in those killed at 24 or 32 days of age and in the adults, HCP first depressed the action of the hind limbs.

Microscopic study of sections of the cerebrum and cerebellum showed that a state of HCP poisoning was accompanied by the formation of vacuoles of varying sizes in the white matter of both regions at all the ages studied from 14 days to adulthood. The grey matter appeared to be structurally normal. In the 8-day-old rats no difference could be detected between the brain histology in the test animals and that in the controls.

DISCUSSION

It has previously been observed (Kimbrough & Gaines. 1971) that, in a state of HCP poisoning, the weight of the brain of an adult rat increases and relatively large vacuoles are formed in the white matter of both the cerebrum and cerebellum. Furthermore, we observed that HCP increased the water content of the brain of the adult rat, a finding which explained the increase in the brain weight. Thus, the vacuoles observed in the microscopic sections were liquid accumulations.

The increase in water content caused by HCP was just as great in the 17-, 24- and 32-day-old groups as in adults. In the same groups, the increase in brain weight was significant and the vacuoles found in the white matter were comparable to those present in the adults. In the 14-day-old rats HCP treatment raised the water content of the brain significantly ($P < 0{\cdot}05$), but the effect was clearly less than in the older groups. Distinct vacuoles were seen in the white matter of the brain. However, no significant increase was observed in brain weight. It must be noted, however, that brain weight is a relatively rough measure. In the 8-day-old rats, HCP treatment slightly lowered the water content of the brain ($P < 0{\cdot}05$), but compared with the controls no changes in the weight or histological structure of the brain were observed.

These results indicate that HCP affects the central nervous system by different mechanisms in 8-day-old rats and in adults. The drop in the water content of the brain in the former group could have been due to the systemic toxicity of HCP. This view was supported by the finding that no signs of central nervous system disturbance (e.g. paralysis) were observed in 8-day-old rats after a toxic dose of HCP. The increase in water content of the brain in rats treated on days 12–13, associated with vacuolation of the white matter and the occurrence of paralysis, demonstrated that HCP began to have a toxic effect on the rat brain between days 8 and 14 after birth.

Myelination begins in the rat at about 10 days of age (Luse, 1956). According to Bass, Netsky & Young (1969) the formation of myelin in the rat brain takes place in two stages. Between days 10 and 30 the accumulation of lipids occurs prior to the formation of histologically detectable myelin. Between days 30 and 40 the formation of compact myelin lamellae takes place. Kimbrough & Gaines (1971) established by electron microscopy that a layer of myelin surrounds the vacuoles caused by HCP in adult rats. We observed that HCP exerted a toxic effect on the brain from about the time that the synthesis of myelin in the brain was starting and thus long before the formation of compact myelin lamellae. Consequently it is logical to think that HCP reacts with one or more substances in the myelin structure to form liquid vacuoles in the white matter.

The acute toxicity of HCP and also the toxicity of a given dose to the brain decreases very quickly in rats after day 10 of life. Possibly the excretion and metabolism of HCP quicken at that stage. In cattle, 60–70% of ingested HCP is excreted, mainly in the faeces (St. John & Lisk, 1972), and the rest is possibly metabolized in the body. It is interesting to observe that in the adult rat the LD_{50} is about half that in a 32-day-old rat. Perhaps the swiftly

occurring synthesis of the myelin components between days 30 and 40 has an effect on HCP toxicity. It must also be noted, that in the young animal the thin bones of the cranium offer less resistance to the expansion of the hydrated brain. Thus cerebral pressure remains at a lower level and there is less interference with the nerve connexions.

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Effet de l'hexachlorophène sur le cerveau du rat pendant l'ontogenèse

Résumé—La toxicité orale aiguë (DL_{50}) de l'hexachlorophène (HCP) est de 9 ± 2 mg/kg chez le rat âgé de 10 jours. Elle diminue ensuite en fonction de l'âge: chez le rat âgé de 32 jours la DL_{50} est de 111 ± 12 mg/kg. Chez le rat adulte, par contre, le DL_{50} ne s'élève plus qu'à la moitié de la DL_{50} à 32 jours. L'HCP commence à exercer un effet toxique sur le cerveau du rat entre 8 et 14 jours d'âge. Cet effet se traduit par une augmentation du poids et de la teneur en eau du cerveau, par l'apparition de vacuoles dans la substance blanche et par des perturbations du fonctionnement du système nerveux central.

Einfluss von Hexachlorophen auf das Rattengehirn während der Ontogenese

Zusammenfassung—Die akute orale Toxicität (LD_{50}) von Hexachlorophen (HCP) betrug bei der 10 Tage alten Ratte 9 ± 2 mg/kg. Danach nahm die Toxicität als eine Funktion des Alters ab, und im Alter von 32 Tagen betrug die LD_{50} bereits 111 ± 12 mg/kg. Bei erwachsenen Ratten jedoch war die LD_{50} nur etwa halb so gross wie bei einer 32 Tage alten Ratte. HCP begann eine toxische Wirkung auf das Gehirn zu haben, wenn die Ratten 8 bis 14 Tage alt waren. Dies zeigte sich an einer Gewichtszunahme und am Wassergehalt des Gehirns, am Auftreten von Vacuolen in der weissen Masse und durch Funktionsstörungen des Zentralnervensystems.

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Study of Long-term Percutaneous Toxicity and Carcinogenicity of Hair Dyes (Oxidizing Dyes) in Rats*

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Abstract—The long-term percutaneous toxicity and carcinogenicity of *p*-toluenediamine, resorcinol and *m*-diaminoanisole, which are all important constituents of hair dyes, were studied in rats. The substances examined, *p*-toluenediamine alone in an appropriate vehicle or a mixture of all three compounds similarly formulated, were applied to the shaved dorsal skin of the rats twice weekly for 2 yr, in doses considerably greater than those used in practice. One group of control animals remained untreated while a second was treated with the vehicle alone. After the application period of 2 yr, surviving animals were observed for another 6 months. There were no indications that the substances examined had any adverse effects. Behaviour, feed intake, body-weight changes, blood counts, activity of serum glutamic–pyruvic transaminase and results of the bromosulphthalein test for liver function were comparable in the control and experimental groups. No pathological formation of Heinz bodies or methaemoglobin was associated with application of the dye components. Similarly, experimental and control animals showed no difference in respect of their mean lifespan or the type and incidence of tumours. No tumours or other skin reactions occurred at the site of application of the substances and histopathological studies of the liver, kidneys and lungs provided no evidence of degenerative change or functional disturbance. Acute studies involving sc or ip injection of the substances confirmed the lack of effect on erythropoiesis and methaemoglobin formation.

INTRODUCTION

Of the aromatic diamines, aminophenols and phenols commonly included in preparations used for dyeing human hair, the most important is the oxidizing dye, *p*-toluenediamine. *p*-Phenylenediamine is another major component in some countries, while other para and the corresponding meta compounds are normally used in much lower concentrations as shading dyes. Reports of tumour induction by some dyes administered to experimental animals by various routes (Saruta, Yamaguchi & Matsuoka, 1962) has provoked some suspicion about the carcinogenicity of substances of this type. Ito, Hiasa, Konishi & Marugami (1969) reported the formation of hepatic carcinomas in Wistar rats fed diets containing 0·1 or 0·06% *m*-toluenediamine.

In the work reported here, the percutaneous toxicity and possible carcinogenic effects of *p*-toluenediamine and of a mixture of *p*-toluenediamine, resorcinol and *m*-diaminoanisole in concentrations used in practice in hair-dyeing to achieve particularly dark shades were studied in rats over almost the whole of their lifespan.

*These investigations were conducted on behalf and with the assistance of Elida-Gibbs GmbH, Hamburg, L'Oreal, Haarkosmetik und Parfümerien GmbH, Karlsruhe, Hans Schwarzkopf GmbH, Hamburg, Thera-Chemie GmbH, Düsseldorf, and Wella AG, Darmstadt.

EXPERIMENTAL

Materials. The substances examined were prepared by incorporating *p*-toluenediamine at a concentration of 4% (model substance T) or levels of 3% *p*-toluenediamine, 0·75% resorcinol and 0·75% *m*-diaminoanisole (substance TRA) in a carboxymethylcellulose (CMC) slime (4% Tylose HT supplied by AKU, Arnhem, The Netherlands), to which 0·5% pure sodium sulphite was added to prevent premature atmospheric oxidation of the amino components. The two dye bases were available commercially as the sulphates from Farbwerke Hoechst AG, Frankfurt a.M., were therefore partly dissolved and converted with 25% ammonia solution in the absence of iron and, with stirring, dissolved completely in the CMC slime to form slightly brown, transparent jellies with a pH of about 10·1. These were filled into aluminium tubes coated inside with a protective lacquer, in which form they were stable for several years. A colourless control jelly (substance O) was similarly prepared, part of the ammonia being replaced by ammonium sulphate in an amount equivalent to that formed in the experimental preparations by neutralization of the basic dye sulphates (Table 1). All three formulations were therefore comparable in free ammonia content and pH, although in each case control analyses showed that part of the free ammonia was lost during preparation and the filling of the tubes (Table 1).

Table 1. *Composition of experimental formulations*

Component/Analysis	Concentration (% w/w) in substance		
	T	TRA	O
Component			
Tylose HT	4·0	4·0	4·0
Sodium sulphite	0·5	0·5	0·5
Ammonia, 25%	13·0	12·5	8·5
p-Toluenediamine*	4·0	3·0	—
Resorcinol	—	0·75	—
m-Diaminoanisole*	—	0·75	—
Ammonium sulphate	as formed by neutralization	as formed by neutralization	3·7
Deionized water	78·5	78·5	83·3
Total....	100·0	100·0	100·0
Analysis			
Ammonia: total (100%), titrated	2·95	2·90	3·03
free (100%)	1·96	1·96	1·99
pH	10·1	10·1	10·2

*Used as sulphate, calculated as free base.

Animals and diet. Sprague–Dawley rats from a closed randomized stock were obtained from Dr. K. E. Møllegard-Hansen, Havdrup-Kopenhagen, Denmark, for the skin-painting study. The rats (about 3 months old and with a mean body weight just under 100 g) were given a 2-wk adaption period before treatment was begun and were maintained throughout on a standard diet (Altromin-R) and water given *ad lib.* Injection studies were carried out on 6-month-old rats of the Wistar–Elberfeld strain bred at Battelle.

Experimental design and conduct

Skin application study. A preliminary 4-wk experiment established that twice-weekly application of the test substances to the skin of rats caused no local irritation. In the main experiment, three groups, each of 50 male and 50 female rats, were treated with substance T or substance TRA or given no treatment, while a fourth group of 25 males and 25 females was treated with the control substance O. To simulate conventional hair-dyeing techniques, substances T, TRA and O were mixed with an equal volume of 6% hydrogen peroxide immediately before application. This was done in batches to ensure that the material was applied to the skin within 10 min of mixing.

The solution (0·5 g) was applied to the shaved dorsal skin over an area of 3 × 3 cm and was left on the skin for 30 min, during which the animals were kept apart and fitted with a rubber collar to prevent any ingestion of the applied material. As under practical conditions, the painted area was then cleaned with a commercial agent and washed with water, and the animal was rubbed with a towel and dried under an infrared lamp. This procedure was repeated twice weekly for 2 yr, after which surviving rats were observed for a further 6 months.

The rats were weighed weekly and observed regularly for any change in behaviour. Red and white cell counts and haemoglobin and methaemoglobin levels were determined in blood samples from ten males from each group at 3-monthly intervals, and at 4-monthly intervals liver function was checked by means of a bromsulphthalein test and the determination of serum glutamic–pyruvic transaminase activity (using the μl-scale Biochemica-Test Combination, Boehringer AG, Mannheim), again in ten males from each group. Transaminase activity was also determined in random samples taken from tumour-bearing animals. At month 7 and 16, blood samples from three animals in each group were examined for Heinz bodies by two methods, the conventional Nile-blue sulphate staining and a fluorescence microscopic-detection method using morin. All rats that died during the experiment or were killed when moribund and all survivors, which were killed by decapitation, were autopsied and all tumours were examined histologically. Sections from the application site, liver, kidneys, lungs and any organ that appeared abnormal at autopsy from a total of nine or ten males and ten females from each group killed randomly and singly at intervals of 6, 9, 12 and 24 months during the study were stained with haematoxylin and eosin for microscopic examination.

Injection study. Heinz-body counts and methaemoglobin determinations were carried out in groups of 2–4 rats given a single sc injection of 0·75–24·0 mg *p*-toluenediamine sulphate/kg, or the same dose repeated daily for 3–5 days, a single ip injection of the same compound in doses of 4–32 mg/kg, or a single sc injection of substance TRA in a 1 : 1 mixture with hydrogen peroxide in a dose equivalent to 1·5 or 3 mg *p*-toluenediamine sulphate/kg (1 mg *p*-toluenediamine sulphate being equivalent to 0·555 mg free base). Sodium sulphite was added to the aqueous solution of *p*-toluenediamine sulphate as in the formulation of substance T, used in the skin painting study, and the pH of the preparation was adjusted to 7 by addition of ammonia.

RESULTS

Growth, food intake and mortality

The behaviour of all the rats was normal and there were no marked differences between the various groups in respect of body-weight changes (Table 2) and food intake. The mean

lifespan and mortality rate during the long-term experiment were comparable in all experimental and control groups.

Table 2. *Mean body weights of groups of control rats and of rats treated with* p-*toluenediamine formulations by twice-weekly applications to the skin for 2 yr*

Treatment	Body weight* (g) at wk			
	0†	52	104	130
		Males		
None (control)	86 ± 15 (50)	456 ± 49 (36)	437 ± 71 (26)	298 ± 35 (9)
Substance O (control)	91 ± 13 (25)	452 ± 45 (18)	421 ± 64 (9)	361 (3)
Substance T	88 ± 15 (50)	443 ± 47 (43)	409 ± 64 (27)	343 ± 63 (8)
Substance TRA	90 ± 17 (50)	446 ± 53 (41)	411 ± 59 (27)	328 ± 35 (9)
		Females		
None (control)	83 ± 13 (50)	289 ± 40 (44)	287 ± 50 (21)	248 ± 41 (7)
Substance O (control)	83 ± 11 (25)	269 ± 33 (19)	258 ± 41 (10)	260 (2)
Substance T	82 ± 14 (50)	282 ± 33 (42)	292 ± 47 (14)	278 ± 59 (6)
Substance TRA	81 ± 16 (50)	280 ± 37 (42)	277 ± 34 (23)	247 ± 31 (7)

O = Vehicle only T = *p*-Toluenediamine formulation
TRA = *p*-Toluenediamine–resorcinol–*m*-diaminoanisole formulation
*Values given are means ($\bar{x}$) ± standard deviation $\{\sqrt{[S(x^2)-\bar{x}S(x)]/(n-1)}\}$ for the numbers of animals (n) indicated in parentheses.
†Beginning of the experiment.

Haematology

All the haematological data recorded were within the normal ranges. No pathological methaemoglobin formation was observed even at 3 min or 4 hr after ip injection of a single highly toxic dose of 32 mg *p*-toluenediamine sulphate/kg. No Heinz bodies were found in animals treated with the experimental substances for 7 or 16 months or in those given a single sc injection of 1·5 mg *p*-toluenediamine sulphate/kg. A very small number of Heinz bodies was found 2–3 days after administration of several of these sc injections and also after the single sc injection of a comparable dose of TRA (1–2/1000 erythrocytes), but even in a dose of 12 or 24 mg/kg given ip, *p*-toluenediamine sulphate caused little or no increase in Heinz bodies. In a positive control experiment, phenylhydrazine dihydrochloride given ip as a 1% aqueous solution in a dose of 3 mg/kg body weight produced an incidence of Heinz bodies of 500–700/1000 erythrocytes.

Liver function

No impairment of liver function was detected in the experimental groups. Bromsulphthalein excretion by the liver was comparable in experimental and control groups and the serum transaminase values were not increased in the treated animals or in any of the random samples from animals with tumours.

Autopsy and histopathology

Autopsies revealed no pathological changes attributable to either of the long-term treatments. The main causes of death were pneumonia and lymphadenitis, the latter having the symptoms of a Klebsiella infection and being associated with abscess formation but not

Table 3. *Tumour incidence in control rats and rats treated with* p-*toluenediamine formulations by twice-weekly applications to the skin for 2 yr*

Treatment	Sex	No. of rats/group	Rats with tumours		Tumours		
			No.	% of group	Total no.	Type (no.)	Time of appearance* (months)
None (control)	M	50	4	8	4	Fibrolipoma	20
						Lymphosarcoma	23
						Hyperkeratotic papilloma	29
						Polymorphocellular sarcoma†	24
	F	50	12	24	15	Round-cell sarcoma†	11
						Round-cell sarcoma	19
						Cystadenomas (2)	22, 29
						Fibroadenomas (8)	16–29
						Adenomas (2)	17, 24
						Fibroma	17
Substance O	M	25	1	4	1	Round-cell sarcoma	18
	F	25	4	16	4	Round-cell sarcoma	24
						Carcinoma solidum	29
						Fibroadenoma	25
						Fibrosarcoma	12
Substance T	M	50	4	8	5	Fibromas (2)	18, 31
						Lipoma	24
						Round-cell sarcoma	21
						Lymphosarcoma	21
	F	50	10	20	11	Fibroadenomas (8)	15–26
						Carcinoma solidum	17
						Adenocarcinoma	23
						Adenoma	28
Substance TRA	M	50	3	6	4	Lymphosarcoma	29
						Adenoma	29
						Fibroma	18
						Adrenal tumour	25
	F	50	14	28	15	Cystadenoma	18
						Fibromas (2)	18, 25
						Fibroadenomas (6)	18–29
						Fusocellular sarcoma	15
						Lymphosarcomas (2)	28
						Hyperkeratotic papilloma	12
						Carcinoma solidum†	21
						Squamous cell carcinoma†	27

O = Vehicle only T = *p*-Toluenediamine formulation
TRA = *p*-Toluenediamine–resorcinol–*m*-diaminoanisole formulation
*Time at which tumour was detected, calculated from beginning of experiment.
†With metastases in the peritoneal cavity.

with any "non-specific irritation" (Huhn, 1966) of the lymph nodes in the form of lymphogenous tumour cell propagation and formation of metastases. Both of these diseases occur frequently in the Sprague–Dawley rats used, but were no more common in the experimental groups than in the controls. Histopathological studies similarly revealed no tissue changes that could be ascribed to treatment with the dyes.

Tumour incidence. Table 3 shows that tumours only became clinically detectable during the second half of the life of the rats. Of the 350 Sprague–Dawley rats used in the study, 52 (15%) developed tumours and six had more than one tumour, but the incidence, the proportion of males and females affected and the types of tumours were comparable in the experimental and control groups (Table 3). The 59 tumours found included 16 different types (Table 4), of which fibroadenomas were by far the most frequent, followed by fibromas, lymphosarcomas and round-cell sarcomas. The two skin tumours (hyperkeratotic papillomas) and ten sc tumours (six fibromas, one fibrolipoma, one fibrosarcoma, one lipoma and one round-cell sarcoma) were not found in the area of application of any of the substances.

Table 4. *Identity and location of tumours in control rats and rats treated with* p-*toluenediamine formulations by twice-weekly applications to the skin for 2 yr*

Type of tumour	Location of tumour	Sex	No. found in: Untreated controls M	Untreated controls F	Animals treated with substance: O M	O F	T M	T F	TRA M	TRA F
Adenocarcinoma (m)	E							1		
Adenoma (b)	E			2				1	1	
Carcinoma solidum (m)	E					1		1		1
Fibroadenoma (b)	E			8		1		8		6
Fibrolipoma (b)	Ms		1							
Fibroma (b)	Ms			1			2		1	2
Fibrosarcoma (m)	Ms					1				
Hyperkeratotic papilloma (b)	ES		1							1
Cystadenoma (b)	E			2						1
Lipoma (b)	Ms						1			
Lymphosarcoma (m)	M		1				1		1	2
Phaeochromocytoma (b)									1	
Squamous cell carcinoma (m)	E									1
Polymorphocellular sarcoma (m)	M		1							
Round-cell sarcoma (m)	M			2	1	1 (s)	1			
Fusocellular sarcoma (m)	M									1
Total number of tumours			4	15	1	4	5	11	4	15

m = Malignant b = Benign E = Epithelial tissue M = Mesenchymal tissue
s = Subcutaneous tissue S = Skin

DISCUSSION

Although the amount of solution applied to the animal and the frequency of application both exceeded those likely to be encountered in practice, no evidence of carcinogenic or other toxic effects was detected in the experimental animals. The relatively high tumour incidence was to be expected in these Sprague–Dawley rats, which are somewhat susceptible

to spontaneous tumour formation and were examined or kept under observation for up to 2·5 yr. The tumour incidence and lifespan were comparable in all the experimental and control groups, however, and no tumours developed at the site of application. The other criteria studied (behaviour, feed consumption, body-weight changes, blood parameters, serum activity of glutamic–pyruvic transaminase and bromosulphthalein tests for liver function) were also comparable in experimental and control groups.

Although there is evidence, including the development of allergic reactions in hypersensitive subjects, that substances of the type examined penetrate through the epidermis and also into the hair shaft, no signs of skin irritation, such as the erythema, oedema or scar-tissue formation described by Carson, Weinberg & Goldhamer (1965), nor any increase in cell proliferation in the skin were observed. Kiese, Rachor & Rauscher (1968) showed that phenylenediamines were absorbed through the skin of dogs when applied in vehicles used in the dyeing of human hair and appeared unchanged in small amounts in the urine. Absorption was reduced when hydrogen peroxide was added to the mixture before application. Kiese & Rauscher (1968) demonstrated the absorption of *p*-toluenediamine through the human scalp during dyeing of the hair with a mixture of *p*-toluenediamine sulphate with resorcinol and hydrogen peroxide. The absorbed material was slowly excreted in the urine, principally as *N,N'*-diacetyl-*p*-toluenediamine. It was not known, however, whether the *p*-toluenediamine was absorbed as such, or was liberated in the body from some compound formed during the oxidation of the *p*-toluenediamine–resorcinol mixture.

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Etude sur le rat de la toxicité percutanée à long terme et des propriétés carcinogènes de teintures capillaires (colorants oxydants)

Résumé—On a étudié sur des rats la toxicité percutanée à long terme et le pouvoir carcinogène de la *p*-toluènediamine, du résorcinol et du *m*-diaminoanisol, produits qui sont d'importants composants de teintures pour les cheveux. On les a appliqués—la *p*-toluènediamine seule dans un porteur approprié ou chaque produit dans des mixtures de compositions similaires—deux fois par semaine, et ceci pendant 2 ans, à la peau rasée du dos des rats. Les doses étaient beaucoup plus fortes que celles utilisées dans la pratique. Un groupe d'animaux témoins n'a pas été traité et un second groupe n'a été traité qu'au seul porteur. Après les 2 années de traitement, on a prolongé de 6 mois l'observation des animaux survivants.

Rien n'a indiqué que les substances examinées eussent un effet nocif quelconque. Le comportement, la consommation de nourriture, l'évolution du poids vif, les numérations globulaires, l'activité de la glutamate-pyruvate-transaminase sérique et les résultats du test par la bromsulfonephtaléine de la fonction hépatique des groupes témoins et des groupes d'essai sont restés comparables. L'application des composants de teinture n'a pas donné lieu à des formations pathologiques de corps de Heinz ou de méthémoglobine. De même, on n'a pas constaté de différences entre les animaux d'essai et les animaux témoins sous le rapport de la longévité moyenne et quant aux types et fréquences de tumeurs. Aucune tumeur ou autre réaction cutanée ne s'est manifestée au site d'application des produits et l'examen histopathologique du foie, des reins et des poumons n'a apporté aucune preuve d'altération dégénérative ou de désordre fonctionnel.

Des études en phase aiguë, comportant l'injection sous-cutanée ou intrapéritonéale des produits, ont confirmé l'absence d'effet sur l'érythropoièse et sur la formation de méthémoglobine.

Langzeituntersuchung der percutanen Toxizität und Carcinogenität von Haarfärbemitteln (oxydierenden Farbstoffe) an Ratten

Zusammenfassung—Die langzeitige percutane Toxizität und carcinogene Wirkung von *p*-Toluoldiamin, Resorcinol und *m*-Diaminoanisol, die alle wichtige Bestandteile von Haarfärbemitteln sind, wurden an Ratten untersucht. Diese Substanzen, entweder *p*-Toluoldiamin allein in einem geeigneten Bindemittel oder eine Mischung von allen drei Verbindungen in ähnlicher Rezeptierung, wurden auf die rasierte Rückenhaut der Ratten 2 Jahre lang zweimal wöchentlich aufgetragen, und zwar in beträchtlich grösseren Dosen, als sie in der Praxis angewendet werden. Eine Gruppe von Kontrolltieren blieb unbehandelt, während eine zweite mit dem Bindemittel allein behandelt wurde. Nach der Anwendungsperiode von 2 Jahren wurden die überlebenden Tiere weitere 6 Monate beobachtet. Es gab keine Anzeichen dafür, dass die untersuchten Substanzen nachteilige Wirkungen hatten. Das Verhalten, der Futterverbrauch, die Körpergewichtsänderungen, das Blutbild, die Aktivität der Serum-Glutaminsäure-Pyruvat-Transaminase und die Ergebnisse der Bromsulphthaleinprobe der Leberfunktion waren in der Kontrollgruppe und in den Versuchsgruppen vergleichbar. Keine pathologische Bildung von Heinzschen Körpern oder Methämoglobin war mit der Anwendung der Färbemittelbestandteile verbunden. Ähnlich zeigten die Versuchs- und die Kontrolltiere keinen Unterschied hinsichtlich ihrer mittleren Lebensdauer oder der Art und Häufigkeit von Tumoren. Keine Tumoren oder anderen Hautreaktionen traten an der Anwendungsstelle der Substanzen auf, und histopathologische Untersuchungen der Leber, der Nieren und der Lungen boten keinen Beweis für degenerative Veränderungen oder funktionelle Störungen. Akute Untersuchungen mit sc oder ip Injektion der Substanzen bestätigten die Wirkungsfreiheit bezüglich der Erythropoese und der Methämoglobinbildung.

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SHORT PAPER

The Effects of Long-term Ingestion of Methimazole on the Thyroids of Rats

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Summary—Harlan rats were fed methimazole in the diet at levels of 0, 5, 30 and 180 ppm for 2 yr. Survival was poor in the rats of the 180 ppm group and their growth was greatly retarded. Hypertrophy and hyperplasia of the thyroid occurred in rats of the 30 and 180 ppm groups but not in those of the 5 ppm group. In rats of the 30 and 180 ppm groups there was a high incidence of thyroid follicular adenoma and a lower incidence of follicular adenocarcinoma. A follicular adenoma was also found in one rat of the control group and one given 5 ppm methimazole, while one rat in the control group had a follicular adenocarcinoma. Incidence and induction time of neoplasias other than of the thyroid were similar in the treated and control groups. The no-effect dietary level of methimazole on the thyroids of rats was found to be 5 ppm.

Introduction

Methimazole (1-methyl-2-mercaptoimidazole; Tapazole®), is an established antithyroid drug in human medicine. Moreover, its inclusion in the feed of meat-producing animals improves carcass quality. In this connexion, we undertook a 2-yr continuous feeding study in rats to establish the effects of long-term exposure to methimazole.

Experimental

Weanling Harlan rats (initial body weight 86–136 g) were obtained from Harlan Industries, Cumberland, Ind., for this 2-yr feeding study. The control group consisting of 50 males and 50 females was fed a complete ration of Lilly mill diet only. Groups of 25 rats of each sex were fed Lilly mill diet containing pure methimazole in finely granular form at levels of 5, 30 or 180 ppm. The rats were kept in individual hanging cages with feed and water always available.

All rats were autopsied. Following gross inspection, samples of thoracic and abdominal viscera, thyroid, brain, pituitary and mammary gland were fixed in 10% buffered formalin and processed for histological study. The tissue sections were stained with haematoxylin and eosin.

Results

Clinical observations

Differences between experimental and control groups occurred only with rats on the 180 ppm methimazole diet. Growth was greatly retarded in this group, the rats being runts,

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with short but stout bodies. After reaching maturity, these runts became obese. Survival was poor, with a 50% mortality during the first year compared with less than 10% for the other groups. Only 6% survived the 2-yr study, whereas 16–20% of the other groups survived.

Pathological findings

Morphological changes of the thyroid are listed in Table 1. Thyroid enlargement occurred in rats fed 30 or 180 ppm methimazole but not in those fed 5 ppm. The lobes and isthmus were uniformly enlarged, up to 2–6 times normal size. Microscopic examination showed hypertrophy or hyperplasia of the follicles. Hypertrophy occurred in rats that died before day 50 of treatment. The follicles were enlarged, the lining epithelium was columnar and the colloid was thin. Hyperplasia was present after 50 days of exposure. There was an increased number of small follicles which, in most cases, contained thin colloid.

Table 1. *Thyroid lesions in rats fed 0–180 ppm methimazole in the diet in a 2-yr toxicity study*

		No. of rats affected in groups given methimazole (ppm)							
		0		5		30		180	
Thyroid lesion	Sex...	M	F	M	F	M	F	M	F
Hypertrophy, mild		0	0	0	0	3	3	3	7
moderate		0	0	0	0	1	0	0	0
Hyperplasia, mild		0	0	0	0	6	7	2	3
moderate		0	0	0	0	7	9	5	4
marked		0	0	0	0	7	2	4	7
Follicular adenoma(s)		1	0	1	0	17	14	5	12
Follicular adenocarcinoma(s)		1	0	0	0	2	3	3	2
Carcinoma		0	0	0	0	0	1	0	0
Light-cell adenoma(s)		3	4	2	1	2	5	1	0

A high incidence of follicular adenomas occurred in rats on the 30 and 180 ppm methimazole regimens and there was also a low incidence of follicular adenocarcinomas in these groups. Some of these lesions were up to 1·0 cm in diameter and could be palpated in the live rat. However, most of them were 0·1–0·3 cm in diameter and were detected by serial sectioning of the thyroid and by microscopic examination. The larger nodules were usually solitary while up to three of the 0·1–0·3 cm nodules could be detected in a single lobe.

The prototype adenoma compressed the surrounding thyroid tissue and was delimited from it by a thin and sometimes incomplete band of connective tissue. The follicles were smaller than those of the adjacent thyroid tissue and the lining cells were smaller and more basophilic. The colloid appeared relatively normal. Variant adenomas contain large pools of colloid, usually in the centre, with sheets of benign follicular cells at the periphery. One rat in the control and one in the 5 ppm methimazole group had a follicular adenoma morphologically similar to the prototype of the other groups.

The follicular adenocarcinomas were similar in appearance to the variant follicular adenomas except that the sheets of follicular cells were relatively undifferentiated and the differentiated follicles were variable in size. All but one of the adenocarcinomas were confined to the thyroid. Metastasis to the lungs occurred in one rat on the 30 ppm methimazole regimen. Concomitant growth of follicular cells in a few of the capsular blood

vessels of the thyroid were detected in some of the rats. One of the rats on the 30 ppm regimen had a thyroid neoplasm classified simply as a carcinoma and consisting of broad sheets of undifferentiated epithelial cells.

The incidences of thyroid adenomas and adenocarcinomas are given in Table 2. The method of calculation was that of Bryan & Shimkin (1941), the percentage being based on the number of animals in the group surviving at the time the first neoplasm was detected. Since there was no indication of sex difference in incidence, both sexes were combined for the purpose of calculation; thus, the susceptible population was counted from the earliest detected tumour in each diet group regardless of sex. The incidence of adenomas was similar in the 30 and 180 ppm methimazole groups, whereas that for adenocarcinomas was greater for the 180 ppm group (21 against 12%). However, the tumour incidence for the 180 ppm group was based on a much smaller group of surviving rats than that for the 30 ppm group. The time when the first tumour was detected (Table 3) was considerably earlier in the 180 ppm group.

Table 2. *Incidence of thyroid neoplasia in rats given 0–180 ppm methimazole in the diet in a 2-yr toxicity study*

Thyroid neoplasia	Incidence* in groups of rats fed methimazole (ppm)			
	0	5	30	180
Follicular adenoma	1/55	1/8	31/55 (56%)	17/32 (53%)
Follicular adenocarcinoma	1/17	0	5/42 (12%)	5/24 (21%)

*Expressed as no. of rats with tumour/no. surviving when first tumour was detected in the group. There was no indication of any sex difference in the incidence of either type of tumour.

Table 3. *Day of onset of thyroid neoplasia in rats given 0–180 ppm methimazole in the diet in a 2-yr toxicity study*

Dietary level (ppm)	Sex	Time (days) of first appearance of	
		Follicular adenoma	Follicular adenocarcinoma
0	M	625*	732*
	F	NT	NT
5	M	732*	NT
	F	NT	NT
30	M	508	508*
	F	461*	566
180	M	368	360*
	F	256*	623

NT = No tumours found

*Determined at autopsy.

The incidence of light-cell adenomas of the thyroid was similar in the control and experimental groups (Table 1) and the time of onset was not affected by treatment with methimazole.

Table 4. *Incidence of neoplasia other than of the thyroid in rats fed dietary levels of 0–180 ppm methimazole for up to 2 yr*

Organ and type of neoplasm	No. of rats examined...	No. of rats with neoplasms							
		Males fed methimazole (ppm)				Females fed methimazole (ppm)			
		0	5	30	180	0	5	30	180
		50	25	25	25	50	25	25	25
Pituitary									
Chromophobe adenoma		5	5	4	1	26	11	12	1
Mammary gland									
Fibroadenoma		0	0	0	0	9	6	8	0
Adenocarcinoma		0	0	0	0	2	2	0	1
Lymphoreticular system									
Reticulum-cell sarcoma		5	4	5	0	4	2	0	0
Lymphosarcoma		1	1	0	0	1	0	0	0
Thymoma		2	0	0	0	0	0	0	0
Skin									
Epidermoid carcinoma of Zymbal's gland		0	0	0	0	1	0	0	0
Epidermoid carcinoma		0	1	0	0	0	0	0	0
Sebaceous-cell adenoma		0	1	0	0	0	0	0	0
Papilloma		0	1	0	0	0	0	0	0
Subcutaneous tissue									
Neurofibroma		1	1	1	0	1	0	0	0
Neurogenic sarcoma		0	0	0	0	0	1	0	0
Liposarcoma		0	0	0	0	1	0	0	0
Fibroxanthoma		0	0	0	1	0	0	0	0
Kidney									
Nephroblastoma		0	0	0	0	0	0	0	1
Adrenal									
Phaeochromocytoma		4	1	2	1	0	0	1	0
Adenoma		0	0	0	0	1	0	0	0
Adenocarcinoma		0	0	0	0	0	0	1	0
Uterus									
Cystadenoma		—	—	—	—	0	0	1	0
Haemangioma		—	—	—	—	0	1	0	0
Prostate									
Fibrosarcoma		0	1	0	0	—	—	—	—
Pancreas									
Adenocarcinoma		0	0	0	0	1	0	0	0
Spleen									
Haemangioendothelioma		0	1	0	0	0	0	0	0
Brain									
Glioma		0	0	0	1	0	0	0	0
Brain neoplasm, unclassified		0	0	0	0	0	0	0	1
Meningioma		0	1	0	0	0	0	0	0
Meningeal neoplasm, unclassified		0	1	0	0	0	0	0	0
Circulatory system									
Granulocytic leukaemia		1	1	0	0	0	0	0	0
Lymphocytic leukaemia		0	1	0	0	0	0	0	0

The only other treatment-related changes were an increase in the severity of fatty metamorphosis of the liver and an increased incidence and severity of fatty degeneration of the kidneys in rats on the 180 ppm methimazole diet. The incidence, induction time and/or severity of neoplasias other than of the thyroid (Table 4) and of naturally occurring disease processes were similar in the treated and control groups. The incidences of pituitary and mammary gland neoplasia were greatly reduced in rats on the 180 ppm methimazole regimen because of the high mortality in this group during the first year of the test, at an age prior to the usual onset of these lesions.

Discussion

It is well recognized that continuous suppression of thyroid function in rats by sufficient doses of a variety of antithyroid agents causes thyroid hypertrophy and hyperplasia and that if such agents are administered for long enough they lead to thyroid follicular neoplasia, although neoplasia does not result when the agents are given intermittently (Griesbach, Kennedy & Purves, 1945; Jukes & Shaffer, 1960).

An important finding in our study was the no-effect level of 5 ppm methimazole in the diet. One of the rats had a follicular adenoma but so did one of the control group, and one of the control group had a follicular adenocarcinoma.

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Review Section

REVIEWS OF RECENT PUBLICATIONS

Evaluation of Certain Food Additives and the Contaminants Mercury, Lead, and Cadmium. Joint FAO/WHO Expert Committee on Food Additives–Sixteenth Report. *Tech. Rep. Ser. Wld Hlth Org.* 1972, **505**, pp. 32. £0.40.

The latest report summarizing the findings and recommendations of the Joint FAO/WHO Expert Committee on Food Additives contains an assessment of the hazard to man resulting from the ever-increasing levels of mercury, lead and cadmium in the environment, and particularly in food. The report is completed with a reassessment of four intentional food additives, amaranth, caramel colourings, diethyl pyrocarbonate and octyl gallate. This publication is valuable for its clear interpretation of a considerable volume of up-to-date toxicological information collected from world-wide sources.

Each of the metal contaminants is examined from the point of view of sources of environmental contamination, its level in foods and total diets, and the hazard it is expected to present to human health. The Committee recognizes that the established practice of setting acceptable daily intakes (ADIs) is not appropriate in the case of heavy metals, since these compounds are essentially cumulative poisons and their levels in diets vary considerably. There are also problems in connexion with the distinction between different chemical forms of these contaminants, varying susceptibilities among different population groups and a lack of facts regarding existing responses to current levels of exposure. Moreover, in some areas, the narrow margin between existing exposure and the levels causing overt symptoms would not permit the imposition of a reasonable safety factor (as normally incorporated in an ADI) and the maintenance of a normal food intake. The estimation of a provisional tolerable weekly intake (PTWI) is therefore considered to be more appropriate, although the Committee still recommends that separate assessments should be made for populations on atypical diets.

In the case of mercury, particular attention is paid to methylmercury compounds, which are considered to be far more toxic than other forms of the element, and also to the aquatic pollution which results from mercury accumulating in edible fish. The PTWI for mercury is set at 0·3 mg total mercury/person, with a maximum of 0·2 mg methylmercury. The Committee recognizes that some fish-eating populations already ingest more than the prescribed limit for methylmercury, but suggests that this could probably be tolerated for a limited period without adverse effect.

The PTWI for lead of 3 mg for an adult (about 0·05 mg/kg body weight) is based on the assumption that only 10% of lead ingested in water and food is absorbed. No attempt is made to establish a tolerable level for infants and children, who are known to be far more sensitive to lead than adults.

In view of the great variation in the extent of cadmium accumulation by adults in different

countries, it is recommended that the suggested PTWI of 400–500 μg/person should be subject to revision when more data become available. The Committee points out that the recommended limit is already exceeded in some areas, and that continuing environmental contamination by the metal is likely further to increase its level in food in the future.

The main difficulty encountered in an evaluation of such ubiquitous pollutants is that the only really effective way of reducing the hazard to health lies in decreasing the contamination from industrial and related sources. So long as such pollution continues to increase, the levels of these metals in food will rise, resulting in the loss of numerous valuable food sources.

Turning to intentional food additives, the Committee recommends, in the light of fresh data, a temporary ADI of 0–0·75 mg/kg for amaranth, a reduction in the acceptable level of treatment with diethyl pyrocarbonate to 250 ppm and a restriction of such use only to soft drinks with a maximum pH of 4, and the cessation of the use of octyl gallate in beverages. In addition the Committee has prepared a revised specification for caramel colourings prepared by the ammonia process. Until this is published, it can be obtained on request from: Food Policy and Food Science Service, Food and Agriculture Organization, 00100 Rome, Italy.

Methods of Aflatoxin Analysis. By B. D. Jones. Tropical Products Institute, London, 1972. pp. v + 58.

This is an authoritative report on the best up-to-date chemical, as distinct from biological, methods for the quantitative determination of aflatoxins. For ease of reference, the various stages in the methodology are set out on the decimal classification system. All the methods given are based on the characteristic fluorescence of aflatoxins under ultraviolet light after their separation from interfering compounds by thin-layer chromatography. Particular consideration is given to procedures suitable for use in overseas laboratories. The book is divided into sections dealing with all stages of aflatoxin analysis, including sampling methods, defatting, extraction of aflatoxins, "clean-up" procedures, assessment of aflatoxins and chemical confirmation. All stages are dealt with exhaustively, with full experimental details and alternative procedures and with adequate explanations of the reasons for each step and of the difficulties likely to be encountered.

Two particularly valuable sections deal with the sequences recommended for the analysis of individual cereals, nuts, oil seeds and other products and with calculations of the aflatoxin content of samples, with examples of the use of each of the recommended procedures. Appendices give details of the apparatus and chemicals required and suppliers of specialized equipment for aflatoxin analyses, some suppliers of aflatoxin standards, bulk sampling methods for aflatoxin analysis and the determination of the concentration and purity of aflatoxin standards.

Analysts engaged in aflatoxin work will find this book of immense value. The forthright style of presentation and the copious cross-references are particularly welcome.

Department of Trade and Industry: Laboratory of the Government Chemist. Report of the Government Chemist 1971. HMSO, London, 1972. pp. v + 176. £1.25.

During 1971 the Laboratory continued its extensive programme of work on the analysis of food additives and contaminants, water, fertilizers and feeding stuffs, tobacco, drugs

and other medical supplies and industrial atmospheres, and the fruits of this labour are presented in the report cited above.

As in previous years, a large amount of effort was devoted to the determination of nitrosamines. The sensitivity of the analytical methods was improved so that the limit of detection in favourable circumstances was reduced to about 1 μg/kg, and the characterization of any nitrosamine detected was made unequivocal through the development of a technique combining gas chromatography with high-resolution mass spectrometry. A preliminary survey involving over 100 food samples confirmed previous findings of dimethylnitrosamine in bacon and salami and identified this contaminant in fresh cod (7/11 samples), hake (3/5 samples) and cheese (6/13 samples). Diethylnitrosamine, 1-nitrosopyrrolidine and 1-nitrosopiperidine were also detected in bacon. Levels were in all cases below 5 μg/kg, except in two bacon samples which contained 1-nitrosopyrrolidine at levels of 15 and 40 μg/kg.

Heavy-metal contamination has also received a large share of attention. The Laboratory collaborated in the analysis of fish and other foodstuffs for mercury, results of which were subsequently published in "Survey for Mercury in Food", the first report of the Working Party on the Monitoring of Foodstuffs for Mercury and Other Heavy Metals (*Cited in F.C.T.*, 1972, **10,** 399). Shellfish in general contained high levels of heavy metals, and cadmium was found at a level of 4 ppm in a sample of scallops. Of other foodstuffs, lamb's liver from the UK and New Zealand showed copper in concentrations ranging from 52 to 135 ppm. In samples of potable water examined, cadmium levels in no case exceeded the WHO European Standard level of 0·01 mg/litre, while lead in excess of the standard value of 0·1 mg/litre continued to be found in about two cases yearly. Such samples are generally associated with infrequently used supplies and further samples taken after thorough flushing reveal no cause for concern. However, significant amounts of lead and cadmium could be extracted from a set of 50 glazed plates with a 4% acetic acid solution, and heating of such a solution in imported glazed pottery casseroles also yielded significant lead levels in some cases. No such problem was evident with plastics culinary ware or enamel-ware in general, although some brightly-coloured imported mugs and plates in the latter category proved to contain high levels of extractable cadmium.

Another investigation was designed to determine whether oil pollution round the British Isles was raising the natural level of polycyclic aromatic hydrocarbons in seafoods. Fresh fish showed no detectable contamination, but shellfish such as clams, cockles, mussels and scallops contained benzo[*a*]pyrene at levels of 4–16 μg/kg, together with smaller amounts of benz[*a*]anthracene and benzo[*g,h,i*]perylene. Levels in river water varied from below the limit of detection in the rivers Tamar and Tavy in Cornwall to 0·012 μg/litre in the Manchester Ship Canal.

A large part of the Laboratory's work has always been devoted to pesticide residue analysis. In 1971 it was decided to discontinue the sampling of British mutton fat and of beef fat from Eire because of the continuing decline in organochlorine residues, but proof of this downward trend was provided by analysis of imported butter and mutton and beef kidney-fats. Pesticide residues in human fat showed evidence of a decline since a previous study was carried out in 1965, with falls of about 30% in dieldrin levels and over 20% in total DDT; heptachlor epoxide had also declined, but BHC levels remained approximately steady. β-BHC was of almost universal occurrence, as were small traces of hexachlorobenzene. In wild birds, the main residues were dieldrin and *p,p'*-DDE, hexachlorobenzene and heptachlor epoxide being relatively uncommon and confined to terrestrial predators. Polychlorinated biphenyls were again common and, indeed, dominant in some samples,

reaching the massive level of 600 ppm in some samples of heron liver. Pesticide residues in Antarctic fish, birds and mammals, brought back to the Laboratory by British Antarctic Survey teams, were low in species having short food chains and little contact with man, but wide-ranging and scavenging birds had acquired significantly higher levels of contamination.

The Laboratory has also been involved in the examination of medical plastics on behalf of the Department of Health and Social Security. Significant amounts of plastics additives could not be extracted from haemodialysis coils, and PVC bottles for storing liquid paraffin and arachis oil did not yield detectable levels of their organotin stabilizer on storage. On the other hand, small amounts of phthalates were found in serum and plasma exposed to PVC blood-transfusion equipment, and work on this subject is continuing.

The above items are but a small proportion of the studies described in the report, which should interest anyone concerned with problems of contamination in a great variety of fields.

Fd Cosmet. Toxicol. Vol. 11, pp. 659–669. Pergamon Press 1973. Printed in Great Britain

BOOK REVIEWS

Food Allergy. Its Manifestations and Control and the Elimination Diets. A Compendium with Important Considerations of Inhalant (Especially Pollen), Drug, and Infectant Allergy. By A. H. Rowe and A. Rowe, Jr. Charles C. Thomas, Springfield, Ill., 1972. pp. xvi + 687. $20.00.

This is very much a manual for the working clinician. The senior author has spent all his working life treating and studying food allergy and published his first book on the subject in 1931. With his son as co-author, he has now produced a revised and amplified version, which gives a detailed and comprehensive account of the Rowe concept and treatment of food allergy in all its manifestations. Theoretical considerations of immunological mechanisms are not entirely ignored, but they are not treated in depth, and many of the references to basic research work in this field are so dated as to give the impression that nothing has been done for the last twenty years or so. Food allergy is a neglected field of research, but not as neglected as this would imply. This, of course, does not necessarily decrease the book's value, as practical medicine is at best a very empirical science.

The authors have no narrow view of food allergy. They consider here not only the immediate and delayed gastro-intestinal symptoms that may follow the ingestion of a food to which the patient is hypersensitive, but also the remote effects on other organs. Asthma, urticaria, hay fever and perhaps migraine have for long been accepted as potential outcomes of such ingestion. Epilepsy, oedema of the Eustachian tube, corneal ulcers and many other symptoms would be less generally accepted without question as being caused by allergy to food. However, in each instance, clinical evidence is cited to uphold the authors' contention in this respect. In many instances, such evidence is based on the results of provocative feeding trials, or the disappearance of symptoms following strict adherence to one of the elimination diets designed primarily by Dr. Rowe, Sr. The unreliability of skin testing is correctly stressed.

The Rowe cereal-free elimination (CFE) diet in fact excludes not only all cereals but also all dairy products, beef, chocolate, coffee and fish. If allergy to fruit is suspected, a fruit-free CFE is substituted. If, after a few weeks on one or other of these diets, the patient becomes symptom-free, the conclusion is drawn that he is suffering from food allergy, whatever his symptoms. At this stage, single foodstuffs can be reintroduced into the diet and, if symptoms recur, that food is considered to be responsible and the patient is advised to refrain altogether from eating it. Other types of contact are also important. Some foods, such as flour, may be readily inhaled and hypersensitive patients may suffer symptoms as a result. Even the smell of the offending food being cooked may cause symptoms.

While the authors are undoubtedly correct in many of their observations, one does sometimes wonder whether the relief of symptoms following the use of their elimination diets may not be coincidental rather than truly indicative of an allergic basis. In one instance this is admitted. Some cases of acne were originally attributed primarily to food allergy

because they cleared markedly when the patients were on an elimination diet. Subsequently, however, it was concluded that the improvement was due to the low fat content of the diet rather than to freedom from some offending allergen.

Further, very little is said about various enzyme deficiencies which may produce gastro-intestinal symptoms similar to those due to allergy. Removal of the indigestible foodstuffs would again produce improvement.

The treatment advocated is clearly very rigorous and some exaggeration may be suspected in statements such as the assertion that kissing a hypersensitive child after eating a food to which he is allergic may be sufficient to produce symptoms. However, anyone who has experienced the day-to-day difficulties involved in bringing up such a child will be less inclined than the uninitiated to dismiss such statements unreservedly.

Undoubtedly many clinicians will disagree with the authors' principles of treatment and with some of their assertions, but most of them will respect the practical knowledge and full account of clinical experiences presented in this book.

Health and Food. Edited by G. G. Birch, L. F. Green and L. G. Plaskett. Applied Science Publishers Ltd., London, 1972. pp. xi + 224. £4.20.

This book presents the proceedings of a symposium held at the National College of Food Technology, University of Reading, in March 1972.

In his opening remarks, Professor E. J. Rolfe, Principal of NCFT, reviewed food processing and production from several different angles and outlined some of the problems involved. The authors of the sixteen papers that followed came not only from the UK, but also from the USA, France, the Netherlands and Poland and included names well known in food science, technology and toxicology. The discussion following each presentation has been published with the papers.

Several contributors mentioned the advantages and disadvantages of "health foods". The reasons given for the increase in demand for these foods included a deep-rooted distrust of the food manufacturer dating from the nineteenth century and the consumer's emotional attitudes to his food. Attention was also drawn to the belief that during the purification and processing of food, many essential components were removed, destroyed or converted into harmful materials, resulting in an increase in the incidence of certain types of disease such as ischaemic heart disease and diverticulosis. Indeed there have been suggestions that a consideration of dietary factors might lead to a possible prolongation of the lifespan. On the other hand, many highly toxic compounds occur naturally in the food we eat. Although these do not necessarily constitute any hazard to man, certain bacterial and fungal toxins exert powerfully adverse effects either in man or on the condition of the foods they contaminate. The discussion of these points at the symposium included suggestions as to means of eliminating such risks.

These days, no self-respecting discussion on food would be complete without a mention of residues of organochlorine pesticides and on this occasion particular consideration was given to the control and limitation of such residues in the ingredients of baby-foods manufactured in Poland.

Intentional food additives featured in several papers. Their legislative control, particularly in the EEC, was reviewed and some of the problems encountered in safety evaluation studies

and the need for a better understanding of the results obtained in scientific studies were outlined.

The inadequacy of world food supplies, the changing living habits of many populations and the increasing realization of the inability of some people to sustain themselves and remain healthy on conventional diets are providing an impetus for the development of new types of foodstuffs. Among the newer developments discussed at this meeting were foods for space travellers, food proteins derived from micro-organisms (the "single-cell proteins"), ready-prepared meals and therapeutic diets now used in the treatment of a variety of diseases.

It is encouraging to see from this well-produced publication that it is still possible under some circumstances for a group of symposium papers together with ensuing discussions and an adequate index to be published by conventional printing methods within 8 months of the meeting. This small book has much to offer to those interested in food not only in a professional capacity but also as consumers. Summing up at the end of the symposium, Professor Morley Kane put much of the preceding discussion into perspective with his comment: "While nutrient content and toxicity are obviously important, it would be a grave error to focus on these parameters and ignore the palatability of food".

Mercury in the Environment. An Epidemiological and Toxicological Appraisal. Edited by L. Friberg and J. Vostal. CRC Press, Ohio, 1972. pp. 215. $33.00.

There has been considerable public concern about the possible hazards that may arise from the mercury present in the environment. The epidemics of methylmercury poisoning in Minamata and Niigata in Japan, which claimed many deaths and caused permanent disabilities, brought home to everyone, laymen and scientists alike, the tragic consequences of failing to deal adequately with the disposal of this highly toxic but industrially valuable metal. A vast amount of epidemiological and experimental work has been carried out on both inorganic and organic mercury. In particular, metabolic studies and the determination of mercury in blood, hair and other tissues have been carried out primarily with the important objective of determining how much environmental mercury contamination man can tolerate without adverse effects on his health.

The editors of the volume named above are to be congratulated on achieving the formidable task of collecting most of these valuable data into one publication. Consideration is given not only to the metabolism of mercury compounds and the toxicological significance of the levels of mercury found in the environment, but also to such important subjects as the analytical methods available for determining mercury in biological materials. The particular problems presented by the analysis of organic mercury compounds are underlined in the latter part. The mutagenic activity of mercury and the clinical signs of inorganic and organic mercury poisoning are also dealt with at some length.

The individual contributors have obviously taken pains to ensure that the coverage of the particular aspect for which they were responsible was accurate and comprehensive. A wealth of data is provided and adequately identified by appropriate references. Moreover, a very detailed Table of Contents goes a fair way towards compensating for the absence of a subject index. There are, however, some important gaps. The pathology of the central nervous system and the kidney in mercury poisoning receives only brief mention. Since clinical manifestations of disease are dependent on the "critical" organ (to borrow a phrase frequently used in the book) one might have expected to see adequate reference to pathological studies

along with the descriptions of clinical signs. Biochemical changes also receive relatively minor consideration. These are two rather serious omissions since a considerable amount of information is available on both topics.

The book also fails to bring into relief the need for teratological studies. Transplacental migration, especially of methyl- and ethylmercury, is mentioned in some detail and a fair amount of space is devoted to mutagenic changes. Clearly there is a gap here which needs urgently to be filled.

Residue Reviews. Residues of Pesticides and Other Foreign Chemicals in Foods and Feeds. Vol. 40. Edited by F. A. Gunther. Springer-Verlag, Berlin, 1971. pp. ix + 231. DM 52.

Volume 40 of this established series deals first with new analytical techniques available for identifying pesticides and their degradation products in animals and soils and secondly with the use of granular pesticides and the subsequent effects of various formulations on soil and plant residues. Also included in this volume is a short chapter dealing with pesticide regulations in Mexico. And for good measure, a comprehensive subject index covering volumes 31–40 is appended.

In the first chapter, F. J. Biros considers the use of mass spectrometry and combined gas chromatographic–mass spectrometric techniques for identifying some pesticides and their metabolites in animals and their photodecomposition products in soils and plants. Data on the electron impact fragmentation of several organophosphorus, organochlorine, carbamate and other pesticides are reviewed and the importance of coupling techniques using thin-layer or gas chromatography for the isolation of individual residues or metabolites is considered. Various collection and trapping devices are mentioned in relation to the separation and identification of DDT, DDE and dieldrin mixtures. Obvious contenders for these combined analyses are mixtures containing several polychlorinated biphenyls, and one experiment is described. It is concluded that combined gas chromatography–mass spectrometry affords a useful approach to the analysis of pesticide residues, because components of a mixture can be positively identified without prior separation at sensitivities compatible with those encountered in the environment.

The use of granular pesticides is reviewed in relation to the resulting residues in plants and soils. Granules are usually composed of an inert material impregnated or fused with the active chemical toxicant, with the result that slow release or concentration of the pesticide at certain sites on the plant can be controlled. Such factors as the type and size of granules, the amount of dust in the formulation, uneven application, drift, the type of application target and soil temperature may all affect residues in crops and soils, and specific examples of these are given for various pesticides. For example, DDT residues in the butter-fat of cows grazed on pastures treated with DDT pellets were five times less than those grazed on pastures treated with DDT superphosphate fertilizer. On the other hand, the application of granules at ground level to avoid the contamination of leaves and fruits associated with aerial spraying can result in a much more prolonged persistence of residues in the trees or plants. However, each case has to be studied separately and residues of long or short duration can be accomplished. The author, who has provided an impressive 18 pages of references, considers that much more attention should be paid to the significant differences between aerial sprays and granules when residues of pesticides on crops and vegetables are considered.

Residue Reviews. Residues of Pesticides and Other Contaminants in the Total Environment. Vol. 41. Edited by F. A. Gunther. Springer-Verlag, Berlin, 1972. pp. vii + 187. DM 49.

Glancing down the contents page of this, the forty-first volume of *Residue Reviews*, the eye immediately alights on two chapters whose subjects, aflatoxin and lindane (γ-BHC), are hardy annuals as far as readers of this journal are concerned.

Over the past few years, many of the contributions to the ever-growing literature on aflatoxin have been concerned with the contamination of particular food products and animal feeds. Although some doubt has been cast on the usefulness of such data (*Cited in F.C.T.* 1971, **9**, 575), this present volume provides a useful source of reference to levels found in oil seeds, nuts, grains and other crops as well as in processed foods. Reference is also made to the reduction of contamination by crop rotation, post-harvest drying and the control of specific insects. Further sections of this chapter describe the effect of feeding various farm livestock and fish on diets containing aflatoxin and consider the residues of the toxin that may be expected in the edible products and tissues of such animals. The last 5 years or so have seen much revision and refinement of methods for the analysis of aflatoxin and numerous procedures have been published, particularly for peanuts and cottonseed products. Also reviewed are methods that have been developed to deal with milk in various forms and with mixed feeds, which contain a variety of plant and animal products and are used in compounding many specialized diets.

For many years lindane has been used throughout the world to protect stored products from insect pests. Concern has been expressed, however, about frequent reports of associations between lindane exposure and human blood disorders and several countries have recently either banned or restricted the use of lindane. The review now published looks at the background to these developments, considering in turn the history of the use and effectiveness of lindane, techniques of residue determination and their adequacy and accuracy, lindane contamination of air, water and food, and its toxicity to man, other mammals, fish and birds. Much information is presented (and backed up by 21 pages of references) and it is the author's opinion that although there does not appear to be any proof of a causal relationship between lindane and blood disorders in man, enough evidence has been generated to raise suspicions and justify more study.

In the United States, Community Pesticide Studies are being carried out in 13 different states, with the aim of determining long- and short-term effects of pesticides on human health. Details are presented of the Hawaii Project, particularly as it relates to potential hazards from the use of pesticides and what is being done to ensure their safety-in-use.

The remainder of this volume is taken up with a consideration of bromophos and bromophos-ethyl residues, including methods of analysis and the decomposition of residues in plant and animal products, and a chapter on the mechanism of ethylene dibromide action in laying hens.

The high standard of presentation that we have come to expect from *Residue Reviews* is maintained in this volume, which must provide a valuable source of reference for all the topics covered.

Microbial Toxins. A Comprehensive Treatise. Vol. VI. Fungal Toxins. Edited by A. Ciegler, S. Kadis and S. J. Ajl. Academic Press, New York, 1971. pp. xviii + 563. £13.55.

Microbial Toxins. A Comprehensive Treatise. Vol. VII. Algal and Fungal Toxins. Edited by S. Kadis, A. Ciegler and S. J. Ajl. Academic Press, New York, 1971. pp. xviii + 401. £10.25.

These two excellent volumes deal with the fascinating subject of fungal and algal toxins, many of which are potential contaminants of staple foods.

The first topic to be considered is the well-known group made up of aflatoxin and related compounds produced by various strains of Aspergillus. The carcinogenic effect of aflatoxin in various animals is now well established, but evidence of a toxic response in man is at best equivocal. The authors suggest that the complexity of the problems raised by mycotoxins warrants a "huge multidisciplinary approach to solving a potentially serious and neglected problem in our society".

Among the fifteen or more kinds of fungi causing mouldy or yellowed rice, Penicillia are the commonest. *Penicillium toxicarium* Miyake is highly toxic and, in animals, causes ascending paralysis and circulatory disturbances resembling beri-beri in man. Two hepatotoxic metabolites, luteoskyrine and cyclochlorotene, have been isolated from rice infected with *P. islandicum*. Both cause serious liver damage, varying from acute atrophy to hepatomas, in rats. Again we have no definite knowledge about toxicity in man, but the similarity between Shoshin-kakke, the acute cardiac beri-beri which causes death in a few days and which occurs among the low-income groups in Japan, and the acute reactions in rats and dogs fed on extracts of rice affected with *P. citreo-viride* Biourge suggests that Shoshin-kakke may be due primarily to eating yellowed rice rather than to vitamin B_1 deficiency.

An oestrogenic mycotoxin from various species of Fusarium has produced outbreaks of vulvovaginitis in swine fed on infected corn. Zearalenone, the major Fusarium metabolite of this type, is a true oestrogen and claims have been made for its growth-promoting activity. A more serious result of the ingestion of grains infected with Fusarium is alimentary toxic aleukia or septic angina. Severe outbreaks of this disease, with a high level of mortality, have occurred from time to time in Russia, particularly among population groups in agricultural areas.

Toxic algae have been responsible for deaths of fish, livestock, waterfowl and man. Those identified as responsible are mostly fresh-water species and all are members of the Pyrrhophyta, Chrysophyta and Cyanophyta. In man, the toxic effects of blue-green algae include gastro-enteritis, asthma, sore throat and papulo-vesicular dermatitis. Since one of the direct results of the indiscriminate use of rivers and other surface waters for waste disposal is the overgrowth of these potential toxin-producers, it could be argued that such relatively minor discomforts are a light punishment. However, the possibility that some strains may have the capacity to cause acute hepatitis and jaundice in man, as they do in some animals, would seem to indicate that the punishment might, in fact, fit the crime.

These books are excellently written and contain a wealth of interesting information. The various contributions maintain a high standard throughout.

Biological Oxidation of Nitrogen in Organic Molecules. Proceedings of the Symposium held at Chelsea College, London, 19–22 December 1971. Edited by J. W. Bridges, J. W. Gorrod and D. V. Parke. Taylor & Francis Ltd., London, 1972. pp. xxi + 269. £6.

This book presents the proceedings of a symposium sponsored by The Royal Society European Science Exchange Programme, supported by the pharmaceutical industry and held at Chelsea College, London, in December 1971. The programme was devoted entirely to the topic of nitrogen oxidation and consisted of forty communications, including some review-type lectures. The main aspects covered were the phenomena, mechanism and biological consequences of nitrogen oxidation, and inevitably there was a considerable overlap of interest and some repetition of information.

An introduction by R. T. Williams delineated the classes of compound that undergo nitrogen oxidation to hydroxylamines, *N*-oxides and oximes and drew attention to the growing awareness of the important biological effects resulting from such biotransformations. The review papers, by acknowledged experts in the field, were of outstanding quality. The metabolic transformation of tertiary amines to *N*-oxides and the interrelated subsequent steps of reduction and dealkylation were lucidly described by M. H. Bickel. H. Uehleke dealt extensively with the *N*-hydroxylation and *N*-oxidation of primary, secondary and tertiary amines, while the biological oxidation of aromatic heterocyclic compounds was reviewed by J. W. Gorrod. Difficulties in the interpretation of metabolic studies of nitrogen compounds were stressed by A. H. Beckett, who pointed out the importance of distinguishing metabolites from 'metabonates', formed from metabolites by non-enzymic breakdown.

An excellent review on the naturally occurring alkaloid *N*-oxides was presented by J. D. Phillipson, who dealt particularly with the toxicity and metabolism of the pyrrolizidine group of alkaloids. This topic was discussed further in three papers by A. R. Mattocks and a colleague. The role of microbial enzymes in mediating the oxidative cleavage of alkyl-nitrogen bonds was described by P. J. Large, and R. Walker and A. J. Ryan mentioned the influence of substituent groups on the azoreductase activity of intestinal microflora. Other reviews dealt with the role of the cytochromes and electron-transport systems in the oxidation of nitrogen compounds. The importance of *N*-hydroxylation in the carcinogenesis of aromatic amines was considered by D. B. Clayson and his group, and some aspects of the mechanism of urethane carcinogenesis were reviewed by K. Williams and R. Nery.

The 'organic nitrogen compound' umbrella covers many drugs and antibiotics as well as other important environmental chemicals, and this publication will undoubtedly be extremely valuable to toxicologists and biochemists concerned with the metabolism of this important chemical group.

Aging in Cell and Tissue Culture. Edited by Emma Holečková and V. J. Cristofalo. Plenum Press, New York, 1970. pp. xx + 163. £5.85.

Advances in medical sciences have resulted in the prolongation of man's lifespan to such an extent that the phenomenon of ageing is, by necessity, becoming a major field of research.

One possible line of investigation is to take the fundamental unit of living organisms, the cell, and investigate ageing by *in vitro* techniques. Several such exercises were reported and discussed at the annual meeting of the European Tissue Culture Society held in Czechoslovakia in May 1969.

Two generally accepted observations point to the relevance of *in vitro* investigations on senescence. Firstly, cell cultures derived from adult tissues have a significantly shorter lifespan than those derived from foetal tissues. Secondly, tissues from adult animals take longer to commence growth *in vitro* than do the same tissues from younger animals. However, within this established framework, areas of controversy abound. For example, is observed senescence *in vitro* dependent on an intrinsic programmed function of the cell or is it due to nutritional deficiencies during cultivation? Another unresolved question is whether the decline in the proliferative capacity of cultures depends on the number of cell generations rather than on the time the cells have been cultured. Other observations, such as the fact that certain rodent cells can proliferate for indefinite periods without variation in karyotype whereas human and avian cells eventually change in chromosome number, reflect the complexity of the system.

The major emphasis in this symposium was on studies of the proliferative capacity of cells after ageing *in vivo* and *in vitro*. In the opening presentation, R. J. Hay (Washington) reviewed the available evidence on these problems and examined the question of species specificity in the limited *in vitro* life of normal cells. J.W.I.M. Simons (Leiden) showed that cell volume and its variability was directly correlated with ageing, while M. Soukupova and her colleagues (Prague) demonstrated a similar heterogeneity in cell structure and activity in primary explant fragments. A. Macieira-Coelho (Villejuif) presented evidence that during their first phase in culture, the growth pattern of adult human fibroblasts resembles that of embryonic fibroblasts that have reached the declining stage of their *in vitro* lifespan.

The symposium also included several papers on changes in various biochemical parameters associated with cell ageing. These included age-related increases in cellular lipids and RNA, a decrease in RNA stability during ageing and the varying activity of certain enzymes. The final papers dealt with the relationship between the growth promoting α-globulin of serum, and the synthesis of pyrimidines during culture.

The discussions prompted by these papers have not been published, and with topics so complex and controversial to contend with, readers will regret this omission as much as did the editors. The standard of presentation has been sacrificed in the interests of speed and economy; certainly there is much to be said for the speedy publication of symposium proceedings, but the result in this case is a disturbing variation in type-face, spacing, table sizes and figure clarity. Nevertheless, the book is adequately indexed and referenced, and in an area of research where definition of problems is still a major factor, it makes a good attempt at the task and points the way to more meaningful *in vitro* investigations of the phenomenon of ageing.

Advances in Cancer Research. Vol. 14. Edited by G. Klein and S. Weinhouse. Academic Press, New York. 1971. pp. xiv + 417. £10.50.

Immunological aspects of cancer dominate the contributions to this volume of a well-known series, but the emphasis is on the diagnosis and treatment of cancer by means of immunological procedures rather than on basic concepts concerning changes in the antigenic pattern of somatic cells when they become cancerous and the host's reactions to these transformed cells.

The first chapter provides an outline of the experimental and clinical procedures available for the study of the immunotherapy of cancer. Specific antigens derived from tumour cells

have been obtained in animals and have been used with a moderate degree of success in the treatment of other animals with the same type of tumour. In man, this has not been completely achieved and most of the efforts concerned with the immunotherapy of cancer have revolved around non-specific methods of treatment which enhance the ability of the host to react to a wide variety of antigenic materials.

Apart from attempts to induce the host to produce antibodies against cancer cells (active immunotherapy), investigations have been conducted on the possibility of transferring antibodies from one affected organism to another. So far, the results of studies in both man and animals are not encouraging, and this approach remains of little more than theoretical interest at the moment.

An important element in immunological responses to foreign antigens is the substance called complement. This is now known to consist of several components which assist the antibodies to adhere to the antigens exposed on the cell surface and thus participate in the destruction of the cell. Complement is now known to be of importance in the host's response to tumour cells and a number of claims have been made regarding the diagnostic and therapeutic applications of techniques involving the utilization of complement.

A major contribution on the application of immunological procedures not involving complement to the diagnosis of cancer is the chapter dealing with α-foetoprotein and its association with malignant tumours. α-Foetoprotein is present in the liver cells during foetal life and the neonatal period. It can be detected in the serum by means of precipitation and agglutination techniques as well as biochemically by a radio-immunoassay procedure. The level of this protein gradually decreases during the period from growth to maturity but it has been shown to reappear in the adult in conditions involving cell proliferation. The amounts produced in non-cancerous conditions are low, whereas substantial amounts are found in spontaneous liver cancer both in man and in experimental animals. In the latter, high levels of α-foetoprotein are also found when tumours are induced chemically. Tumours in other tissues, notably in the gastro-intestinal tract, also produce antigens that are normally identifiable only in the foetal and neonatal periods and that have potential value in the diagnosis of malignant disease. Together with hepatic α-foetoprotein, these antigens form a group known generically as the carcino-embryonic antigens.

Two chapters in the book are devoted to viral oncogenesis. One of these deals with the hybridization techniques available for the detection of viral DNA in the genome of the transformed cell. The detection of viruses in transformed cells is only rarely successful by other techniques relying on the production of infectious virus by the transformed cell. The other chapter on viral oncogenesis reviews experimental results demonstrating the changes that occur in the nuclear and plasma membranes during oncogenic transformation.

An important chapter in this book comes right at the end. The author, who has been interested in radiation carcinogenesis in man for a number of years, reviews the evidence for cancer induction by low doses of radiation. Drawing her clinical material from individuals irradiated *in utero* during radiological investigation of some pelvic abnormality in the mother and from the Japanese atom-bomb victims, the author draws the conclusion that, contrary to the widely held view, exposure to low doses of radiation increases the risk not only of leukaemia but also of other forms of cancer.

Progress in Experimental Tumor Research. Vol. 14. Inhibition of Carcinogenesis. Edited by B. L. van Duuren and B. A. Rubin. S. Karger, Basel, 1971. pp. x + 241. DM 72.

This volume concentrates on the present status of research on 'anticarcinogenesis', considering those aspects of carcinogenesis that are amenable to inhibition or control.

In the first contribution, R. T. Prehn examines the evidence for immunosurveillance in neoplasia in higher animals, and concludes that it is probably inefficient, slow in response and unreliable and hence that it might be susceptible to improvement by chemical or immunological intervention. He then speculates that neoplasia in higher vertebrates is analogous to limb regeneration in amphibians, where a process of dedifferentiation precedes controlled differentiation. Higher vertebrates have lost the ability to control dedifferentiated or undifferentiated (tumour) cells.

The possibility of monitoring the presence and progress of tumour development is suggested and amplified by D. L. Morton and his colleagues and by P. Gold. The former group demonstrates a close correlation between the level of circulating antitumour antibodies to the tumour antigens of malignant melanoma, and the localization, diffusion or resection of the diseased tissue. A low level of antibodies accompanies a large or recurring tumour, and a high level is found following resection or regression. Similarly, Gold describes the so-called carcino-embryonic antigens (CEA), which are present in the normal human embryonic digestive system and in the malignantly transformed human gut, but are not found in children or healthy adults. A radioimmunoassay for circulating CEA, capable of detecting these antigens in nanogram quantities, was positive in 33 out of 34 patients with cancer of the colon or rectum. Successful removal of localized bowel tumours resulted in the disappearance of CEA from the serum, while it remained high in patients in whom tumours had metastasized.

A long contribution by T. L. Dao assembles the current knowledge on the initiation, promotion and inhibition of mammary tumours induced by carcinogens. Briefly, any agency which causes mammary hyperplasia (pregnancy, lactation or the injection of oestrogens) is likely to be highly effective in preventing future induction of tumours by carcinogen administration. Oestrogen may, however, act as an initiator and a promoter, and mammary tumours seem unable to commence in its absence.

Induction of microsomal-enzyme activity results in a decreased carcinogenic response to a variety of carcinogens. L. W. Wattenberg explores this potential means of obtaining increased protection against a number of carcinogenic substances, some of which are environmental. In particular, his studies on polycyclic hydrocarbon hydroxylases occurring in rat lung and intestine (the two major portals of entry of foreign substances) suggest that most or all of the activity of these enzymes is due to incidental exposure to exogenous inducers. On balance it seems that microsomal enzyme induction is beneficial, protecting against rather than enhancing the tumorigenic action of most carcinogens.

The importance of prevention rather than cure of cancers is stressed by H. L. Falk at the beginning of a long but very readable review chapter, in which he considers all the potential anticarcinogenic agencies that might offer promise for human protection. These include nutritional factors (dietary protein, vitamins and trace metals), competitive inhibition (perhaps less appropriate for man) and enzyme induction and inhibition.

In contrast, the final contribution to this collection is more of a laboratory cook-book of carcinogenicity testing than a positive summary of progress and prospects. Full of complicated detail, this lengthy chapter by B. A. Rubin on the value of the homograft tolerance

assay in determining carcinogenicity serves to obscure an already esoteric branch of immunology. A rich crop of abbreviations abounds, so that either an outstanding memory or a home-made glossary of terms is essential if the reader is to keep track of the author's meaning. However the summary, which terminates the chapter, is a model of insight and clarity, bringing out far more clearly than the preceding pages the innovations that the author has made. In his hands, the mouse homograft tolerance test has proved to be a rapid, sensitive and accurate method for detecting potential carcinogens and anticarcinogens among polycyclic hydrocarbons. When this was combined with the results of studies on the induction and enhancement of leukaemias in DBA/2 mice, a high degree of certainty in detecting polycyclic carcinogens was achieved. Great reliance must clearly have been placed on accurate technique and careful husbandry and record-keeping by the staff responsible for the animals. The "cook-book" approach used in this chapter should enable other laboratories to attempt to duplicate and extend the results reported.

The complexities of this book are ably drawn together and summarized in a preface by the Series Editor. The indexes provided are cumulative ones, covering all the volumes that have so far appeared in this series.

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Information Section

ARTICLES OF GENERAL INTEREST

REFLECTIONS FOR SMOKING MOTHERS

A wide range of maladies can be linked directly or indirectly with tobacco smoking, the most impressive being bronchial carcinoma and possibly other associated tumours (*Cited in F.C.T.* 1970, **8,** 570). More recently the emphasis has shifted to the effects that smoking during pregnancy may have upon the welfare of the offspring. The *British Medical Journal* (1968, **4,** 339) has reminded its readers that human studies have shown quite conclusively that the average birth weight of infants of smoking mothers is significantly lower than that of infants of non-smokers, and that the extent to which the infant's growth rate is depressed depends upon the amount the mother smokes.

In a prospective report from Scandinavia, Kullander & Källén (*Acta obstet. gynec. scand.* 1971, **50,** 83) reviewed 6363 pregnancies in which the mothers' smoking habits were recorded. Mean birth weight was reduced by an average of 170 g and the prematurity rate was increased by 50% among infants of smokers, compared with non-smokers. Body length and head and shoulder circumference were also less in infants of smokers. Smoking was associated with a reduction in placental weight and an increase in the ratio of placental weight to body weight at birth, with an increase in the infant mortality rate during the first year to 1·6 times the control rate, and with more frequent abruptio placentae among infants dying within the first week of life. The increased risk of spontaneous abortion detected among smoking women was associated with unwanted pregnancy, however, and the evidence that tobacco smoking itself might have a slight abortifacient effect was not conclusive. On the credit side of the picture, Kullander & Källén (*loc. cit.*) found a reduction in the incidence of pre-eclampsia and morning sickness in the smoking women.

Butler *et al.* (*Br. med. J.* 1972, **2,** 127) have reported that, among a British population, smoking during pregnancy went hand in hand with a reduction in birth weight of 170 g and an increase of 28% in the combined foetal and neonatal mortality rate. They calculated that if all the women in the UK who smoke after month 4 of pregnancy ceased to do so, some 1500 babies might be saved each year in England, Scotland and Wales. On the other hand, working with a population of nearly 10,000 white and more than 3000 Negro women in America, Yerushalmy (*Am. J. Epidemiol.* 1971, **93,** 443) failed to demonstrate any increase in neonatal mortality among smokers, although he confirmed the decrease in birth weight. On the contrary, he found a lower risk of neonatal mortality and congenital abnormalities among infants of low birth weight among smokers than among non-smokers. Yerushalmy (*loc. cit.*) unearthed some strange correlations. Thus, the vulnerability of infants of low birth weight was highest where the wife did not smoke and the husband did. The mode of life of non-smokers, which tended to be less strenuous and stressful than that of the smokers, also influenced the findings. Moreover, for some unexplained reason,

smoking mothers showed a lower average age of menarche than the non-smokers. Thus there must (according to Yerushalmy) be doubts as to whether cigarette smoking necessarily introduces an exogenous factor which interferes with intro-uterine development.

Some support for this contention was offered by Hickey *et al.* (*Lancet* 1973, **i,** 270), who suggested the possible existence of a sub-group of women constitutionally more likely to smoke, to have small babies and to be particularly vulnerable to the effects of some environmental mutagenic hazards and thus more liable to spontaneous abortions. The question of biased case selection, together with faulty data analysis, was also raised by Meyer & Comstock (*Am. J. Epidemiol.* 1972, **96,** 1), in discussing inconsistencies in reports of connexions between mortality rates and maternal smoking.

Nevertheless, there is considerable evidence that maternal smoking not only reduces birth weight but also increases foetal and neonatal mortality. The mechanism behind these effects is, however, not altogether clear. Little difference between smokers and non-smokers has been noted in maternal body weight half way through pregnancy, or in maternal weight gain during the latter half of pregnancy, so that it is not reasonable to attribute a reduction in birth weight to an impairment in maternal nutrition. This conclusion is supported by animal experiments. For example, Haworth & Ford (*Am. J. Obstet. Gynec.* 1972, **112,** 653) performed parallel studies in rats exposed to cigarette smoke and in others deprived of food to an extent equivalent to the loss of appetite in the first group. Offspring of dams exposed to smoke had significantly reduced body and organ weights compared with those in a control group, whereas those of the food-restricted group did not.

It is more likely that foetal oxygen supplies, and therefore foetal nutrition, are impaired either by restriction of placental blood flow as a result of the familiar pharmacological effect of nicotine or by some other factor. Meyer & Comstock (*loc. cit.*) point to the hypoxia resulting from an increase of carboxyhaemoglobin (CHb) in the blood of both mother and foetus as a possible cause. There may be a parallel here between the observed reduction in birth weight and increase in perinatal mortality seen in infants of smoking mothers and similar differences observed in births at high altitude. An increase in the ratio of placental weight to foetal weight is another finding common to both classes of births. Such an explanation is supported by the findings of Astrup *et al.* (*Lancet* 1972, **ii,** 1220) who have reported that in rabbits exposed to 180 ppm carbon monoxide (producing a level of 16–18% CHb) during pregnancy the birth weight of offspring fell by 20%, and neonatal mortality rose to 35% compared with 1% in unexposed controls. Furthermore, Astrup *et al.* (*loc. cit.*) found that birth weights of the infants of 176 smoking and 177 non-smoking women were on average 2990 and 3225 g respectively and that a negative correlation appeared between birth weight and the mean CHb concentration in the mother's blood.

Reports on levels of CHb in the human foetus are conflicting. Cole *et al.* (*J. Obstet. Gynaec. Br. Commonw.* 1972, **79,** 782) reported that the level of CHb was three times higher in the blood of smokers than of non-smokers and was about twice as high in the foetal circulation as in that of the mother, but earlier work (Young & Pugh, *ibid* 1963, **70,** 681; Bjure & Fällström, *Acta paediat., Stockh.* 1963, **52,** 361) failed to detect any significant differences between maternal and foetal concentrations.

The complexities of the problem have been demonstrated further by Younoszai *et al.* (*Am. J. Obstet. Gynec.* 1969, **104,** 1207), who exposed rats to smoke from various types of cigarette for periods of 4 minutes five times daily between days 3 and 22 of pregnancy. Blood CHb saturation was taken as the index of inhalation. Compared with controls, foetuses from all the 'smoked' rats were growth retarded. The most marked retardation appeared

after exposure to tobacco smoke (15 mg nicotine/cigarette), and milder effects, which were in proportion to the decrease in maternal food consumption, were seen with lettuce-leaf cigarettes, with or without added nicotine. Food consumption was reduced in all the 'smoked' rats, but in the rats exposed to tobacco smoke, foetal weight was reduced more than would have been expected solely on the basis of the decrease in maternal food intake. Since rats in the groups exposed to similar amounts of carbon monoxide and nicotine in the lettuce-leaf smoke showed less reduction in birth weight, the marked effect of tobacco smoke on foetal development may be due to some other unidentified factor in tobacco.

Studies in sheep have suggested that, at least in the short term, foetuses are protected to a considerable degree both from the carbon monoxide and the nicotine present in the maternal circulation as a result of exposure to tobacco smoke. Kirschbaum *et al.* (*Obstet. Gynec., N. Y.* 1970, **35,** 527) studied gaseous exchange in blood flow in the placenta and foetus of pregnant ewes implanted with an endotracheal tube to facilitate inhalation of smoke from cigarettes. For comparison, iv injections of nicotine (2·5 μg/kg) were also given. When the ewes were injected, they showed an initial reduction and subsequently a more prolonged increase in blood pressure, as was to be expected from purely pharmacological considerations. Uterine blood-flow rates paralleled the variations in maternal blood-pressure, but no changes in foetal circulation were recorded. When injected directly into the foetal circulation, nicotine provoked similar but far less marked changes in blood pressure. Smoking significantly increased the CHb concentration of the maternal blood but had no effect on the foetal Hb complex.

Interest has also centred on the possible involvement of cyanide in the production of small babies. A report from McGarry & Andrews (*Br. med. J.* 1972, **2,** 74) indicates that in pregnancy the serum level of vitamin B_{12} is lower in women who smoke than in those who do not. Anaemic women and those who have smaller-than-average babies tend also to show lower serum vitamin B_{12} levels. Studies of tobacco amblyopia have shown that cyanide in tobacco smoke may disturb vitamin B_{12} metabolism and lead to inability to detoxicate cyanide, thus setting up a vicious circle (*Cited in F.C.T.* 1970, **8,** 574). The detoxication of cyanide in the body relies upon three main mechanisms, the combination of cyanide with body reserves of hydroxycobalamin, combination with cystine and other essential amino acids, and metabolism to thiocyanate. Thus it seems reasonable to suppose that the anaemia of pregnancy associated with tobacco smoking may stem from the cyanide content of the smoke.

While a high blood concentration of CHb has been reported to increase the incidence of malformations in rabbit foetuses, it has been concluded that the levels of CHb in the blood of women smokers would not normally be high enough to lead to serious developmental defects in human infants (Astrup, *Br. med. J.* 1972, **4,** 447). However, the possibility of an association between congenital heart disease and the smoking habits of mothers has been investigated by Fedrick *et al.* (*Nature, Lond.* 1971, **231,** 529), who report a 50% increase in this condition (from 4·7 to 7·3/1000 live births) among infants of smokers compared with those of non-smokers. This difference was statistically independent of maternal age, parity and social class and appears to warrant further examination.

Neutal & Buck (*J. natn. Cancer Inst.* 1971, **47,** 59) made a perinatal mortality survey involving 89,302 babies who survived at least 7 days and whose mothers' smoking habits were recorded carefully. In this group there were 65 deaths from cancers and 32 survivors of cancers between birth and the age of 7–10 years. The data obtained indicated that the risk of cancers of all sites among children of smoking and of non-smoking mothers was in

a ratio of 1·3:1, but no dose–response relationship could be established. These authors assert that while their findings do not support the contention that exposure to tobacco smoke *in utero* may have a generally carcinogenic effect on the foetus, a carcinogenic response confined to one specific tissue or effective over a narrow range of infant age cannot be discounted altogether.

The question has also been raised from time to time as to whether the offspring of women who smoke may show some evidence of intellectual impairment. Reports have been conflicting, but in a recent study in the USA, Hardy & Mellits (*Lancet* 1972, **ii**, 1332) found no evidence of physical or mental handicap at 7 years of age in the children of smoking mothers. In their study of body and organ weights in the foetuses of rats exposed to cigarette smoke, Haworth & Ford (*loc. cit.*) found that cell numbers were reduced in the foetal hindbrain, as they were in the carcass. In contrast, the forebrain showed no deficiency in numbers of cells although it weighed some 12% less than in control animals.

While the details of the foetal responses to maternal smoking are still far from clear, the balance of available evidence has prompted the *British Medical Journal* in a recent editorial (*ibid* 1973 **1**, 369) to recommend an increase in efforts to persuade women to give up smoking as soon as they become pregnant.

HOPE FOR NTA?

There have been several ups and downs in the career of nitrilotriacetic acid (NTA), which was proposed as a replacement for polyphosphates when these were implicated in the eutrophication of lakes and rivers. The phasing out of phosphate builders in detergents was planned for 1972 in America, but this was prevented by reports that NTA caused nephrosis in rats after massive doses and was associated with an increase in embryotoxicity when administered concurrently with cadmium (Cd) or mercury (Hg) (*Cited in F.C.T.* 1971, **9**, 889). The soap industry in America agreed in turn to phase out the use of NTA, in view of this evidence of its embryotoxicity in conjunction with heavy metals and its ability to form non-degradable heavy-metal complexes, although studies on NTA itself had produced no evidence of teratogenicity or embryotoxicity in rats and rabbits (Nolen *et al. Fd Cosmet. Toxicol.* 1971, **9**, 509).

Since then several new papers have been published to supplement the data on NTA. We referred recently (*Cited in F.C.T.* 1973, **11**, 521) to a 2-year feeding study in rats, in which a dietary no-effect level of 0·03% was established for NTA, and mention was also made of a report that 200 mg NTA/kg appeared to have a markedly protective effect when administered with a dose of 64 mg $CdCl_2$/kg.

Extensive studies of the fate of NTA in rats, rabbits, dogs and monkeys (*ibid* 1971, **9**, 888) have been supplemented by a paper on the absorption, distribution, biotransformation and excretion of NTA in dogs by Budny (*Toxic. appl. Pharmac.* 1972, **22**, 655). Beagle dogs were given NTA, labelled with ^{14}C in the carboxyl group, in an oral dose of 20 mg/kg. Blood and urine were collected by cannula and catheter during the first 4 hours, and then urine, faeces and blood were collected at 24, 48 and 72 hours after dosing. The animals were killed and autopsied at 72 hours. The NTA was rapidly absorbed, the peak level in the blood serum occurring about 75 minutes after treatment. Most of the compound absorbed in 72 hours was found in the urine, with traces in the gastro-intestinal tract, liver and kidneys.

Some 96% of an iv dose was found in the urine and only a trace in the faeces, suggesting a virtual absence of enterohepatic circulation. Tissue analysis showed slight deposition of NTA in bone (2–3 μg NTA/g bone). It has previously been indicated that the deposition of up to 8 μg NTA/g bone would have no demonstrable effect on bone development (*Cited in F.C.T.* 1971, **9,** 888). This low tissue deposition is a function of the rapid and extensive clearance that occurs with NTA. Analysis of the metabolites showed that no biotransformation occurred in the dog, a finding consistent with earlier findings in the rat (Michael & Wakim, *Toxic. appl. Pharmac.* 1971, **18,** 407).

Budny (*loc. cit.*) also tested the cardiovascular effects of NTA by measuring blood-pressure changes after iv administration. Rapid injection of 40 mg NTA/kg in dogs anaesthetized with pentobarbitone was followed firstly by a sharp drop in blood pressure and then by a rapid return to normal. A lower dose (30 mg/kg) infused over a longer period (12 minutes) did not invoke the same response, probably because there was sufficient time for the release of bound calcium into the serum pool to compensate for the rapid drop in serum calcium ions that was thought to be the cause of the blood-pressure fall following rapid NTA injection.

An autoradiographic study by Tjälve (*ibid* 1972, **23,** 216) also showed rapid absorption of NTA in pregnant and non-pregnant mice treated orally or iv with [^{14}C]NTA (5 μC/mouse) and then killed for autoradiography 5 or 30 minutes or 1, 2, 4, 8, 24 or 48 hours after dosing. However, this author reported a high skeletal uptake of NTA, greater in the epiphyses than the diaphyses. The radioactivity appeared rapidly in the bone and was still present after 48 hours. In the mice treated with [^{14}C]NTA on day 18 of pregnancy, a high uptake of radioactive material was demonstrated in the foetal skeleton and there was a marked accumulation in the maternal uterine wall. Nevertheless, a small teratology study, in which ten mice were given 0·2% NTA in the drinking-water on days 6–18 of pregnancy, showed that NTA at this level had no significant effect upon foetal resorptions and did not cause foetal abnormalities. Tjälve (*loc. cit.*) noted that other chelating agents also had a high affinity for bone.

Further to the problem of the effect of a chelating agent on the toxicity and teratogenicity of heavy metals, Nolen *et al.* (*ibid* 1972, **23,** 222) studied the effects of NTA with $CdCl_2$ and methylHg in rats. Chernoff & Courtney (Progress Report, National Institute of Environmental Health Sciences, 1 December 1970) had reported that NTA heightened the embryotoxic effect of Cd and Hg, but Nolen *et al.* (*loc. cit.*) felt that the data from these studies were somewhat ambiguous, as different solvents were used for the metal compounds alone and for the NTA/metal complexes, making it difficult to determine whether the changes in absorption and thus in the toxicity of the metal were due to NTA or to the solvent. Also, the Cd salt was given sc, which had little relevance to oral ingestion of the metal. They therefore calculated the maximum probable intakes of NTA, Cd and Hg by a pregnant woman under adverse conditions and used these as the basis for a set of realistic doses given to rats in the drinking-water in a teratogenicity study. The top doses of Cd and Hg given were the possible minimum effect levels. The dose levels used were 0·1 and 20 mg NTA/kg, with 0, 0·01, 1 and 4 mg Cd/kg as $CdCl_2$ and 0, 0·02, 0·2 and 4 mg Hg/kg as methylHg chloride. NTA was given at a level of 0, 0·1 and 20 mg/kg at each level of the metal tested. The 24 groups of 20 Charles River rats used were treated from days 6 to 14 of gestation, and a normal teratology study was carried out with, in addition, analyses for Cd and Hg in selected tissues from five animals in each group.

The highest dose levels of the metals produced some toxic effects in the dams, and

correspondingly some increase in foetal damage. However, these effects were not increased by the administration of NTA. In fact, NTA appeared to exert a slight protective effect on the maternal toxicity of Cd. Tissue analyses showed that very little Cd crossed the placenta and, in the dams, the liver content of Cd was increased significantly only at the highest dose level. Following methylHg treatment, there was an increase in Hg levels in the foetus as well as in the maternal liver, kidneys and brain, but the quantities found were again unaltered by the presence of NTA.

The same group followed this paper with a second study (Nolen *et al. ibid* 1972, **23,** 238), in which they compared the effects of Cd or Hg administered in conjunction with NTA, with trisodium citrate, another chelating agent, or with a mixture of NTA and ferric chloride. It had been found that NTA would bind preferentially to ferric ions (Fe^{3+}) rather than to Cd or Hg, and because of the ubiquitous occurrence of Fe^{3+} it was felt that the effect of its NTA complex on Cd and Hg toxicity might be important. Groups of rats were given drinking-water containing $CdCl_2$ or methylHg chloride, to provide a dose of 4 mg Cd or Hg/kg/day, either alone or together with trisodium citrate (20 mg/kg/day) or NTA (20 mg/kg/day) with or without $FeCl_3$ (7 mg/kg/day) on days 6–15 of gestation.

This dose of Cd or methylHg when given alone was again toxic to the dams, and produced some foetal abnormality. Again NTA appeared to exert some protective effect when given with Cd, although it did not affect Hg toxicity. While evidence for a protective effect was very limited, in no case did NTA or sodium citrate heighten the toxic or teratogenic effects of these heavy metals. There was some evidence that the administration of $FeCl_3$ and NTA with methylHg reduced the latter's teratogenicity, but this could have been a result of treatment differences or of individual variations in the test animals. There appeared to be no correlation between the tissue concentrations of the metals and either their toxic effects or the presence of NTA.

Thus this study reinforces the conclusion that NTA does not enhance the toxic or teratogenic effects of Cd or Hg, and that the influence of another chelating agent, trisodium citrate, does not differ significantly from that of NTA. Furthermore, the authors suggest that the slight protection against Hg toxicity afforded by the ubiquitous ion, Fe^{3+}, is unaffected by NTA.

Further evidence that ingested NTA does not enhance the toxicity or teratogenicity of methylHg in rats has been presented by Scharpf *et al.* (*Nature, Lond.* 1973, **241,** 461), who administered methylHg hydroxide orally in doses of 1·5–8·0 Hg/kg on days 6–19 of gestation, together with either sodium sulphate or NTA. Simultaneous treatment with NTA did not affect the 100% mortality caused by the highest methylHg dose, but at other dose levels of Hg fewer animals died in the NTA-treated groups than in the corresponding groups given sodium sulphate. Foetotoxic and teratological effects were generally directly related to the Hg dose given. The incidence of external foetal abnormalities was lower in the NTA-treated groups, but this trend was not reflected in the occurrence of internal abnormalities, and where differences between the groups were indicated, the numbers involved were insufficient to permit any valid demonstration of a protective action by NTA against methylHg.

The possibility that NTA might increase the effectiveness of generally non-toxic levels of ingested lead (Pb) has also been studied (Mahaffey & Goyer, *Archs envir. Hlth* 1972, **25,** 271). Since NTA increases the retention of iron in the body, it seemed possible that ingestion of a Pb–NTA chelate might result in higher tissue concentrations of Pb than would follow ingestion of inorganic Pb. In view of the reported association of NTA with renal damage

and the known effects of Pb on that organ, particular attention was paid to the kidneys of rats given 0·01, 0·1 or 1% NTA in the drinking water with or without Pb acetate (200 μg Pb/ml). Those given 1% NTA with or without Pb died or were moribund by wk 4 and were found to have marked vacuolization of the lining cells of the renal tubules, with hyperglycaemia and glycosuria. The other groups were treated for 10 wk, but no histological changes were found in the kidneys (or in the liver, brain or pancreas) and the administered NTA did not increase the renal deposition of ingested Pb. Indeed in rats given NTA alone, the Pb levels in the kidneys were lower than those in untreated controls. Hyperglycaemia has not apparently been associated before with administration of NTA. It was confirmed in this study in a second strain of rat, but the mechanism behind this finding remains to be established.

It would seem that NTA is gradually being cleared of suspicions laid at its door. Investigation of its metabolism has indicated that it is rapidly absorbed and is excreted unchanged. Furthermore, while some bone deposition does occur, it appears to be insufficient to cause foetal damage, although more extensive substantiation of this may be desirable. Data has been presented to show that NTA does not enhance the toxic or teratogenic effects of Cd or methylHg at practical levels of exposure, and, in fact, NTA may exert some protective effect against the toxicity of Cd. It may be necessary to investigate further exactly what does happen to NTA in the environment and whether it can produce nitrosamines under certain adverse conditions (*Cited in F.C.T.* 1971, **9,** 889), but in general the future of NTA looks a little more promising in the light of recent work.

THE TORTUOUS PATH OF BILIARY EXCRETION

We have previously touched upon the complexity of factors governing the extent to which foreign compounds may be excreted in the bile (*Cited in F.C.T.* 1972, **10,** 713), but interest in this elimination of substances directly from the liver into the intestine by way of the gall bladder appears to have been relatively limited. One group in London, however, has been studying this mode of excretion for some time and several papers relating to this work are now available.

Early studies showed that the extent to which an organic compound is excreted in the bile of any one species was regulated by several physico-chemical factors, including its molecular weight, polarity, chemical structure and lipid solubility (Abou-El-Makarem *et al. Biochem. J.* 1967, **105,** 1289; Millburn *et al. ibid* 1967, **105,** 1275 & 1283). It appeared that an appreciable degree of biliary excretion occurred in the rat only with compounds with a polar anionic group and a molecular weight above 325. To confirm these findings and determine the influence of chemical structure on the excretion of anions by this route, Hirom *et al.* (*Xenobiotica* 1972, **2,** 205) studied a series of sulphanilamide derivatives, in which one *N*-position was substituted with various carboxyacyl groups and another with an acetyl or 2-thiazole structure. The compounds were chosen as being unlikely to be extensively metabolized, and in fact 16 of the 23 compounds studied appeared in the bile unchanged.

The compounds were administered ip to female rats in a dose of 100 μmoles/kg dissolved in dimethylsulphoxide. Bile was collected for the following 24 hours from the cannulated

common bile duct and chromatographed on paper using four different solvent systems, the compounds present being identified by their R_F values and colour reactions. The amounts of the original compounds in the bile were estimated spectrophotometrically. Six of the compounds studied had molecular weights in the 172–314 range, and with these less than 5% of the dose was excreted in the bile. Significantly greater proportions (7–74%) of the remaining 17 compounds (molecular weights 319–719) were eliminated by this route. Thus confirmation was obtained in the female rat for a threshold molecular-weight range of about 320–370, above which biliary excretion became significant (20–70% of the dose). Within the threshold region, considerable variation was apparent, and even above this range biliary excretion did not increase uniformly with molecular size. There appeared to be some tendency for the more lipophilic compounds to be excreted most readily in the bile, but the relationship was not a simple one and there were one or two notable exceptions. The authors concluded, therefore, that marked differences in the biliary excretion of substances of similar molecular weight were due to structural differences, which might affect either the shape of the molecule or its lipid solubility. This fits in with the findings of other workers (Jones *et al. Fd Cosmet. Toxicol.* 1966, **4,** 411), who reported that the presence of a carboxyl group and a sulphonic acid group in defined positions on the molecule effectively blocked the biliary excretion of the phenylazopyrazolone dyes.

At a fairly early stage in this work, it became apparent that biliary excretion varied not only with the physico-chemical properties of the foreign compound but with the species of animal involved. Further studies of this aspect have been reported by Abdel Aziz *et al.* (*Biochem. J.* 1971, **125,** 25P). The eleven compounds they used included several sulphathiazole derivatives, phenolphthalein salts and tartrazine, bromophenol blue, bromocresol green and indocyanine green, and all had molecular weights between 354 and 752. The biliary excretion of the compounds was compared in female rats, guinea-pigs and rabbits following iv injection into animals with cannulated bile-ducts. For the six compounds with the lowest molecular weights (354–511) a marked species difference was observed, with biliary excretion being greatest in the rat and lowest in the rabbit. The recovery of the dose of hexahydrophthalylsulphathiazole (molecular weight 408), for example, was 80% in the rat, 44% in the guinea-pig and 8% in the rabbit. One compound showed little difference between the rat and guinea-pig, and the four compounds with molecular weights of 580 and above were excreted in the bile of all three species to the extent of over 70% of the dose. It seems, therefore, that species differences tend to disappear with compounds of molecular weight above about 550.

These studies were later extended to include four other sulphathiazoles and lissamine fast yellow, all within the same molecular-weight range (Hirom *et al. ibid* 1972, **129,** 1071). The combined results indicated that the minimum molecular weight for appreciable biliary excretion (over 10% of the dose) was about 400 in the guinea-pig and about 475 in the rabbit, compared with the already mentioned 325 in the female rat. This suggests the possibility of marked species differences in the patterns of elimination of endogenous compounds as well as compounds derived from external sources. Thus, the bile may be an important excretory pathway for steriod hormones, but while oestrone and progesterone, which form polar conjugates in the body with molecular weights of 350–500, should be extensively excreted in rat and guinea-pig bile, this is not likely to be the case in the rabbit. It has in fact been demonstrated (Sandberg *et al. Am. J. Physiol.* 1967, **213,** 1138) that the rat and guinea-pig excrete about 60% of an injected dose of oestrone in the bile, whereas the rabbit excretes only 17%. Unpublished work on drugs (cited by Hirom *et al. Biochem. J.*

1972, **129,** 1071) has presented a similar picture. Morphine is largely converted *in vivo* into its 3-glucuronide, with a molecular weight of 461, and whereas 50–60% of an injected dose is excreted in the guinea-pig or rat, only about 8% is excreted in the rabbit bile.

In an earlier issue (*Cited in F.C.T.* 1972, **10,** 713), we reported this same group's finding that within 3 hours of parenteral administration, females excreted 2–3 times as much of a dose of tartrazine in the bile as did males, which excreted most of the dose in the urine. A threefold difference between the sexes was still observed in the 3-hour bile collection when the renal pedicles of the rats of both sexes were sealed to prevent urinary excretion, indicating that the difference could not be explained simply in terms of a more rapid urinary excretion of the dye by the males. In female rats given daily testosterone injections for 1 month prior to iv injection of tartrazine, the amounts of the administered dye excreted in the bile and urine were 14 and 63%, respectively, compared with 31 and 47% in untreated females and 12 and 64% in normal males. Testosterone pretreatment of females thus altered the pattern of tartrazine excretion to that found in males.

A similar sex difference is not found, however, in the guinea-pig, in which 30–40% of an iv dose of tartrazine was excreted within 3 hours by both sexes. A further complication arises from an earlier demonstration (Hart *et al. Am. J. Physiol.* 1969, **217,** 46) that whereas the biliary excretion of some compounds is higher in female than in male rats, the reverse is the case with certain other compounds (e.g. chlorothiazide). It is therefore not a question of the minimum molecular weight for biliary excretion being for some reason higher in the male than the female rat.

Clearly, no single explanation based on the variation of one parameter is applicable to the question of what determines the relative importance of the bile as a route of excretion. The fact that administration by the oral route was not included in these investigations may have over-simplified the situation, in that for food additives and drugs taken by mouth the extent to which intestinal absorption competes with the effect of gut micro-organisms will inevitably affect the nature and relative amounts of the compound or its metabolites excreted in the bile. It may prove illuminating to look into speculations that a relationship may exist between the nature of protein binding in the liver and the degree of biliary excretion of an organic compound, but many different approaches will probably be necessary before the complex nature of the interplay between the parameters affecting biliary excretion of organic compounds can be fully understood.

THE GUT FLORA OF MAN AND LABORATORY ANIMALS

Although the mammalian gut is sterile at birth, it rapidly becomes colonized by a multiplicity of types of bacteria, the total numbers of which reach about 10^{10} viable organisms/g faecal sample within the first week of life (Smith & Crabb, *J. Path. Bact.* 1961, **82,** 53). The importance of the intestinal microflora to an animal's nutrition and response to ingested foreign compounds cannot be overestimated. In addition to its high metabolic activity, potentially as great as that of the liver (Drasar *et al.* in *Metabolic Aspects of Food Safety*, edited by F. J. C. Roe, p. 245, Blackwell Scientific Publications, Oxford, 1970), the intestinal flora has a unique capacity for adaptation, since its composition (and hence its range of

metabolic activity) can be altered by exposure to a foreign compound which promotes or inhibits the multiplication of one or more specific types of bacteria.

The gut flora is capable of performing a wide variety of reactions, including reduction, decarboxylation, hydrolysis, acetylation, dehydroxylation and ring fission. In view of this wide range of metabolic activity, it is not surprising that micro-organisms of the intestinal flora metabolize many food additives and drugs and in doing so modify their toxicological or pharmacological effects. Clearly, it is important in any study of the toxicity of a food additive to take into account the possibility that the gut flora may alter the structure and properties of the compound.

Numerous studies have been concerned with the identification and enumeration of the component organisms of the gut flora of animals and man (Cregan & Hayward, *Br. med. J.* 1953, **i,** 1356; Drasar *et al. Gastroenterology* 1969, **56,** 71; Drasar *et al.* 1970, *loc. cit.*; Dubos *et al. J. exp. Med.* 1965, **122,** 67; Haenel & Müller-Beuthow, *Zentbl. Bakt. ParasitKde Abt. I. Orig.* 1957, **167,** 123; Kalser *et al. New Engl. J. Med.* 1966, **274,** 500; Moore *et al. J. infect. Dis.* 1969, **119,** 641; Nath *et al. J. Bact.* 1948, **56,** 783; Raibaud *et al. Annls Inst. Pasteur, Paris* 1966, **110,** 568 & 861; Smith, *J. Path. Bact.* 1965, **89,** 95). Early studies were hampered by the difficulty of cultivating the dominant organisms in the gut (the non-sporing anaerobes), many of which are oxygen-sensitive and require considerable care in their culture. However, Hungate (*Bact. Rev.* 1950, **14,** 1) and Drasar (*J. Path. Bact.* 1967, **94,** 417) devised relatively stringent anaerobic techniques permitting the isolation of many more members of the gut flora. It has been shown that anaerobic bacteria constitute more than 99% of the total viable bacteria in the large intestine and faeces of man and most animals, whereas *Escherichia coli*, the organism commonly used as an indicator of faecal contamination of rivers and water supplies, normally comprises less than 0·1% of the faecal flora (Drasar *et al.* 1969, *loc. cit.*).

In general, the micro-organisms usually found in the mammalian gut comprise lactobacilli, bifidobacteria, bacteroides, enterobacteria, streptococci, staphylococci, yeasts and veillonellae. The relative numbers of these organisms in the gut are, however, drastically influenced by a variety of factors, including the type of animal, the region of the intestine and the animal's diet, eating habits and age.

Type of animal

Major differences exist between the gut flora of different mammalian species. If one considers the region of the small intestine nearest to the stomach (the 'proximal ileum') in man and some laboratory animals, these differences are particularly clear. Both in terms of total numbers of organisms and in the number of bacterial species, the proximal ileum of the rat, mouse, monkey and hamster harbours a far more abundant flora than that of man or the rabbit (Smith, *loc. cit.*; Drasar *et al.* 1970, *loc. cit.*). In man, few organisms are isolated from this region of the intestine, especially in periods between eating. Contrary to several previous reports, however, Moore *et al.* (*loc. cit.*) stated emphatically that at no time and under no conditions did they find the stomach or proximal ileum of man or any animal to be sterile.

In terms of bacterial numbers, the flora of the rabbit intestinal tract (unlike that of the rat and mouse) is comparable to that of the human gut, but major qualitative differences between the two species are found. In the rabbit, the flora of this region consists almost exclusively of bacteroides and bifidobacteria, while in man lactobacilli and streptococci

also make an important contribution to the total. In view of the increasing interest in the ferret as an experimental animal for toxicological studies, it would be useful to establish the differences and similarities between its gut flora and that of man.

Region of intestine

In the rat and mouse, the bacterial population of the gut remains fairly constant in numbers and species through the length of the intestine. The stomach of both rodents harbours approximately 10^7–10^9 organisms/g contents and this number is maintained in the small intestine, large intestine and caecum, rising to about 10^9–10^{10} organisms/g sample in the rectum and faeces (Drasar *et al.* 1970, *loc. cit.*; Smith, *loc. cit.*; Raibaud *et al. loc. cit.*). The predominant organisms in all regions of the rat and mouse gut are the lactobacilli (10^6–10^9/g sample), yeasts (10^6–10^7/g sample) and streptococci (10^2–10^6/g sample). The enterobacteria form a smaller portion (10^2–10^6/g sample), their numbers increasing gradually from the stomach to the large intestine (Smith, *loc. cit.*). According to Dubos *et al.* (*loc. cit.*) and Smith (*loc. cit.*), bacteroides are found in large numbers only in the large intestine and caecum, where they are the predominant organisms, but Drasar *et al.* (1970, *loc. cit.*) reported high numbers of bacteroides and bifidobacteria throughout the intestinal tract of the rodents.

The situation in man is very different from that described above for the rat and mouse, since there is a progressive increase in the bacterial population (from 10^1 to 10^{10} organisms/g sample) from the stomach to the large intestine (Drasar *et al.* 1970, *loc. cit.*). In addition, each region of the human gut has a characteristic flora dominated by one or two different bacterial types, although reports often differ on the identity of those predominating.

Except after a meal, the human stomach is colonized by very few micro-organisms, usually no more than 10^1–10^2/g sample (Moore *et al. loc. cit.*). Food passing into the stomach takes micro-organisms with it, so immediately after a meal the number of bacteria in the stomach rises sharply to about 10^4 or 10^5/g sample, but it declines again within about 1 hour to its former value (Moore *et al. loc. cit.*; Drasar *et al.* 1969, *loc. cit.*). Usually the predominant and most persistent bacteria are the lactobacilli and some yeasts, both of which tolerate the acid conditions (pH 1–2) prevailing in the stomach.

In the small intestine of man, the degree of colonization increases with the distance from the stomach. The proximal regions of the ileum contain relatively few bacteria, only about 10^5/g sample after intake of food, but numbers rise to 10^7/g in the distal portion of the ileum (Drasar *et al.* 1969, *loc. cit.*; Moore *et al. loc. cit.*). Reports vary considerably as to the commonest organisms in the small intestine. Kalser *et al.* (*loc. cit.*) found *Aerobacter aerogenes* in the greatest numbers in the proximal and distal ileum and streptococci and *E. coli* were sometimes found in large numbers in the distal region. Significant numbers of lactobacilli, clostridia or bacteroides were seldom isolated. In contrast, Moore *et al.* (*loc. cit.*) reported large numbers of lactobacilli and enterococci in both proximal and distal regions of the ileum, whereas Drasar *et al.* (1969, *loc. cit.*) isolated a wide variety of micro-organisms (enterobacteria, streptococci, bifidobacteria and bacteroides) from this part of the gut, with bifidobacteria and bacteroides predominating. Lactobacilli were present in variable numbers.

In the distal region of the human ileum, the bacterial flora is more abundant and permanent than in the proximal portion and as the terminal ileum is approached bacterial numbers increase and the flora begins to resemble qualitatively the flora of the faeces, in which

anaerobic bacteria, bacteroides and bifidobacteria (10^9–10^{11} organisms/g sample) predominate (Drasar *et al.* 1969, *loc. cit.*). Enterobacteria and lactobacilli contribute less than 0·1% of the total viable bacteria in the faeces (Moore *et al. loc. cit.*; Drasar *et al.* 1969, *loc. cit.*). Clostridia, veillonellae and yeasts are also found in small numbers. The bacterial flora of the large intestine of man has not been well studied, but it is usually assumed that it bears a close resemblance to that of the faeces.

The conflicting reports of the flora of the stomach and proximal small intestine of man are probably due partly to the transient nature of the flora in these regions. In man, the bacteriostatic action of the acid gastric juice (pH 1–2) is without doubt a prime cause of the low bacterial population in the stomach and upper ileum under conditions of fasting. In this respect man is similar to the rabbit which, unlike the rat and mouse, with stomach contents of pH approximately 4·4 and 4·0 respectively, has a highly acid (pH 1·9) gastric juice (Smith, *loc. cit.*). As mentioned earlier, total bacterial numbers in the rabbit stomach and proximal ileum are comparable to those in man, but although the intestinal flora of the rabbit is distributed in a similar fashion to that of man, considerable qualitative differences exist, notably in numbers of lactobacilli, which are virtually absent from the rabbit gut. Also it must be noted that the upper alimentary tract of the rabbit, although sparsely populated with bacteria, harbours a more permanent flora than that of man, since the rabbit feeds more continuously (Drasar *et al.* 1970, *loc. cit.*).

Although for much of the time the upper intestinal tract of man harbours very few bacteria, it would be imprudent to assume that no bacterial metabolism of food and food additives occurs in this region. When food is eaten, bacterial numbers in the stomach and upper ileum increase and therefore food additives and other foreign compounds ingested with the food are exposed to a fairly large population of micro-organisms.

Effect of diet and eating habits

The habit of eating more or less continuously is thought to play a part in the maintenance of large bacterial populations throughout the gut of most types of animal, in contrast to the almost sterile conditions in the upper alimentary tract of man, who feeds sporadically. Smith (*loc. cit.*), using rats prevented from indulging in coprophagy (eating of faeces), found distinct changes in the gut flora when the rats were starved for 24 hours. Only small numbers of lactobacilli were isolated from the stomach and small intestine of such rats, compared with the abundant flora of enterobacteria, streptococci, yeasts and lactobacilli present in normal rats.

Coprophagy may play an important role in maintaining the gut flora of rats and guinea-pigs. Gustafsson & Fitzgerald (*Proc. Soc. exp. Biol. Med.* 1960, **104**, 319) found that in rats prevented from indulging in coprophagy, the number of lactobacilli in the caecal contents and faeces was considerably reduced. In addition, signs of vitamin K deficiency have been noted in such rats (Barnes & Fiala, *J. Nutr.* 1959, **68**, 603). Smith (*loc. cit.*) was unable to find these changes in rats, reporting only a possible slight decrease in lactobacilli in the stomach and ileum, but he noted that coprophagy seemed essential for the maintenance of the lactobacilli population in guinea-pigs.

In rats, the diet significantly affects the composition of the gut flora. A marked reduction in the number of lactobacilli is found in the intestine of rats fed on diets lacking cereals, or on diets of starch or casein, compared with rats fed on a standard diet (Smith, *loc. cit.*). Yeasts, normally present in numbers of 10^4–10^5/g sample, are virtually absent in rats fed

on a high protein diet and are much reduced in starch-fed rats. Numbers of *Clostridium welchii* increase dramatically in rats fed on raw pork, even though the meat itself is free from this organism.

Effect of age

Dubos *et al.* (*loc. cit.*) reported that the gut flora of mice was essentially unaffected by the age or sex of the animals, but changes in the flora with age have been noted by Smith & Crabb (*loc. cit.*) and by Smith (*J. appl. Bact.* 1961, **24,** 235). The latter found that the faecal flora of all the animals he examined (calf, lamb, piglet, rabbit and human baby) were similar in early life, despite the fact that after birth a wide variety of bacteria constantly entered the alimentary tract. However, differences became apparent as the animals aged, certain bacteria becoming predominant in some species while disappearing almost completely from others. The total population of bacteria declined with age in some animal species. In the calf, for example, there were 10^4 times fewer bacteria in the faeces at 6 months than when the calf was a few weeks old. *E. coli* and *Cl. welchii* were the first bacteria to colonize the alimentary tract of animals, but they became less numerous as the animals aged. Lactobacilli and bacteroides colonized the alimentary tract somewhat later but persisted in large numbers for a longer time than *E. coli* and the clostridia.

Conclusions

To summarize, although there are some qualitative similarities between the intestinal flora of man and the common laboratory animals, great differences exist in the relative numbers of the different types of bacteria. Bifidobacteria and bacteroides, two strictly anaerobic species, are the dominant organisms in the faeces of all animals studied. In most animals, the alimentary canal is more densely colonized than that of man. Of the laboratory animals studied, the rabbit is the only one in which the distribution of micro-organisms in the gut is similar to that in man, but even here there are distinct qualitative differences. Diet and eating habits are major factors determining the composition of the gut flora.

In view of the somewhat conflicting reports about the component organisms of the gut flora of laboratory animals and the strong influence of diet on the character of the bacterial population, a careful study of the flora of the particular strain of animal concerned is an essential prerequisite of any assessment of the possible contribution of the gut flora to the metabolism of foreign compounds.

Fd Cosmet. Toxicol. Vol. 11, pp. 685–710. Pergamon Press 1973. Printed in Great Britain

TOXICOLOGY: ABSTRACTS AND COMMENTS

COLOURING MATTERS

2541. Tartrazine sensitivity

Juhlin, L., Michaëlsson, G. & Zetterström, O. (1972). Urticaria and asthma induced by food-and-drug additives in patients with aspirin hypersensitivity. *J. Allergy* **50,** 92.

Reactions to food colourings in hypersensitive patients have been recognized for some time (*Cited in F.C.T.* 1968, **6,** 95). Various hypotheses have been put forward for the mechanism of aspirin hypersensitivity, and opinions differ as to whether it is an allergic response or has some kind of non-allergic basis. The fact that a response may also be promoted by substances other than aspirin, such as indomethacin, antipyrine and tartrazine, suggests a non-allergic mechanism.

This paper reports a study of the sensitivity of eight aspirin-sensitive patients to other agents. When they were symptom-free, or as nearly symptom-free as possible, the patients were challenged with 1 or 0·75 mg tartrazine in solution or with a gelatin capsule containing 1–500 mg aspirin, 1–500 mg purified acetylsalicylic acid (anhydride-free), 5–100 mg acetylsalicylic acid anhydride, 50 mg 2-hydroxybenzyl alcohol, 50 mg 4-hydroxybenzoic acid or 250 mg sodium benzoate. Lactose was the only other constituent of the capsules, and capsules containing 100 mg lactose were used as placebos. Only one drug was tested each day and no test was started until any symptoms from the previous test had disappeared.

The cases are all reported individually. Not one of the patients reacted to the placebo but seven of the eight were highly sensitive to tartrazine, reacting with asthma, urticaria or both. Cross-sensitivity to benzoate was less common, but all the patients showed significant sensitivity to 4-hydroxybenzoic acid. Asthmatic patients tended to respond to lower doses of aspirin than did the urticarial patients, although it is uncertain whether different mechanisms were involved.

The reported incidence of tartrazine sensitivity has not been as high in previous studies as in the current work. Samter & Beers (*J. Allergy* 1967, **40,** 281), for example, found a reaction to tartrazine in only 8% of their 182 aspirin-sensitive patients, but it is not clear whether provocation tests were carried out on each patient. The authors of the present study consider that tartrazine should not be used as a colouring in foods and pharmaceuticals without information to this effect being available to the consumer.

[It is probably as a result of this paper that the National Foods Administration of Sweden now requires the presence of tartrazine in a food substance to be declared. As we pointed out when this problem arose before (*Cited in F.C.T.* 1968, **6,** 95), this type of allergic response is likely to be limited to a few hypersensitive individuals, and most consumers have no cause for alarm.]

FLAVOURINGS, SOLVENTS AND SWEETENERS

2542. Elucidation of allyl halide metabolism

Kaye, C. M., Clapp, J. J. & Young, L. (1972). The metabolic formation of mercapturic acids from allyl halides. *Xenobiotica* **2,** 129.

The toxicity of allyl halides has been known for some time, but their metabolism has not been studied extensively before. In this study, male CFE rats were dosed sc with allyl chloride (1 ml of a 10% v/v solution in arachis oil), with allyl bromide or iodide (0·5 ml of a 5% v/v solution in arachis oil), or with one of their possible metabolic intermediates, *S*-allylglutathione (SAG; 1 ml of a 10% w/v aqueous solution) and *S*-allyl-L-cysteine (SAC; 3 ml of a 15% w/v aqueous solution).

The urines were collected separately from the faeces and examined for sulphur-containing compounds, amino acids and mercapturic acids by descending paper chromatography using three different solvent systems. Some of the rats were fed 5% ^{35}S-labelled yeast in the diet so that sulphur-containing metabolites in the urine could be detected by means of chromatography and a radiochromatogram scanner. Bile from animals treated with allyl chloride was also examined for sulphur-containing metabolites.

All three allyl halides gave similar results, allylmercapturic acid (AMA), 3-hydroxypropylmercapturic acid (3-HPMA) and AMA sulphoxide being found in the urine after their administration. Only AMA and its sulphoxide were found after dosing with SAG or SAC. AMA in the urine accounted for only 1·7% of the allyl chloride administered but for 16·3% of the SAC dose. No additional sulphur-containing metabolites were found in the urine of the rats fed ^{35}S-labelled yeast and given allyl chloride, but in the bile, SAG and SAC were found in addition to AMA.

Thus the allyl halides form AMA and its sulphoxide. The results in this paper support the hypothesis that mercapturic acids are formed by interaction of glutathione and the foreign compound (or a derivative) to give *S*-substituted glutathione, and that this is broken down to *S*-substituted cysteine, which in turn is acetylated to mercapturic acid.

The results of this study support previous findings that certain allyl halides lead to the urinary excretion of hydroxyalkylmercapturic acids. There are a number of pathways by which this mercapturic acid could have been formed, and the specific route has not been established. Possibilities include formation via the 3-halogenopropanol, or via SAG and AMA, but since previous work has shown only slight traces of 3-HPMA in rat urine after administration of AMA and since, in the present study, no 3-HPMA was found after SAG or SAC administration, it seems more likely that some of the allyl halide is converted to allyl alcohol, which then gives rise to 3-HPMA. This acid has been found in rat urine after allyl alcohol treatment.

2543. Rat behaviour and neonatal MSG

Pradhan, S. N. & Lynch, J. F., Jr. (1972). Behavioral changes in adult rats treated with monosodium glutamate in the neonatal stage. *Archs int. Pharmacodyn. Thér.* **197,** 301.

We recently reviewed some of the more recent studies on the neurotoxicity and other effects of monosodium glutamate (MSG) on experimental animals, particularly in the neonatal period (*Cited in F.C.T.* 1973, **11,** 309). The study now cited was designed to detect possible behavioural abnormalities in adult rats treated neonatally with MSG.

Three groups of new-born rats were given MSG in intragastric doses of 1·25, 2·5 or 5 g/kg, daily for 5 days (from day 5 to day 10 after birth). A control group received an equivalent volume (10 ml/kg) of water only. Weight gain, measured during the first 3 months of the experiment, was less in the MSG-treated rats than in controls, the difference being most marked in the group given 5 g MSG/kg. There was also evidence of the development of behavioural defects in the rats treated neonatally with MSG, particularly in connexion with discrimination aptitude. Spontaneous motor activity was significantly less than in controls only in the 5 g/kg group, but maze-learning capacity was depressed at all dose levels. On the other hand, training time in a fixed-ratio food reinforcement test was not significantly affected.

2544. Lethal effects of cyclohexylamine in mice

Lee, I. P. & Dixon, R. L. (1972). Various factors affecting the lethality of cyclohexylamine. *Toxic. appl. Pharmac.* **22,** 465.

Cyclohexylamine (CHA), a compound with numerous industrial uses, is now well known as a metabolite of the artificial sweetener, sodium cyclamate. In previous issues we have referred to the pharmacological action of the amine and shown that it can produce cardiovascular effects by the stimulation of receptors responsible for the release of catecholamines (*Cited in F.C.T.* 1970, **8,** 587). The current report examines these and other effects of CHA in mice in relation to the toxicity of the amine.

Toxic ip doses of CHA resulted in hyperactivity and other amphetamine-like symptoms, which were accentuated both by an increase in environmental temperature and by aggregation of the mice. The degree of toxicity of CHA doses up to 400 mg/kg could be correlated with the increase in body temperature that followed CHA treatment and that may have been a reflection of an increase in metabolic rate. The LD_{50} of CHA was increased by drugs that affect normal adrenergic function. Subsequent treatment with chlorpromazine, for example, increased the ip LD_{50} of CHA by nearly 50%, a protective effect attributed in part to a decrease in hyperactivity as well as to a reduction in body temperature. On the other hand, the microsomal-enzyme inhibitor, SKF 525-A, significantly increased CHA toxicity in mice, suggesting that microsomal enzymes normally metabolize CHA to less toxic metabolites.

Carbon-labelled acetate was used to demonstrate that CHA administration increased the metabolic rate in mice. While death from doses up to 480 mg/kg apparently correlated with hyperthermia, the lethal effects of higher doses were considered to be more probably linked with other mechanisms related to the sympathomimetic action of CHA.

ANTIOXIDANTS

2545. Promotion of fat oxidation by BHT

Pascal, G. et Terroine, T. (1972). Effets de l'ingestion d'un additif alimentaire, le di-tertio-butyl-hydroxy-toluène (BHT) sur les échanges respiratoires du rat mâle. *Annls Nutr. Aliment.* **26,** 33.

One of the authors named above has already reported that butylated hydroxytoluene (BHT) fed to rats at a dietary level of 0·1 or 0·5% decreased food consumption and growth

rate and reduced hepatic levels of fat and DNA but not of protein nitrogen or RNA (*Cited in F.C.T.* 1971, **9**, 583). At the 0·5% dietary level, changes in lipid metabolism have been seen, their nature depending on the fat content of the diet (*ibid* 1969, **7**, 541). The effects of BHT in diets of varying fat content have now been further explored.

BHT was incorporated into the diet of growing male rats at a level of 0·5% and dietary fat was provided by lard or maize oil at levels of 8 or 16%. Compared with control rats fed these diets *ad lib.* without BHT, animals receiving BHT showed a slight reduction in growth rate. Weight gain was lowest in rats given the diets containing maize oil, particularly at the 16% level, and was low even when no BHT was included, food consumption being markedly reduced with this diet. Nitrogen balance was unaffected by BHT administration, but the respiratory quotient (carbon dioxide expired/oxygen consumed) was decreased in rats receiving BHT and 8 or 16% lard or 16% maize oil. This decrease was attributed to a considerable increase in lipid oxidation, calculated as 121% in the case of the 16% lard diet and as 56% in the case of the 16% maize-oil diet. Carbohydrate oxidation, on the other hand, was apparently decreased slightly. None of these parameters was affected in a further group of rats maintained on a diet containing only 0·1% BHT and 11% groundnut oil, a finding somewhat in conflict with previous results (*ibid* 1971, **9**, 583).

The authors speculate that the greater effect of lard on lipid oxidation may have been due simply to the greater food intake and growth rate of rats on this regime, compared with those receiving maize oil, or may have been a reflection of a protective effect exerted by linoleic acid, of which maize oil is a rich source. BHT's capacity to increase lipid oxidation may be associated with its ability to uncouple oxidative phosphorylation. The authors have demonstrated this *in vitro* in a study still in progress.

PRESERVATIVES

2546. Urethane from diethyl pyrocarbonate

Fischer, E. (1972). Über die Bildung von Carbaminsäureäthylester (Urethan) in Getränken nach Behandlung mit Pyrokohlensäurediäthylester. *Z. Lebensmittelunters. u.-Forsch.* **148**, 221.

Swedish workers have reported relatively high levels of urethane in wines, beers and soft drinks treated with diethyl pyrocarbonate (*Cited in F.C.T.* 1972, **10**, 588) but these findings have been disputed by others in the field (*Food Chemical News* 1972, **14** (5), 45). In view of the earlier indications that the amounts of urethane formed depended not only on the pH of the treated beverage but also on the ammonium-ion content, the author cited above carried out urethane analyses on ammonium chloride solutions treated with 300 mg diethyl pyrocarbonate/litre after various pH adjustments as well as on wine and fruit juices similarly treated. Isotope dilution analysis was used as before and the diethyl pyrocarbonate was labelled with ^{14}C in the carbonyl group. The urethane levels of the treated ammonium chloride solutions were 0·0098, 0·03 and 0·086 mg/litre at pH 3·0, 3·5 and 4·0, respectively. Treated orange juice (pH 3·5) contained 0·014 mg/litre, grapefruit juice (pH 3·0) 0·012 mg/litre and Moselle wine (pH 3·2) 0·04 mg/litre.

These values are much lower than those previously reported from Sweden, and it is suggested that the difference may be attributable to inadequate purification of the extracted urethane in the earlier analyses.

MISCELLANEOUS DIRECT ADDITIVES

2547. Metabolic fate of a modified starch

Ryan, A. J., Holder, G. M., Máté, Colleen & Adkins, G. K. (1972). The metabolism and excretion of hydroxyethyl starch in the rat. *Xenobiotica* **2**, 141.

The attainment of desired properties by chemical modification of the starch molecule has been widely utilized in food technology. The possibility that toxicological properties may also be modified has been the subject of numerous investigations in animals, particular emphasis being placed on the metabolic fate of these modified compounds in comparison with that of starch itself. In the study cited above, the fate of hydroxyethyl starch was examined in rats. This material is used principally as a blood-plasma substitute but its metabolic fate following ingestion is also of interest.

Within 96 hr of ingesting 20 mg [1,2-^{14}C]hydroxyethyl starch, rats had excreted some 60% of the dose in the faeces, 20% as carbon dioxide and 15% in the urine. This pattern of metabolism differed significantly from that of starch itself, which gave rise to a much higher proportion of radioactivity in the expired air.

After iv injection of 5 mg of the labelled material no significant biliary excretion of metabolites occurred within 6 hr, and an ip dose of 20 mg was excreted almost completely in the urine within 24 hr. The urinary metabolites from orally and ip administered [^{14}C]-hydroxyethyl starch were analysed using a paper-chromatographic technique, and 6-*O*-hydroxyethylglucose was identified together with traces of oligomeric hydrolysis products.

Clearly hydroxyethyl starch is absorbed only slowly from the gastro-intestinal tract. The striking difference between the amounts of radioactivity excreted as carbon dioxide following oral and ip administration is interpreted by the authors as indicating that the gut flora may be active in the oxidation of hydroxyethyl starch or its hydrolysis products, either directly or by initiating conversion to other substances that can be absorbed prior to complete metabolism.

[If specific gut flora are actively involved in the oxidation of this and possibly other modified starches, some doubt must be cast on the relevance to the human situation of the extensive animal data available on the metabolism of these materials, in the absence of supportive data from human studies.]

AGRICULTURAL CHEMICALS

2548. Dichlorvos metabolism in rodents

Hutson, D. H. & Hoadley, Elizabeth C. (1972). The metabolism of [^{14}C-methyl]dichlorvos in the rat and the mouse. *Xenobiotica* **2**, 107.

We have previously discussed the metabolic fate of the important organophosphorus insecticide, dichlorvos (2,2-dichlorovinyldimethyl phosphate), in several species. It is

readily detoxified, particularly in the liver (*Cited in F.C.T.* 1966, **4,** 353), and following its oral or parenteral administration, tissue residues are very low and disperse rapidly (*ibid* 1963, **1,** 290). The present and more detailed report confirms this finding in rats and mice, and illustrates the involvement of glutathione in dichlorvos metabolism.

[^{14}C-Methyl]-labelled dichlorvos was administered to rats and mice by the oral route. In both species, metabolism was almost complete within 24 hr, two-thirds of the dose being excreted in the urine and about 15% being metabolized completely to carbon dioxide.

About 50% of the dose was hydrolysed to dimethyl phosphate, the major urinary metabolite, suggesting that hydrolytic cleavage at the P-O-vinyl bond is an important route for detoxication of the insecticide. Elimination of 2,2-dichlorovinyl monomethyl phosphate (desmethyldichlorvos) showed that demethylation is another important mechanism of detoxication. *S*-Methylcysteine, *S*-methylcysteine oxide and *N*-acetyl-*S*-methylcysteine present in small amounts in the urine of both species, but in somewhat greater proportion in the mouse, were taken as clear evidence of the involvement of glutathione in the dealkylation reaction, *S*-methylcysteine being derived from *S*-methylglutathione by hydrolysis.

2549. DDT prolongs reproductive life in rats

Ottoboni, Alice (1972). Effect of DDT on the reproductive life-span in the female rat. *Toxic. appl. Pharmac.* **22,** 497.

The reproductive performance of mice over five generations has been found to be unaffected by DDT at a dietary level of 3 ppm (Tarján & Kemény, *Fd Cosmet. Toxicol.* 1969, **7,** 215) but in another study some impairment was noted with 7 ppm (*Cited in F.C.T.* 1969, **7,** 535). In female rats, DDT fed at dietary levels up to 200 ppm appeared to have an adverse effect on matings of marginal fertility, but, in contrast, seemed to improve reproductive performance in animals nearing the end of their reproductive span (*ibid* 1969, **7,** 535). The present study was designed to establish whether prolonged exposure to low levels of DDT does, in fact, extend the reproductive life of the female rat beyond the normal menopausal age of 15–18 months.

Twelve pairs of littermate female rats were divided so that one of each pair was included in the test and the other in the control group. Rats in the test group were fed DDT at a dietary level of 20 ppm from weaning and were mated with males of proven fertility at 3 months and every 2 months thereafter, until 11 breedings had been accomplished. Except during the mating periods, the males received untreated diets. Feed consumption, feed efficiency, growth and adult body weight were unaffected by DDT, as were litter size and viability. However, average reproductive life-span was increased to 14·55 months, as compared with only 8·91 months in the controls. Eight of the littermate pairs survived beyond 17 months and of these all eight DDT-fed females became pregnant once or more after this age, producing a total of 11 litters, while only one pregnancy was recorded in the control rats of the same age.

The reasons for the various and apparently conflicting effects of DDT on reproduction are not clear. Oestrogenic activity has been demonstrated in the case of *o,p*-DDT, technical DDT and, to a much smaller extent, *p,p'*-DDT (*ibid* 1970, **8,** 444), while the metabolites *p,p'*-DDD and *p,p'*-DDE appear to possess anti-oestrogenic properties (Welch *et al. Toxic*

appl. Pharmac. 1971, **19,** 234). In the present study the level of DDT administered was apparently below that required to have an antifertility effect, but was sufficient to protect against the impact of ageing on the female reproductive processes.

2550. Unplumbed depths in lindane metabolism

Chadwick, R. W. & Freal, J. J. (1972). The identification of five unreported lindane metabolites recovered from rat urine. *Bull. env. contam. & Toxicol. (U.S.)* **7,** 137.

The metabolism of γ-benzene hexachloride (γ-BHC; lindane), at least in rats, now appears to be considerably more complex than was previously thought.

Female rats were fed 400 ppm lindane in their diet. After 7 days of treatment, gas chromatography of benzene extracts of the acidified urine from these animals demonstrated at least five hitherto unrecognized metabolites, in addition to the already reported 2,3,5- and 2,4,5-trichlorophenols. The new compounds were identified as 3,4-dichlorophenol, a minor metabolite, 2,4,6-trichlorophenol, 2,3,4,5- and 2,3,4,6-tetrachlorophenols and 2,3,4,5,6-pentachloro-2-cyclohexen-1-ol. The last four compounds were excreted in greater quantities than either 2,3,5- or 2,4,5-trichlorophenol, and therefore rank as major metabolites.

It is evident that current ideas regarding the metabolic fate of lindane in mammals need revision to take account of these findings, since the metabolites now isolated do not fit in with the metabolic pathway already postulated.

2551. Lipoproteinaemia following pesticide exposure

Carlson, L. A. & Kolmodin-Hedman, Birgitta (1972). Hyper-α-lipoproteinemia in men exposed to chlorinated hydrocarbon pesticides. *Acta med. scand.* **192,** 29.

The affinity of chlorinated hydrocarbon pesticides for a variety of lipids, demonstrated by their observed accumulation in adipose tissue, suggests that these compounds may complex with lipids in the serum and elsewhere. In view of suggestions that the pesticides may bind to lipoproteins, a study was designed to investigate plasma lipoprotein metabolism in subjects exposed to chlorinated insecticides. This study revealed a high incidence of a rare abnormality, hyper-α-lipoproteinaemia, in such workers.

Lipoproteins were classified according to their ultracentrifugal properties into those of very low density (VLDL or pre-β), low density (LDL or β) and high density (HDL or α), and the amounts of various serum lipoproteins in these categories were studied in 22 clinically healthy men exposed regularly to pesticide sprays, mainly of lindane and DDT. Control groups consisted of healthy men not directly exposed to insecticides.

Of the 22 test subjects examined, five were found to have hypertriglyceridaemia, with increased levels of VLDL. One had slight hypercholesterolaemia with an increase in LDL. Nine of the exposed men had raised levels of HDL α-cholesterol or phospholipid and, of these, five showed an increase in both cholesterol and phospholipid. In this group with hyper-α-lipoproteinaemia, comprising some 40% of exposed workers, cholesterol and phospholipid levels up to three times as high as the normal levels were found.

Control subjects showed no evidence of hyper-α-lipoproteinaemia, which is, as stated above, a very rare abnormality. It was concluded that the changes observed in the serum

lipoproteins of subjects exposed to chlorinated insecticides could either represent a transport mechanism for the pesticides, or reflect subclinical liver damage induced by these hydrocarbons.

2552. Pulmonary phospholipids unaffected by paraquat

Fletcher, K. & Wyatt, I. (1972). The action of paraquat on the incorporation of palmitic acid into dipalmitoyl lecithin in mouse lungs. *Br. J. exp. Path.* **53,** 225.

Several authors have associated the effects of paraquat in animals with a reduction in the surfactant materials in the lungs (*Cited in F.C.T.* 1972, **10,** 704). On the other hand, no change in the composition of lung phospholipids, to which the surface activity of lung extracts is mainly due, was detected by the authors cited above in an earlier study of rats treated with paraquat (*ibid* 1972, **10,** 704). However, in that study the major component of these phospholipids, dipalmitoyl lecithin (DPL), was not specifically studied, an omission that has now been rectified.

Synthesis of DPL was found to be very rapid under normal conditions, with maximum labelling of the phospholipid occurring within 30 sec of the injection of radioactively-labelled palmitic acid. In mice given 125 mg paraquat/kg by stomach tube, the incorporation of palmitic acid into DPL measured at intervals from 5 to 144 hr did not differ significantly from that in control mice. Neither was there any significant difference between treated and control mice in the fatty acid composition of the DPL. Moreover, treatment of mice with paraquat for 6 days had no effect on the rate of breakdown of DPL in the lungs. The authors therefore conclude that their results confirm their previous observations and indicate that the biochemical action of paraquat is not associated with phospholipid metabolism in the lung.

2553. Metabolism rates for parathion analogues

Wolcott, R. M. & Neal, R. A. (1972). Effect of structure on the rate of the mixed function oxidase catalyzed metabolism of a series of parathion analogs. *Toxic. appl. Pharmac.* **22,** 676.

An understanding of the mechanism underlying the toxicity of the dialkylaryl phosphorothioates, an important group of cholinergic organophosphate insecticides, requires a detailed knowledge of their mode of metabolism. This is illustrated by the finding that certain microsomal-enzyme reactions increase the cholinergic toxicity of these compounds, whereas others lead to the formation of non-toxic products (*Cited in F.C.T.* 1972, **10,** 245). The present paper examines the effect of chemical structure on the rate of oxidation of these phosphorothioates to the corresponding phosphates.

A number of phenyl-substituted diethyl phosphorothioates were incubated in various concentrations with a rabbit-liver microsomal preparation, and the rates of formation of the phenyl-substituted diethyl phosphates and diethyl phosphorothioic acid were determined, so that the V_{max} and K_m values for the reactions could be calculated, the K_m value being the concentration of phenyl-substituted diethyl phosphorothioate that gave half the maximal velocity (V_{max}) for the metabolite formation. The graph of log V_{max} values against the electron-withdrawing capacity of the substituted phenyl groups showed no direct correla-

tion between rate of metabolism and electron affinity, although this has been reported with some substrates containing substituted phenyl groups. Statistical analysis of the K_m and V_{max} values for the various oxidase-catalysed reactions did, however, reveal certain structure-dependent differences. The differences found most consistently in different experiments were the relatively high V_{max} values for the metabolism of *p*- and *m*-nitro compounds to the corresponding phosphates and to diethyl phosphorothioic acid and the relatively high K_m values in the formation of the corresponding phosphates from the diethyl, *p*-methyl and *m*-methylphenyl phosphorothioates.

2554. Lethality of pyrethrins to rats

Verschoyle, R. D. & Barnes, J. M. (1972). Toxicity of natural and synthetic pyrethrins to rats. *Pestic. Biochem. Physiol.* **2,** 308.

Natural pyrethrins, which have been used in insect control for many years, combine powerful insecticidal activity with low oral and dermal toxicity in laboratory animals and a good record of safety in use. They exert a paralytic effect on the nervous system of insects, and mammalian nervous tissue is also sensitive to them, their selective toxicity stemming apparently from differences in their metabolic fate in mammals and insects (*Cited in F.C.T.* 1972, **10,** 111). The effects of oral and iv administration of pyrethrins to rats have now been compared, to shed further light on the nature of this selectivity and provide background for current work on the synthesis of pyrethrin analogues.

Several natural pyrethrins, together with five synthetic analogues, were each administered to rats orally and iv at various dose levels. All the compounds tested were of far greater acute toxicity when injected iv than when given orally, although for each route the toxicity of the different compounds varied widely.

Oral doses generally evoked signs of nervous hypersensitivity after a time lag of 1 or 2 hr and with lethal doses this was followed by convulsive twitching, prostration, coma and death between 3 and 24 hr after dosing. When death did not occur, recovery from the severe effects of most compounds generally occurred within 24–36 hr. Reactions to iv injection was very rapid. Animals collapsed with convulsive tremors immediately and most deaths occurred within 10 min. Signs of improvement were evident in the survivors within an hour or so. This rapid action indicates that the toxic action is evoked by the pyrethrins themselves and that no metabolic conversion is required. However, the relatively transient effect of an almost lethal iv dose suggests the existence of a rapid detoxication process accompanied by membrane repair.

2555. Targets for dioxin

Buu-Hoï, N. P., Pham-Huu Chanh, Sesqué, G., Azum-Gelade, M. C. & Saint-Ruf, G. (1972). Enzymatic functions as targets of the toxicity of "dioxin" (2,3,7,8-tetrachlorodibenzo-*p*-dioxin). *Naturwissenschaften* **59,** 173.

Buu-Hoï, N. P., Pham-Huu Chanh, Sesqué, G., Azum-Gelade, M. C. & Saint-Ruf, G. (1972). Organs as targets of "dioxide" (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) intoxication. *Naturwissenschaften* **59,** 174.

'Dioxin' (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) is a toxic and teratogenic contaminant of herbicides derived from 2,4,5-trichlorophenoxyacetic acid (*Cited in F.C.T.* 1972, **10,** 722).

The first paper cited above reports the administration of a single ip dose of 10 mg dioxin/kg to rats and the examination of blood samples 10 days later. Dioxin severely disturbed several enzyme systems. The most marked changes were a decrease in serum cholinesterase activity and an increase in serum glutamic–oxalacetic transaminase. Significant increases in lactic dehydrogenase and hydroxybutyric dehydrogenase also occurred. The main effects of dioxin on other components of the serum were increases in total lipids and in bilirubin. Such findings point to the liver as a prime target of dioxin toxicity.

Following up these observations, the second paper cited describes the histopathological changes produced in rat liver by the same ip dose of dioxin (10 mg/kg). Examined at intervals up to 10 days after treatment, all the animals showed hepatic lesions, but these were significantly more severe in females than in males. Centrilobular stasis and focal hyperplasia of the Kupffer cells were prominent by day 6. The thymus also revealed lesions, ranging from diffuse pyknosis of the thymocytes to their migration towards the medulla or total involution. These lesions were evident from day 5, and offer an explanation for the pronounced toxicity of dioxin to young animals. The heart showed evidence of valvulitis with endocardial erosion on the ventricular face of the valves, fibroblastic proliferation and fibrinoid thrombi. Finally, the lungs showed foci of alveolitis, with periarteriolar fibroblastic reactions and fibrinoid thrombi.

Similar though less severe reactions were evoked by a smaller dose (1 mg dioxin/kg).

FEED ADDITIVES

2556. Coping with arsanilate feeds

Selye, H., Kourounakis, P. & Szabo, S. (1972). Protection against the neurolathyrism produced by arsanilic acid in the rat. *Expl Neurol.* **25,** 513.

Al-Timimi, A. A. & Sullivan, T. W. (1972). Safety and toxicity of dietary organic arsenicals relative to performance of young turkeys. 1. Arsanilic acid and sodium arsanilate. *Poult. Sci.* **51,** 111.

We have noted that pigs given excessive doses of arsanilic acid (I) in their feed developed tremor and limb incoordination of the fore and hind limbs (*Cited in F.C.T.* 1969, **7,** 390). The first paper cited above describes neurological disturbances resembling those of neurolathyrism in rats given daily sc injections of 100 mg I/kg dissolved in DMSO. In an attempt to counteract this neurotoxicity, doses of one of a variety of steroidal and non-steroidal compounds were given twice daily for 9 days, the treatment being started 4 days before the first injection of I and continued throughout the period of I administration. Pregnenolone-16α-carbonitrile (PCN), potassium 9α-fluoro-11β,17-dihydroxy-3-oxo-4-androstene-17α-propionate, ethylestrenol, spironolactone, norbolethone, oxandrolone, prednisolone, progesterone, oestradiol, desoxycorticosterone and hydroxydione were given in 10 mg doses and triamcinolone in doses of 1 mg. L-Thyroxine in a single daily dose of 200 μg and serotonin in a dose of 2 mg given twice daily were injected sc, and phenobarbitone and phenytoin were each given by oral intubation twice daily in doses of 6 mg.

PCN and ethylestrenol aggravated the neurotoxicity of I, whereas triamcinolone, desoxycorticosterone and thyroxine offered considerable protection and phenobarbitone and serotonin gave less but still significant protection. The countertoxic effects observed here are noteworthy in view of the accepted opinion that I does not undergo significant biotransformation either to more or to less active metabolites and that in the absence of pretreatment with other compounds it appears to be excreted unchanged. On these grounds the protective action would appear to be due to an enhancement of resistance to I rather than to a promotion of its chemical detoxication.

The authors of the second paper cited found that turkey poults fed from 1 to 28 days of age on a diet containing 0·03% I showed a depression in weight gain at both 14 and 28 days. Greater weight reductions occurred with higher dietary levels of I. The mortality rates with feeds containing 0·08, 0·09 and 0·12% I were 55·6, 62·5 and 87·5% respectively. The LD_{50} for diets fed for 28 days was thus slightly below 0·08%.

In similar experiments sodium arsanilate, with a 10% lower content of arsenic, proved somewhat less toxic than I. However, the difference was such that the toxicity of the two compounds could be expected to be similar for feeds compared on a molar-equivalent rather than on a percentage-weight basis.

2557. Gossypol and cottonseed protein

Smith, F. H. (1972). Effect of gossypol bound to cottonseed protein on growth of weanling rats. *J. agric. Fd Chem.* **20,** 803.

Cottonseed is an important source of protein for animal feeds, but contains the toxic phenol, gossypol (1,1′,6,6′,7,7′-hexahydroxy-3,3′-dimethyl-5,5′-diisopropyl-2,2′-dinaphthyl-8,8′-dicarboxaldehyde). The rate at which this is metabolized and excreted apparently determines its toxicity to different animal species (*Cited in F.C.T.* 1971, **9,** 921). During the processing of animal feed, gossypol is largely detoxified by condensation of its aldehyde groups with free amino groups of the cottonseed protein. This process, though it lowers toxicity, also decreases the nutritive value of the protein, as the study cited above shows.

Weanling rats were fed for 4 wk on diets containing 10% cottonseed protein incorporating different levels of bound gossypol. The four preparations used contained 0·23, 0·71, 1·20 and 1·77% total gossypol and 0·01, 0·03, 0·07 and 0·12% free gossypol respectively. Bound gossypol up to a level of 0·68% had little effect on growth, but an adverse effect was apparent with bound-gossypol levels above 0·75–0·80%. Using diets with protein levels varying between 10 and 20%, it was shown that when the level of bound gossypol was raised from 0·445 to 1·30% an increase of at least 25% in protein consumption was required to maintain weight gain in the face of the additional gossypol. This effect probably reflects a reduction in the availability of amino acids following their binding to gossypol. It is calculated that cottonseed could be utilized more efficiently when fed to animals if its content of bound gossypol could be reduced to 0·75% or less.

THE CHEMICAL ENVIRONMENT

2558. Arsenate, arsenite and the embryo

Hood, R. D. & Bishop, Sally L. (1972). Teratogenic effects of sodium arsenate in mice. *Archs envir. Hlth* **24,** 62.

Hood, R. D. (1972). Effects of sodium arsenite on fetal development. *Bull. env. contam. & Toxicol. (U.S.)* **7,** 216.

Although arsenic is a widespread environmental contamination on account of the use of the element or its compounds in agriculture, paint and chemicals manufacture and smelting, the effects of arsenicals on mammalian development have not been extensively studied. The two papers cited above are concerned with the teratogenic effects of two arsenic compounds in Swiss–Webster mice.

Single ip injections of a barely sublethal dose (45 mg/kg) of sodium arsenate given to mice on one of days 6–12 of gestation produced a variety of malformations on the surviving foetuses and significantly increased foetal resorptions (especially when treatment was given on day 12). Except in mice treated on day 12, foetal weights were reduced, particularly after treatment on day 7 or 9. The malformations induced were also dependent on the time of treatment, and included exencephaly, shortening of jaws (micrognathia), exophthalmos, naked eye, umbilical hernia, shortened tail, and rib and vertebral defects. Moderate hydrocephalus was a common internal defect.

It has been thought that arsenate is reduced *in vivo* to arsenite before it exerts its toxic effect in animal tissues, and on this basis the teratogenic effects of the two types of compound would be expected to be similar. However this view has been challenged, on the grounds that *in vivo* the redox equilibria favour oxidation, making reduction to arsenite unlikely, and that arsenate and arsenite appear to exert their toxic effects in mammals by different mechanisms, arsenite by reaction with sulphydryl groups and arsenate by the uncoupling of oxidative phosphorylation and interference with phosphorus metabolism. Despite these differences in mode of action, sodium arsenite and sodium arsenate had similar teratogenic effects in mice, although the defects caused by the former were less numerous than those caused by a similarly toxic dose of sodium arsenate. Injections of arsenite (10 or 12 mg/kg) given ip to mice on one of days 7–12 of gestation resulted in a high rate of foetal death and decreased foetal weights. The most common malformations were exencephaly, micrognathia, open eye, tail defects and rib and vertebral abnormalities.

The similarity in the developmental defects produced by arsenate and arsenite and the fact that arsenite is effective at a much lower dose level than arsenate appear to support the original theory that arsenate is reduced to arsenite before it exerts its toxic action, but it is important to remember that similar terata can be caused by agents that differ markedly in mode of action but yet, in their different ways, damage actively dividing cells in the same area of the embryo at the same period of development.

2559. Factors controlling the carcinogenicity of metals

Webb, M., Heath, J. C. & Hopkins, T. (1972). Intranuclear distribution of the inducing metal in primary rhabdomyosarcomata induced in the rat by nickel, cobalt and cadmium. *Br. J. Cancer* **26,** 274.

Weinzierl, Susan M. & Webb, M. (1972). Interaction of carcinogenic metals with tissue and body fluids. *Br. J. Cancer* **26,** 279.

Webb, M. & Weinzierl, Susan M. (1972). Uptake of $^{63}Ni^{2+}$ from its complexes with proteins and other ligands by mouse dermal fibroblasts *in vitro*. *Br. J. Cancer* **26,** 292.

The carcinogenic potential of nickel compounds in rat and mouse muscle has been considered in a previous issue of *Food and Cosmetics Toxicology* (1969, **7,** 693). The type of tumour produced by intramuscular injection of this and related metals appears to be species-specific (*ibid* 1966, **4,** 212), the predominant type in rats, for example, being a primary rhabdomyosarcoma. The first work cited above examines the intranuclear distribution of powdered metallic carcinogens injected into rat muscle, to help elucidate the biochemical mechanisms underlying tumour production.

Following intramuscular administration of powdered nickel, cobalt or cadmium to rats, the greater part (70–90%) of the injected metal became associated with the nucleus. At least half of the nuclear material was found to be bound to the nucleolus, the remainder being taken up in equal proportions by the nuclear sap and chromatin. The affinity of the metals for these intranuclear fractions may lead to structural modifications which could affect nucleic acid function and protein synthesis.

It is well known that when carcinogenic metals are incubated with horse serum or rat-muscle homogenates, the metal dissolves and complexes with various ligands. In the second study cited above, horse serum was used as an incubation medium and it was demonstrated that both carcinogenic and also certain non-carcinogenic metals dissolved to form products complexed with both proteins and small diffusible molecules. Copper and cobalt had comparable dissolving properties, their solubilities being enhanced by oxygen, but the solubility of cadmium, zinc and nickel was unaffected by the presence of oxygen. The process of adsorption was held largely responsible for the uptake of oxygen by metallic cobalt. Cadmium, cobalt and nickel all dissolved readily when incubated with rat-muscle homogenate (and homogenates of other tissues), being bound in the main by a variety of small diffusible molecules, as is probably the case *in vivo*.

Since non-carcinogens also dissolved in both the media used, it was concluded that the carcinogenicity of nickel and related metals was associated with the biochemical effects of the dissolved element after intracellular incorporation. The extent of the reactivity of carcinogenic ligand-bound metals with mammalian cell types is the subject of the third report cited above.

Uptake of $^{63}Ni^{2+}$ by cultured mouse dermal fibroblasts was studied, using nickel complexed both with serum proteins and with small diffusible molecules from a rat-muscle autolysate. Intracellular incorporation was observed, the extent of binding to various fractions decreasing from a maximum in the nuclei, through cell sap and mitochondria to the microsomes, irrespective of the nature of the carrier. About half of the nuclear radioactivity was centred in the nucleoli, as had been found *in vivo* following nickel implantation. It was concluded that interference with nucleolar function was possibly a common feature of the action of metallic carcinogens *in vitro* and *in vivo*.

2560. Effect of manganese on the rabbit brain

Chandra, S. V. & Sur, R. N. (1970). Early brain changes in rabbits induced by manganese chloride. *Envir. Res.* **3,** 417.

Chandra, S. V. (1972). Histological and histochemical changes in experimental manganese encephalopathy in rabbits. *Arch. Tox.* **29,** 29.

Mustafa, S. J. & Chandra, S. V. (1972). Adenosine deaminase and protein pattern in serum and cerebrospinal fluid in experimental manganese encephalopathy. *Arch. Tox.* **28,** 279.

Epidemiological studies on workers exposed to manganese (Mn) have shown that the metal can induce certain neurological disorders in man (*Cited in F.C.T.* 1970, **8,** 468). Earlier studies in rats (*ibid* 1971, **9,** 300) suggested that damage to the brain cells of laboratory animals could be caused directly by soluble Mn, and work by the same group has now probed more deeply into the mechanism of Mn neurotoxicity by examining the sites at which the metal reacts with the brain of experimental animals.

The early signs of Mn poisoning were investigated in rabbits by injecting 0·5 mg Mn chloride in 0·1 ml normal saline directly into one of the lateral ventricles of the brain on alternate days for 2 wk. The convulsive state which resulted was monitored by electrocorticography, which showed an arousal pattern at a frequency of about 20 Hz. Convulsions did not occur in controls given only saline by the same technique. Animals were killed at intervals between 12 hr and 15 days. Those killed up to day 8 showed mild congestion of the brain, but after day 9 pinpoint haemorrhages appeared. Pathological changes observed under the light microscope included signs of neuronal degeneration in the cerebral and cerebellar cortices, together with small haemorrhagic areas visible in the pons and medulla oblongata. White matter and spinal cord tissue were generally devoid of pathological changes. Brain pathology of the saline-treated animals was normal.

Later, a more prolonged study was made of changes following the intratracheal inoculation of 400 mg Mn dioxide dust suspended in normal saline. After 18–24 months, the animals developed paralysis of the hind limbs and were killed. Chandra (cited above) reports that widespread neuronal degeneration and loss in the cerebral and cerebellar cortices were again evident, but the putamen, caudate nucleus and substantia nigra were also affected. Histochemical studies revealed markedly decreased acid-phosphatase and adenosine-triphosphatase activity in the degenerated neurones, although alkaline-phosphatase and 5′-nucleotidase activities were not affected. It was considered that the reductions in acid phosphatase and adenosine triphosphatase could stem from a disturbance of enzyme-protein catabolism or from destruction of the organelles responsible for synthesis of these two enzymes, namely the lysosomes and mitochondria, respectively.

In animals sacrificed after 24 months, adenosine deaminase activity was determined in cerebrospinal fluid (CSF), whole brain and serum (third paper cited above). Raised levels were found only in the CSF. Examination of this enzyme pattern was thus regarded as a potential basis for a clinical test for chronic Mn intoxication. Total protein patterns were also monitored. Enhanced globulin levels were responsible for a marked increase in serum protein in the Mn-treated rabbits. Serum albumen levels remained unaffected, but there was a marked reduction in the concentrations of both albumens and globulins in the CSF. It was thought that the increase in serum globulins might have been due to formation of a Mn-globulin complex, in line with the formation of caeruloplasmin by the complexing of copper with globulin.

[The extent to which these Mn-induced changes may be mirrored in man is largely unknown. Certain manifestations, such as the early period of excitement and the cerebral lesions following Mn administration, have been noted in both man and experimental animals, but there are conflicting reports about the effects of Mn on protein patterns.]

2561. Sensitivity to nickel

Fisher, A. A. (1972). Safety of stainless steel in nickel sensitivity. *J. Am. med. Ass.* **221,** 1279.

Kellum, R. E. (1972). Safety of stainless steel in nickel sensitivity. *J. Am. med. Ass.* **221,** 1282.

Hutchinson, F., Raffle, E. J. & Macleod, T. M. (1972). The specificity of lymphocyte transformation *in vitro* by nickel salts in nickel sensitive subjects. *J. invest. Derm.* **58,** 362.

Hypersensitization to nickel salts, estimated to occur in about 5% of the general population (*Cited in F.C.T.* 1965, **3,** 124), has been identified as a contributory factor in the incidence of cement eczema (*ibid* 1966, **4,** 544) and cases of contact dermatitis in housewives (*ibid* 1971, **9,** 760).

More recently, a dermatitic condition arising from the implantation of a stainless-steel screw into a patellar tendon of a nickel-sensitive patient was alleged to have been due to the nickel present in the steel screw at a level of 14%. This inference has, however, been disputed on several counts by the first two authors cited above. It is suggested that the condition observed could have been the result of primary irritation caused by the metal screw rather than an allergic reaction. In addition, it is stressed that the nickel present in stainless steel is so firmly bound that it cannot be detected by the dimethylglyoxime spot test used to determine "available" nickel, and cannot be leached out by body fluids or perspiration. Most nickel alloys apart from stainless steel give a positive result with dimethylglyoxime and induce reactions in nickel-sensitive patients. To substantiate his view, the first writer points out that stainless steel used in surgical equipment and, for example, in jewellery has never been shown by other workers to produce an allergic reaction. Kellum (cited above) supports this contention by pointing out that the reported appearance of a reaction within 4 hr of implantation seems incompatible with an allergic response of the delayed hypersensitivity type, which would require at least 12–18 hr to develop.

An *in vitro* investigation of delayed hypersensitivity induced by nickel salts, using the lymphocyte transformation test, is described by Hutchinson *et al.* (cited above). Transformation of peripheral lymphocytes from a sensitive donor is normally effected by addition of an appropriate antigen. This transformation can be measured by uptake of tritiated thymidine, an index of the capacity of the cells to synthesize DNA and undergo mitosis. Blood samples taken from eight subjects shown clinically and by patch testing to suffer from delayed hypersensitivity to nickel and from seven unaffected control subjects were used for lymphocyte transformation tests using nickel sulphate and nickel acetate as antigens at a level of 10^{-4} m-equiv. Ni/ml. Both salts specifically increased transformation in the blood samples from the sensitive individuals, the effectiveness of the stimulant action being similar with either salt, but no clues were revealed as to the mode of reaction between metallic salts and the antigen receptors on the lymphocytes.

2562. Triethylaluminium toxicity

Miura, T., Nakajima, T., Hara, I., Hashimoto, K., Kusumoto, S., Kanai, R. & Uda, K. (1972). Studies on the toxicity of triethylaluminium (TEA). *Int. Arch. Arbeitsmed.* **29,** 189.

There appear to have been no previous reports of experimental studies on the toxicity of triethylaluminium (TEA), which is used as a polymerization catalyst and as a rocket fuel.

The present studies were designed to investigate the effects of single inhalation exposures to TEA in mice, rats and rabbits and also to determine the effects of TEA applied directly to the skin of rabbits. In order to avoid spontaneous ignition, TEA/air mixtures were carefully prepared by diluting TEA with air and nitrogen before increasing the oxygen concentration to 20–21%. However, an unidentified 6-carbon combustion product of TEA in air was discovered on analysis of the mixture using gas-chromatographic techniques.

Results showed that exposure to a TEA–air or a TEA–toluene mixture for 30–90 min resulted in congestion, haemorrhage and oedema of the lungs in rats and mice. Blackish-brown particles were found in the bronchioles of most animals and in some animals these were also seen in the alveoli. Some leucocyte and histiocyte infiltration was evident. The greater severity of the TEA-toluene exposure was attributable to the toluene component. When two drops of TEA–toluene (1:4, v/v) solution were applied to the shaved skin of rabbits, severe scaling and oedema of the skin occurred. No abnormalities were seen on examination of the urine of experimental animals and determinations for methaemoglobinaemia proved negative.

The authors conclude that these results are comparable to some extent with reports of adverse effects in some workers handling TEA. Clinical symptoms seen in such patients have included fever, blood-tinged sputum, haemorrhagic cystitis and haemorrhagic inflammation of the upper respiratory tract, together with dizziness and headache.

2563. Skin reaction to nigrosine in carbon paper

Calnan, C. D. & Connor, B. L. (1972). Carbon paper dermatitis due to nigrosine. *Berufsdermatosen* **20,** 248.

Nigrosine (C.I. (1956) no. 50415B; Solvent Black 7) is a complex mixture of substances prepared by heating nitrobenzene and aniline with iron or copper at 180–200°C. 'Nigrosine spirit soluble' (Solvent Black 5) is the equally complex product of heating nitrophenol or nitrocresols, aniline and aniline hydrochloride with the same metals at the same temperature. Nigrosine base constitutes about 0·5% of special carbon copying papers used in computer printers.

A civil servant who was in the habit of handling a special computer carbon paper containing nigrosine developed seborrhoeic dermatitis of the nasolabial folds, eyebrows, forehead and ears, and later suffered from contact dermatitis of the hands and fingers. Patch testing of the paper was positive, but testing of the various individual ingredients (carbon black, a printing-ink oil, two waxes and two dyes) showed a positive reaction only to nigrosine (1% in petrolatum).

Contact dermatitis to coated carbon paper is very rare. The authors suggest that it is probably the soiling of the fingers which suggests to many office workers who develop a hand dermatitis that carbon paper is the cause of their affliction.

[In view of the complex mixture which is called nigrosine, further breakdown of the ingredients to determine which compound is responsible may be indicated.]

2564. Patch testing of a cetylpyridinium salt

Kleinhans, D. (1972). Epikutantestungen mit Cetylpyridiniumchlorid. *Berufsdermatosen* **20,** 217.

A comparison of patch testing with 1% cetylpyridinium chloride in three different vehicles,

anhydrous eucerin, yellow petrolatum and water, demonstrated a high proportion of primary irritancy which varied with the vehicle, being greatest with water and least with eucerin. Thus in a group of patients attending a skin clinic, reddening of the skin occurred in only 8·2% of the subjects when eucerin was used as the vehicle, but the incidence rose to 50·5 and 86·8% when the material was applied in petrolatum and in water respectively. Aqueous solutions of 0·8 or 0·4% cetylpyridinium chloride produced a papular-vesicular reaction in a group of six patients, and caused erythema at a concentration of 0·2% in all 20 of a group of healthy controls. Only two of these were affected (by a very slight reddening) at 0·1% however. With a 0·8% concentration in eucerin, only one control reacted within 48 hr, while 14 of 20 reacted to 0·2 and 0·8% in petrolatum. None reacted to 0·1% in this base.

These findings indicate that in tests for allergic sensitivity to cetylpyridinium chloride a concentration of 0·1% in petrolatum is suitable, followed if necessary by the cautious application of 0·1% in water if there is no reaction to the first test.

2565. Renal response to dieldrin and methylmercury

Fowler, B. A. (1972). The morphologic effects of dieldrin and methyl mercuric chloride on pars recta segments of rat kidney proximal tubules. *Am. J. Path.* **69,** 163.

As environmental contaminants both dieldrin and methylmercury (MeHg) are widespread and can be concentrated in biological systems. Many studies of dieldrin toxicity have been concerned with its effects on the liver, while toxicological investigations of MeHg have tended to concentrate on changes in the central nervous system. Nevertheless, both dieldrin and MeHg accumulate in the kidney, the latter after its conversion, at least in part, to inorganic mercury, which is highly toxic to the proximal tubules.

The nephrotoxic action of these two chemicals given separately and in combination has now been studied in rats over a period of several weeks. Groups of rats were given a diet containing dieldrin (5 ppm) or MeHg (2 ppm Hg as methylmercuric chloride) or both compounds together at these same levels. Half the treated animals were killed after 84 days and half after 142 days on the test diet, and kidneys were sectioned for light and electron microscopy.

Under these conditions, dieldrin caused no histological changes in the kidney, but ultrastructurally the smooth endoplasmic reticulum (SER) in the proximal tubular cells was increased. This occurred in both sexes but males showed a greater relative increase in SER aggregates than females. In rats given MeHg, histological changes were found only in females and consisted of a flattening of the cells of the proximal tubules with consequent dilatation of the lumen, which contained blue-staining masses in haematoxylin–eosin sections. These masses consisted of SER aggregates, and a considerable increase in SER and dense bodies (lysosomes) was seen in the flattened cells. Ultrastructural changes in males were limited to SER hypertrophy in the proximal cells. Following simultaneous treatment with both dieldrin and MeHg, no changes could be seen by light microscopy. Electron microscopy revealed virtually no changes in males, and only SER hypertrophy in the females.

All the changes observed were present at both 84 and 142 days, but were more severe at the latter stage. However, the severity of the effects depended more on the sex of the treated animals than on the duration of treatment. The finding that simultaneous administration

of dieldrin counteracted the pathological changes caused in female rats by MeHg is probably related to the induction of drug-metabolizing enzymes. Dieldrin induces such enzymes in the liver and may also do so in the kidney, particularly since the proximal tubular cells of dieldrin-treated animals showed pronounced SER hypertrophy, a change known to accompany hepatic microsomal-enzyme induction by dieldrin (Hutterer *et al. Science, N.Y.* 1968, **161,** 1017). Similarly, the pronounced difference in the responses of males and females to dieldrin and MeHg may be due to sex differences in the levels of drug-metabolizing enzymes in the kidney tubules.

2566. Thyroid tumours from ETU

Ulland, B. M., Weisburger, J. H., Weisburger, Elizabeth K., Rice, J. M. & Cypher, R. (1972). Thyroid cancer in rats from ethylene thiourea intake. *J. natn. Cancer Inst.* **49,** 583.

Ethylene thiourea (ETU) is of interest to the toxicologist not only because it is used as an accelerator in the compounding of rubber but also because it is a breakdown product of ethylene bisdithiocarbamates, which are used as fungicides. In a comprehensive screening programme on a large number of pesticides and other chemicals, ETU produced liver tumours (*Cited in F.C.T.* 1970, **8,** 229), but this could not be considered conclusive evidence. ETU and related compounds have also been associated with the production of thyroid cancer in rats, and preliminary reports of an FDA feeding study (*Food Chemical News* 1971, **13** (26), 17) revealed a high incidence of thyroid carcinoma in rats fed a dietary level of 500 ppm, a lower carcinoma incidence at 250 ppm and nodular hyperplasia of the thyroid at 125 ppm. At a later stage (*ibid* 1972, **14** (34), 29) rats fed 'low dietary levels' were reported to be still free from thyroid tumours.

A brief report has now been published of another study in which technical grade ETU was fed to Charles River rats at dietary levels of 350 and 175 ppm for 18 months. At the end of this period, five males and five females from the group on the higher dose level were killed and the rest were maintained on a control diet for a further 6 months, unless they became moribund earlier. Each group consisted of 26 males and 26 females. In the group given 350 ppm ETU, thyroid carcinoma was found in 17 males and eight females, with pulmonary metastases in two males. Three males and three females on the lower dietary level also had thyroid carcinoma. The first tumour was found at wk 68 in the 350 ppm group.

In the same (350 ppm) group, 17 males and 13 females had hyperplastic goitre with extreme thyroid enlargement, and it is possible that serial sections of these glands might have revealed further carcinomas. Hyperplastic goitre also occurred in nine males and six females of the other test group. In addition, there was a scattered incidence of solid-cell adenoma and a simple goitre, and also of hyperplastic liver nodules, but no liver tumours were seen. Apart from one simple goitre, none of these lesions occurred in a large group of control animals.

2567. More diphenyl metabolites

Raig, P. u. Ammon, R. (1972). Nachweis einiger neuer phenolischer Stoffwechselprodukte des Biphenyls. *Arzneimittel-Forsch.* **22,** 1399.

An *in vitro* study on the metabolism of diphenyl in a range of mammalian and non-mammalian species showed that all eleven species converted the compound principally to

4-hydroxydiphenyl, while only a few, including mice, young rats and rabbits, hamsters and cats, also produced the 2-hydroxy derivative (*Cited in F.C.T.* 1966, **4,** 225). The 3,4- and 4,4′-dihydroxy derivatives have also been reported, but since then gas-chromatographic analysis of urine from rabbits given diphenyl orally has revealed the existence of three previously unreported metabolites (Raig & Ammon, *Arzneimittel-Forsch.* 1970, **20,** 1266).

Of these, one has now been identified by gas chromatography and infra-red spectrophotometry as 3-hydroxydiphenyl, and the others, previously isolated as a single metabolite, as 3-hydroxy-4-methoxy- and 4-hydroxy-3-methoxydiphenyl. These last two metabolites probably result from *O*-methylation of 3,4-dihydroxydiphenyl.

2568. Species variations in phenol metabolism

Capel, I. D., French, M. R., Millburn, P., Smith, R. L. & Williams, R. T. (1972). Species variations in the metabolism of phenol. *Biochem. J.* **127,** 25P.

Notable species variations found in the metabolism of several simple organic compounds prompted these investigators to study the degradation of phenol in 19 animal species. Animals were dosed orally or ip with [^{14}C]phenol (usually 25 mg/kg), and urine was collected over 24 hr and analysed by paper chromatography and radiochromatogram scanning.

A wide species variation ranging from 30 to 95% was observed in the radioactivity recovered in the urine. The lower and upper extremes were found in the squirrel monkey and the rat, but in most other species the urinary recovery was in the 50–70% range. Eight of the species, including the rat, mouse, guinea-pig and man, excreted the sulphate and glucuronic acid conjugates of both phenol and quinol, but six other species, including the squirrel and capuchin monkeys and the ferret, dog and rabbit, excreted only three of these metabolites in significant amount. All of this group excreted phenyl sulphate and phenyl glucuronide: the two New World monkeys showed some predominance of glucuronide over sulphate conjugation and excreted quinol glucuronide as the third metabolite, but quinol sulphate was the third major excretory product in the other four species, which varied in their utilization of the two conjugation mechanisms and in the extent of phenol oxidation to quinol. Of the remaining species, the cat utilized the glucuronic acid mechanism only to a very small extent, while the pig excreted over 90% of the total 24-hr ^{14}C excretion as phenyl glucuronide.

2569. PEG effects of vasopressor responses

Heilman, R. D., Bauer, E. W. & DaVanzo, J. P. (1972). Effect of polyethylene glycol on the cardiovascular response of the dog to autonomic agents. *Toxic. appl. Pharmac.* **23,** 263.

Water-soluble, moderately viscous, colourless polyethylene glycols (PEG) have solvent properties that make them obvious candidates for use as vehicles in pharmacological studies.

However, while PEGs 200, 300, 400 and 600, which fit into this category, were found to have no direct effect on the blood pressure of anaesthetized dogs, they did enhance significantly the vasopressor response of the animals to adrenaline and noradrenaline, and to a lesser degree to acetylcholine. In the case of PEG 600, the enhancement of pressor sensitivity to the two catecholamines appeared to be due to a direct cardiac action and not to a

peripheral one, whereas the effect on response to acetylcholine was mediated by a peripheral vascular mechanism.

These observations indicate that PEGs may be unsuitable as vehicles for compounds that are to be injected iv in the course of studies on their effects on peripheral autonomic or cardiovascular systems.

NATURAL PRODUCTS

2570. Metal contamination of alcoholic drinks

Reilly, C. (1972). Zinc, iron and copper contamination in home-produced alcoholic drinks. *J. Sci. Fd Agric.* **23,** 1143.

During recent years, the high incidence of oesophageal cancer in certain sharply defined areas of Africa (*Cited in F.C.T.* 1970, **8,** 337) has focussed attention on locally prepared foods and drinks as possible sources of carcinogens. Popular targets for research have been the alcoholic beverages, home-brewed and home-distilled, which differ widely in different localities because of the variety of starting materials and methods used in their preparation. Earlier reports of nitrosamines in locally distilled spirits may have been due to analytical problems (*ibid* 1972, **10,** 883) but other studies have pointed to a possible association of a high incidence of oesophageal cancer with local beers fermented from maize rather than from millet, sorghum or bananas (*ibid* 1972, **10,** 883), with certain epoxide compounds in some potable spirits (*ibid* 1973, **11,** 165) and with the presence of some trace metal contaminant (McGlashan, *Lancet* 1967, **i,** 578).

In many African localities, oil drums and other metal containers have largely replaced the clay pots and gourds traditionally used for the domestic brewing of alcoholic drinks. This change has led to the contamination of drinks with heavy metals leached from the containers by the action of acids produced during the fermentation. Distillation is not always effective in removing the metals. In a series of analyses carried out on various African home-produced alcoholic drinks, metal contamination was largely independent of the starting materials and was highest in beers and spirits produced in galvanized metal drums, the levels of copper, zinc and iron in such drinks being in the ranges of 0·1–8·3, 1·6–19·4 and 2·5–140 ppm, respectively. Lead contamination of the drinks was rare.

Home-brewed drinks from other countries also showed evidence of metal contamination, as did some commercial alcoholic products. Three samples of home-brewed alcoholic drinks from Ireland were found to contain 7·0–26·2% copper, for example, and a Spanish commercial sherry contained 0·3 ppm copper, 7·5 ppm zinc and 2·5 ppm iron. A high level of metallic contamination has also been reported in illicit spirits drunk in the USA (Hoffmann *et al. J. Ass. off. analyt. Chem.* 1968, **51,** 580).

[While the evidence for heavy-metal contamination of drinks prepared in unsuitable equipment may be building up, there is as yet no evidence linking the ingestion of these metals with tumour induction.]

2571. Effect of culinary processes on bracken carcinogenicity

Hirono, I., Shibuya, C., Shimizu, M. & Fushimi, K. (1972). Carcinogenic activity of processed bracken used as human food. *J. natn. Cancer Inst.* **48,** 1245.

The carcinogenic effects of bracken in rats, mice and farm animals are well documented (*Cited in F.C.T.* 1971, **9,** 920; *ibid* 1972, **10,** 603) and it has been suggested that the use of bracken as a food in Japan may be a factor in the high incidence of stomach cancer in the population of that country. The young bracken fronds generally used for human food are processed before use by various procedures involving immersion in boiling water. The work now reported is an extension of a previous study of the relative carcinogenicity of unprocessed and processed brackens.

One group of rats was fed unprocessed bracken, while other groups were given bracken processed in one of three popular ways, namely with boiling water containing wood ash, with boiling water containing sodium bicarbonate or with boiling water alone after storage of the bracken in sodium chloride. The rats, which were 1–1·5 months old at the start of the study, were fed the test diets for 4 months and then given a standard diet until the experiment was terminated about 16 months from the start. A control group was given the standard diet throughout. Two further groups were treated for 9 months, one with the bracken stored in sodium chloride and one with the basal diet containing added sodium chloride.

The results indicated that the carcinogenic activity of the processed bracken was much lower than that of the unprocessed material, although some activity was retained even after processing. The incidence of intestinal tumours in rats fed the unprocessed plant was 78·5%, compared with 25, 10 and 5% in the groups fed bracken treated with wood ash, sodium bicarbonate and sodium chloride, respectively. In the groups given the salted bracken, the incidence was not increased by the longer period of feeding. No intestinal tumours occurred in groups given only the basal diet, with or without sodium chloride. Few of the intestinal tumours seen in the test group were located more than 20 cm from the ileocaecal junction and a high proportion were found in the terminal 3 cm of the ileum. While the rats fed unprocessed bracken generally developed multiple tumours, those on the processed forms had single tumours, almost all of which were adenomas.

2572. Veratrum effects on the foetus

Binns, W., Keeler, R. F. & Balls, L. D. (1972). Congenital deformities in lambs, calves, and goats resulting from maternal ingestion of *Veratrum californicum*: Hare lip, cleft palate, ataxia, and hypoplasia of metacarpal and metatarsal bones. *Clin. Toxicol.* **5,** 245.

In some areas, grazing can be a hazardous business for sheep and other domestic animals, particularly in relation to foetal development. It is well known that not only locoweed (*Cited in F.C.T.* 1973, **11,** 344) but also the plant known as *Veratrum californicum* (*ibid* 1970, **8,** 86) contains teratogenic components, and a wide range of effects induced by the latter plant has now been reported. Consumption of veratrum by ewes has already been reported to cause cyclopian deformities, which are indicative of a selective inhibition of mitosis at the neural plate stage of embryogenesis. These and other types of deformity are

correlated in the paper cited above with the gestation time at which the dried roots and leaves of the plant were fed to the test animals.

In ewes fed veratrum roots (50–70 g), roots and leaves (125–150 g) or leaves (130–140 g) on 2–6 days between days 14 and 36 of gestation there was an overall incidence of deformities in lambs of about 35%. Cleft palate was a common finding in lambs whose dams were fed veratrum on days 25–30 of gestation and bilateral harelip occurred less frequently in this group. Shortening of the metacarpals and metatarsals and shortening and thinning of body bones was associated with treatment between days 25 and 36, and some of the lambs from dams fed veratrum on days 25–30 showed multiple deformities particularly involving the skeleton. All lambs showing cyclopian deformity were from ewes treated around day 14 of gestation.

In goats fed veratrum leaves (80–100 g) on days 13–15 there were two abortions among five pregnancies, and examination of the remaining pregnancies at 60 days revealed two sets of twin foetuses with cyclopian deformity and one degenerated dead embryo.

Two calves from dams fed veratrum leaves (220–340 g) from days 12 to 34 of gestation showed posterior motor-nerve paralysis, similar to that seen in two lambs from ewes treated on days 17–18. Cyclopian deformity was found in two aborted foetuses exposed to veratrum between days 8 and 25.

The teratogenic effects of veratrum are therefore not confined to the previously observed cyclopian deformities, but are likely to include skeletal abnormalities as well as motor-nerve lesions if exposure occurs at a somewhat later stage of development.

COSMETICS, TOILETRIES AND HOUSEHOLD PRODUCTS

2573. Photosensitizing potential of cosmetics components

Schorr, W. F. (1971). Cosmetic allergy. A comprehensive study of the many groups of chemical antimicrobial agents. *Archs derm.* **104,** 459.

Kligman, A. M. (1972). Allergenic aromatic amines. *Archs Derm.* **105,** 459.

The cosmetics chemist faces many problems in connexion with the formulation and marketing of new products. One of these problems is the prevention of microbial deterioration of cosmetic formulations, and the suitability and safety-in-use of available preservatives therefore demands much consideration. Schorr (cited above) has reviewed the literature on the sensitization potential of several antimicrobial preservatives, comparing the sensitization index (SI) of each preservative with a standard index of 0·8% determined for parabens by the author in 1968.

In general, it appears that the following SIs can be applied to the major chemicals used as cosmetics preservatives. The SI for 2% solutions of sorbic acid is approximately 0·5%. Furthermore, sorbic acid can be used in the presence of nonionic surfactants, which are incompatible with several other preservatives. Hexachlorophene is a relatively weak sensitizer with an SI of 0·3%, whereas some other phenolics and formaldehyde pose greater potential hazards, the SI for formaldehyde being as high as 5%. The dermatological hazards of mercurials as allergens and irritants can be considered greater than those associated with

parabens, sorbic acid and phenolics. Quaternary ammonium compounds and ethylenediaminetetraacetic acid are classed as weak sensitizers. Dimethoxane has recently been reported to have a (Draize test) sensitization potential of 25%, which would counteract the value of its effectiveness in the presence of nonionic surfactants. The author comments on the failure of cosmetics manufacturers to list the ingredients of their preparations on the container. Such a practice would assist the dermatologist in identifying the sensitizer in cases of contact dermatitis and would give consumers a chance to avoid preparations containing materials to which they are sensitive.

Kligman (second paper cited above) considers the difficulties encountered by manufacturers in predicting the incidence of sensitization to new products in the general population. He cites the case of hydro-alcoholic solutions of aminobenzoic acid, which act as an excellent sunscreen particularly in cases of light-sensitive dermatoses. In small-scale experimental studies using the maximization test, 20% aminobenzoic acid in petrolatum produced no reaction in 25 subjects tested, and in extensive testing carried out over a number of years, no cases of contact sensitization were encountered. However, since formulations based on this material have been marketed, several cases of sensitivity to aminobenzoic acid have been reported. In such situations, a decision about use must be based on the risk-benefit ratio.

METHODS FOR ASSESSING TOXICITY

2574. Species and site variations in percutaneous absorption

Maibach, H. I., Feldmann, R. J., Milby, T. H. & Serat, W. F. (1971). Regional variation in percutaneous penetration in man. Pesticides. *Archs envir. Hlth* **23,** 208.

Bartek, M. J., LaBudde, J. A. & Maibach, H. I. (1972). Skin permeability *in vivo*: Comparison in rat, rabbit, pig and man. *J. invest. Derm.* **58,** 114.

These two papers describe studies designed to compare the readiness with which applied substances are absorbed through various types of skin. In the first paper cited, the comparison is between the skin of different areas of the human body, while the second compares percutaneous absorption in several species, including man.

A pesticide (parathion, malathion or carbaryl) labelled with ^{14}C, was applied to the skin of one of thirteen regions of the body of male volunteers. The dose, applied at a level of 4 $\mu g/cm^2$, the vehicle and other factors were kept constant throughout, the site of application being the only variable. The treated sites were not occluded and were not washed for 24 hr. Urine was collected for 5 days, the samples for day 1 being collected separately for three 4-hr periods and one 12-hr. Absorption was estimated from the percentage of the dose of radioactivity found in the urine in 5 days, approximately corrected for incomplete urinary recovery as determined from an iv dose. The absorption at each site was related to that on the forearm.

About 8·6% of the applied dose of parathion was absorbed from the forearm site and slightly greater penetration was recorded for the ball of the foot and palm of the hand. Twice as much absorption occurred on the abdomen and back of the hand as on the forearm, four times as much on the scalp, at the angle of the jaw, in the postauricular area and on the forehead and seven times as much in the axillary region. The scrotum allowed almost

total absorption. With malathion, absorption from the forearm site was slightly less (6·8%) than with parathion, penetration through the palm and the ball of the foot was no higher than that on the forearm, and axillary absorption was only four times higher. Carbaryl was almost totally absorbed on the forearm, so meaningful comparison with other sites could not be made. Two main points emerged from this study: despite the greater thickness of its stratum corneum, the skin of the palm of the hand and ball of the foot presented no greater barrier to the penetration of these insecticides than the forearm skin, and the more permeable skin areas tended on the whole to be those richly supplied with hair follicles.

The second paper cited compares the percutaneous absorption of six substances in the rat, rabbit, miniature pig and man. The compounds used were haloprogin, *N*-acetylcysteine, cortisone, testosterone, caffeine and butter yellow, labelled with ^{14}C or ^{35}S. Only the first two were tested in man in this study, previously published data (Feldmann & Maibach, *J. invest. Derm.* 1969, **52**, 89; *idem, ibid* 1970, **54**, 399) being used for the other compounds. The compounds were applied in acetone in a dose of 4 $\mu g/cm^2$, to the ventral surface of the forearm for all the human studies and to the clipped backs of rats and rabbits and the clipped and chemically depilated backs of pigs. A non-occlusive foam pad was used for the rabbits and pigs and a metal ring sealed to the skin with petroleum jelly for the rat. Radioactivity was estimated in urine samples collected for 5 days after application of the compound, and was corrected for incomplete recovery as described above. The conditions of the test were not strictly comparable throughout, as the human volunteers were allowed to wash the application site after 24 hr, whereas in the other species the sites were left undisturbed for the full 5 days. However, in all cases, at least 80% of the total urinary radioactivity was found in the first 24-hr sample.

With the exception of *N*-acetylcysteine, the penetration of which was very low in all species, percutaneous absorption of all the compounds was greatest in the rabbit. Absorption in the pig was closest to that in man, but was generally slightly greater, and that in the rat was between the values for the pig and rabbit. The authors conclude, therefore, that in some respects the miniature pig is a more suitable animal than the rabbit for tests to be used as a basis for predicting dermal toxicity in man.

[From a consideration of these two papers together, however, a different conclusion might be drawn. The first paper demonstrates that absorption occurs much less readily through the skin of the human forearm, which is frequently used for skin tests, than through the skin of various regions of the head, where cosmetics are most used. The use of occlusive or semi-occlusive patches in human patch testing may compensate for this difference to some extent, but as far as animal testing is concerned, the data from these two papers indicate that skin absorption in the human head regions approximates most closely to that demonstrated on the rabbit back. On this basis, therefore, the rabbit rather than the pig seems the more suitable test animal for cosmetics products.]

BIOCHEMICAL PHARMACOLOGY

2575. Enzyme inhibition by piperonyl butoxide and related compounds

Friedman, M. A., Arnold, E., Bishop, Y. & Epstein, S. S. (1971). Additive and synergistic inhibition of mammalian microsomal enzyme functions by piperonyl butoxide, safrole and other methylenedioxyphenol derivatives. *Experientia* **27**, 1052.

Conney, A. H., Chang, R., Levin, W. M., Garbut, A., Munro-Faure, A. D., Peck, A. W. & Bye, A. (1972). Effects of piperonyl butoxide on drug metabolism in rodents and man. *Archs environ. Hlth* **24,** 97.

Several synthetic methylenedioxyphenyl (MDP) derivatives, such as piperonyl butoxide (PB), are used as insecticide synergists and act by inhibiting the microsomal enzymes responsible for metabolizing certain pesticides in insects (*Cited in F.C.T.* 1970, **8,** 714). Recently, concern has been expressed that by exerting a similar effect on mammalian microsomal enzymes, these MDP derivatives may inhibit the detoxication of a variety of foreign compounds, including environmental pollutants, drugs and carcinogens in man and other mammals.

The first report cited above investigates the possibility that PB and similar pesticide synergists interact with low levels of MDP derivatives already present in foodstuffs to effect additive or synergistic inhibition of liver microsomal enzymes. Low doses (10 mg/kg) of PB, methyl eugenol (ME), safrole (SAF), methylenedioxyaniline (MDA), and vanillylamine (VA) were injected ip into male albino mice, either separately or in various combinations at the same dose levels. The mice were killed 1 hr after treatment and the livers were used to prepare microsomal suspensions for enzyme assays.

As the number of compounds injected together was increased, the activity of the microsomal enzyme biphenyl 4-hydroxylase decreased in a linear fashion. The additive effect of the compounds was less clear with aminopyrine demethylase, but with this enzyme a synergistic inhibition clearly occurred when PB and SAF were injected together in doses of 50 and 10 mg/kg respectively. No such synergistic effect on aminopyrine demethylase occurred when PB was given with the structurally related but inactive piperonylic acid (PA). It was concluded therefore that the inhibitory interactions seen with these compounds depended on functional rather than structural factors.

The second study cited above was undertaken to ascertain whether current levels of PB used in crop-spraying present a toxicological hazard to workers in contact with the spray. The inhibitory effect of PB was gauged by its effect on the metabolism of a potential carcinogen, benzo[*a*]pyrene, and on antipyrine metabolism. In rats, effects were obtained only at high doses, but mice proved to be far more sensitive. An ip dose of 1000 mg/kg caused only a slight depression in the biliary excretion of benzo[*a*]pyrene metabolites in rats and a PB dose of 100–500 mg/kg was required to produce a significant inhibition of antipyrine metabolism in this species, but a single oral or ip dose of as little as 1 mg PB/kg was sufficient to cause a marked inhibition of antipyrine metabolism in the mouse. Prolongation of the action of barbiturates and of zoxazolamine, another index of the inhibitory action of PB, also showed marked variation between these species, and direct studies in man were therefore undertaken. PB was given to normal volunteers in a single oral dose of 50 mg (equivalent to a mean dose of 0·71 mg/kg). This dose level, which was estimated to be more than 50 times greater than the daily exposure received by individuals who spray this compound, did not affect antipyrine metabolism, and it was therefore concluded that the inhibitory effect of PB on human microsomal enzymes was unlikely to constitute a health hazard at present levels of exposure.

2576. Mechanism of enzyme induction by cortisone

Agarwal, M. K. (1972). Lack of correlation between RNA synthesis and hormonal induction of liver enzymes in carbon tetrachloride treated mice. *Experientia* **28,** 259.

It has recently been postulated that in the mammalian liver the adrenal glucocorticoid hormones act by primary stimulation of hepatic RNA synthesis, which is then followed by enzyme induction (Schmid *et al. Biochim. biophys. Acta* 1967, **134**, 80). The present report investigates this mechanism by using carbon tetrachloride (CCl_4) to modify the induction of particular enzymes in mouse liver and noting whether such modification can occur independently of hormonally increased RNA synthesis.

In mice, sc injection of CCl_4 alone, or together with the hormone cortisone, inhibited precursor ([^{3}H]orotic acid) incorporation into RNA within 3 hr, whereas cortisone alone increased the rate of RNA synthesis. Some 4 hr after administration of CCl_4 alone, the activity of tryptophan oxygenase (TO) was largely unchanged and that of tyrosine transaminase (TT) was significantly increased in intact mice. After 20 hr, however, TT levels were nearly normal and TO was almost completely eliminated. Thus, in the early stages of the reduction in RNA synthesis induced by CCl_4, the release of endogenous corticoids, also mediated by CCl_4, permitted normal homeostasis of TO and some induction of TT, although both processes were adversely affected later.

The induction of both TO and TT by cortisone proceeded without interference 4 hr after CCl_4 was administered simultaneously with the hormone, but when RNA synthesis had been suppressed by treatment with CCl_4 for 16 hr prior to cortisone administration, the hormone failed to induce either enzyme. This indicates that some kind of RNA synthesis is required for enzyme induction, although induction of selected enzymes by a corticosteroid can occur at a time when the stimulatory effect of the hormone on total RNA synthesis is completely eliminated.

These results contradict the idea of a mass-action effect on enzyme synthesis due to a greatly increased accumulation of total RNA resulting from the hormone administration. One idea presented is that cortisone may induce the production of specific mRNA species for each enzyme as a forerunner of enzyme induction and general ribosomal RNA synthesis. Thus, the fact that TT, but not TO, is induced by cortisone after CCl_4 administration may mean that the former enzyme has a more stable mRNA species.

Fd Cosmet. Toxicol. Vol. 11, p. 711. Pergamon Press 1973. 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:*

Short-term toxicity of amyl cinnamic aldehyde in rats. By F. M. B. Carpanini, I. F. Gaunt, M. G. Wright, P. Grasso and S. D. Gangolli.

Short-term toxicity of isoamyl alcohol in rats. By F. M. B. Carpanini, I. F. Gaunt, Ida S. Kiss, P. Grasso and S. D. Gangolli.

Long-term toxicity of sodium cyclamate in mice. By P. G. Brantom, I. F. Gaunt and P. Grasso.

Effect of waxy corn starch modification on growth, serum biochemical values and body composition of Pitman–Moore miniature pigs. By T. A. Anderson, L. J. Filer, Jr., S. J. Fomon, D. W. Andersen, R. L. Jensen and R. R. Rogers.

Long-term feeding and reproduction studies on Emulsifier YN in rats. By P. G. Brantom, I. F. Gaunt, Joan Hardy, P. Grasso and S. D. Gangolli.

Metabolism of the phenolic antioxidant 3,5-di-*tert*-butyl-4-hydroxyanisole (Topanol 354). I. Excretion and tissue distribution in man, rat and dog. By J. W. Daniel, T. Green and P. J. Phillips.

Metabolism of the phenolic antioxidant 3,5-di-*tert*-butyl-4-hydroxyanisole (Topanol 354). II. Biotransformation in man, rat and dog. By J. W. Daniel, T. Green and P. J. Phillips.

Metabolism of the phenolic antioxidant 3,5-di-*tert*-4-hydroxyanisole (Topanol 354). III. The metabolism in rats of the major autoxidation product, 2,6-di-*tert*-butyl-*p*-benzoquinone. By J. W. Daniel, T. Green and P. J. Phillips.

Lipid and enzyme changes in the blood and liver of monkeys given butylated hydroxytoluene and butylated hydroxyanisole. By A. L. Branen, T. Richardson, M. C. Goel and J. R. Allen.

The formation of carcinogenic nitroso compounds from nitrite and some types of agricultural chemicals. By R. K. Elespuru and W. Lijinsky.

Induction of liver and lung tumours in rats by the simultaneous administration of sodium nitrite and morpholine. By P. M. Newberne and R. C. Shank.

Dietary copper and the induction of neoplasms in the rat by acetylaminofluorene and dimethylnitrosamine. By W. W. Carlton and Peggy S. Price.

Hyperplasia of hepatic bile ducts in mice following long-term administration of butylated hydroxytoluene. By N. K. Clapp, R. L. Tyndall and R. B. Cumming. (Short Paper).

Dimethylnitrosamine in Chinese marine salt fish. By Y. Y. Fong and W. C. Chan. (Short Paper).

The effect of pH on dimethylnitrosamine formation in human gastric juice. By R. P. Lane and M. E. Bailey. (Short Paper).

Safety regulation in the real world. By P. B. Hutt. (Review Paper).

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

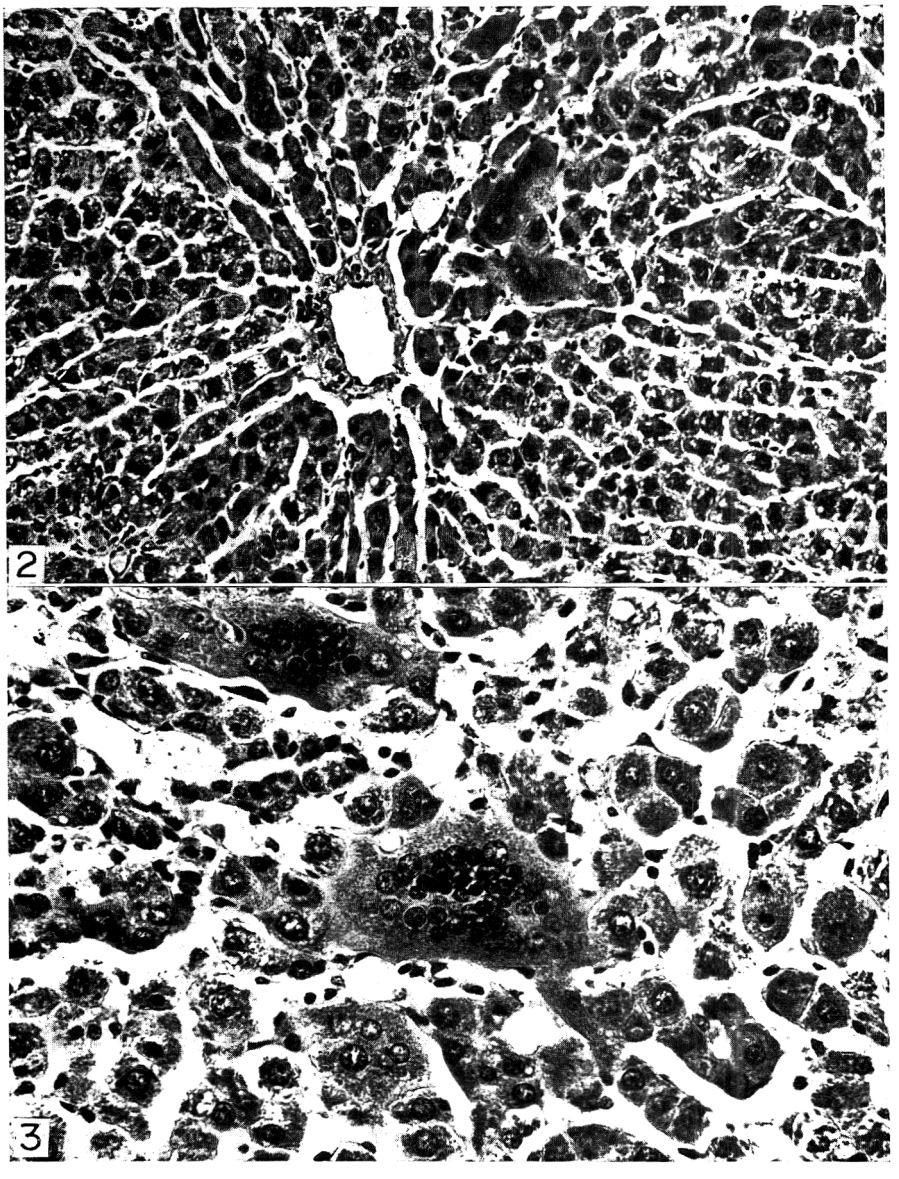

FIG. 2. Liver taken 60 days after treatment from a rat given an oral dose of 100 μgdioxin/ kg, showing thickening of the wall of the central vein and two multinucleate cells. Haematoxylin and eosin $\times$200.

FIG. 3. Multinucleate cells in rat liver 60 days after oral administration of 100 μg dioxin/kg. Other features illustrated are variation in cell and nuclear size and the presence of parenchymal-cell mitoses in these livers Haematoxylin and eosin $\times$380.

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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.

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e.g. Hickman, J. R., McLean, D. L. A. & Ley, F. J. (1964). Rat feeding studies on wheat treated with gamma-radiation. I. Reproduction. *Fd Cosmet. Toxicol.* **2**, 15.

References to books should include the author's name followed by initials, year, title of book, edition, appropriate page number, publisher and place of publication:

e.g. Dow, E. & Moruzzi, G. (1958). *The Physiology and Pathology of the Cerebellum.* 1st Ed., p. 422. The University of Minnesota Press, Minneapolis.

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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.