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／# FOOD AND COSMETICS TOXICOLOGY

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Research Section

LONG-TERM TOXICITY OF INDIGO CARMINE IN MICE

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(*Received 29 August 1974*)

Abstract—Groups of 30 male and 30 female mice were fed diets containing 0·2, 0·4, 0·8 or 1·6% indigo carmine for 80 wk. A group of 60 male and 60 female mice served as controls. The treatment had no effects on the death rate, body-weight gain, organ weights or the results of the histopathological examination, including the incidence of tumours. There was a slight anaemia in mice given diets containing 0·8 or 1·6% indigo carmine. It is concluded that the feeding of indigo carmine to mice at dietary levels of up to 1·6% did not exert any carcinogenic effect. The no-untoward-effect level in this study was 0·4% of the diet.

INTRODUCTION

Indigo carmine, also known as Indigotine or FD & C Blue No. 2 (C.I. (1971) no. 73015; EEC no. E132) is the disodium salt of 1-indigotin-5,5′-disulphonic acid. It is permitted for use in food in the UK at present (Statutory Instrument 1966, no. 1203) and this use may continue after July 1974 (SI 1973, no. 1340). Indigo carmine is permitted for use also in the EEC countries and has a provisional listing in the USA (Sec. 8.501 of the Code of Federal Regulations). It has been used clinically for renal function tests, to aid in urological examinations and to determine blood flow (Campbell, 1963). Nevertheless, a need for further information concerning its safety-in-use was expressed by the Food Standards Committee (1964) and by the Joint FAO/WHO Expert Committee on Food Additives (1965).

Indigo carmine does not appear to be readily absorbed from the gastro-intestinal tract or to be extensively decomposed within the body. Using ^{35}S-labelled material, Lethco & Webb (1966), showed that 10% of an iv dose in rats was present in the bile and 63% in the urine within 6 hr, the majority as the unchanged colouring. On the other hand, 60–80% of an oral dose was present in the faeces and this was considered to indicate a lack of absorption rather than biliary excretion. A similar lack of absorption and decomposition was indicated in studies in the pig reported by Gaunt, Kiss, Grasso & Gangolli (1969), who found blue-coloured intestinal contents, faeces and urine. Nevertheless some metabolism does take place, since Lethco & Webb (1966) found isatin-5-sulphonic acid and 5-sulphoanthranilic acid in the bile and urine of rats following oral or iv doses of the colouring. These same products are known to be produced by oxidation in solutions of the colouring (Jones, Harrow & Heinke, 1955), but the process is much accelerated *in vivo* since both products were found within 2 hr in the urine of iv-treated rats whereas the 5-sulphoanthranilic acid occurred only after 12 wk in solution (Lethco & Webb, 1966). These latter authors also established that the colouring decomposed *in vitro* in contact with rat

intestinal contents and that *in vivo* 5-sulphoanthranilic acid was more rapidly absorbed than the intact colouring. Therefore, it seems likely that some of the indigo carmine may be oxidized in the intestine and that the products may be preferentially absorbed.

Indigo carmine is not acutely toxic when administered orally, the LD_{50} values being in excess of 2·5 and 2·0 g/kg in mice and rats, respectively (Lu & Lavallée, 1964; FDA unpublished data cited by the Joint FAO/WHO Expert Committee on Food Additives, 1970). The LD_{50} was 405 mg/kg after sc injection in mice and 93 mg/kg after iv injection in rats (FDA unpublished data cited by the Joint FAO/WHO Expert Committee on Food Additives, 1970). Rats given 250 mg indigo carmine twice daily by sc injection for 3 days lost weight, but no oestrogenic activity was detected (Graham & Allmark, 1959). No Heinz bodies were detected in cats given 0·1 g/kg/day for 7 days (Oettel, Frohberg, Nothdurft & Wilhelm, 1965) and Bär & Griepentrog (1960) found no sensitization activity in guinea-pigs.

In man, iv injection of indigo carmine (10 ml of 0·8% aqueous solution) was shown to have a vasopressor effect probably mediated by the sympathetic nervous system (Kennedy, Wirjoatmadja, Akamatsu & Bonica, 1968). This finding was confirmed by Wu & Johnson (1969) using only 5 ml of 0·8% solution and they suggested that indigo carmine should be used with caution for renal function studies and urological studies in hypertensive or cardiac patients.

The short-term toxicity of indigo carmine does not appear to have been studied in the rat, but Gaunt *et al.* (1969) found a no-effect level of 450 mg/kg/day in a 90-day study in pigs. However, a short-term feeding study with isatin-5-sulphonic acid, a metabolite of indigo carmine, established a no-effect level of 2% in the diet of rats, equivalent to an intake of approximately 1000 mg/kg/day (FDA unpublished data cited by the Joint FAO/WHO Expert Committee on Food Additives, 1970).

In long-term studies no evidence of carcinogenicity was found in 20 male and 20 female rats given diet containing 1% indigo carmine for 2 yr (Oettel *et al.* 1965) or in groups of 24 rats given diets containing, 0, 0·5, 1·0, 2·0 or 5·0% indigo carmine for 2 yr (Hansen, Fitzhugh, Nelson & Davis, 1966). Growth of male rats in the latter study was significantly inhibited at 2·0 and 5·0% but no change in mortality, organ weights, haematology or the incidence of pathological abnormalities was observed.

Hansen *et al.* (1966) also fed indigo carmine to groups of four beagle dogs at dietary levels of 1 and 2% for 2 yr but the outcome was equivocal since two dogs at the upper level and one at the lower level died. The deaths were attributed to virus infections and there were no clinical signs, gross lesions or microscopic pathology attributable to the colouring.

No difference in tumour incidence was detected between groups of 50 control mice and 50 mice receiving weekly sc injections of 2·5 mg indigo carmine as a 1% solution (Hansen *et al.* 1966). Similarly, there were no local tumours and only one fibroma in the axillary region, remote from the site of injection, among a group of 20 rats given 55 sc injections of indigo carmine for 7 months in decreasing doses from 1 ml of 2% to 0·5 ml of 0·5% (Oettel *et al.* 1965). However, 14 out of 80 rats injected weekly with 2% aqueous solutions of indigo carmine developed fibrosarcomas at the site of sc injection (Hansen *et al.* 1966). Nevertheless this evidence cannot be taken to indicate that indigo carmine is a carcinogen, since it is now well established (Grasso & Golberg, 1966a) that local sarcomas developing after repeated injection of a substance possessing physico-chemical properties capable of producing cell necrosis is not valid evidence of the carcinogenicity of that substance. In-

digo carmine has been shown to have such properties, and when 1 ml of 2% solution was injected twice weekly for 10 wk it produced a progressive lesion at the injection site, accompanied by macrophage necrosis and persistent fibroblastic proliferation (Gangolli, Grasso & Golberg, 1967). This type of lesion was shown to lead to local sarcoma formation as a result of prolonged interference with connective tissue repair (Grasso & Golberg, 1966b).

No mutagenic effect was detected when *Excherichia coli* was grown in a medium containing 5 mg indigo carmine/ml (Lück & Rickerl, 1960). As no evidence was available concerning the effects of long-term feeding of indigo carmine in mice, the present study was undertaken as part of the BIBRA safety evaluation programme.

EXPERIMENTAL

Material. Indigo carmine was provided through the Food Colours Committee of the Chemical Industries Association and conformed to the following specification of the British Standards Institution (1967):

> Indigo carmine consists essentially of disodium indigotin-5,5′-disulphonate but may also contain an appreciable amount of the disodium indigotin-5,7′-disulphonate. Dye content, min. 85%; subsidiary dyes, max 1·0% (excluding disodium indigotin-5,7′-disulphonate); isatin-5-sulphonic acid, max 1·0%; matter volatile at 135°C, max 8·0%; matter insoluble in water, max 0·2%; matter soluble in diisopropyl ether, max 0·2%; chloride and sulphate (as sodium salts), max 7·0%; copper, max 10 ppm; arsenic, max 1 ppm; lead, max 10 ppm; heavy metals (as sulphides), not producing a colour more intense than the reference standard.

Animals and diet. Mice of the Charles River CD1 strain, obtained from a specified-pathogen-free colony, were used for this study. They were housed in a room maintained at 21 ± 1°C with a relative humidity of 50–60% and allowed unlimited access to a basic diet, consisting of reground Oxoid pasteurized breeding diet supplemented with vitamin K, and to water.

Experimental design and conduct. Groups of 30 male and 30 female mice were fed diets containing 0·2, 0·4, 0·8 or 1·6% indigo carmine for 84 wk. Groups of 60 male and 60 female mice served as controls. Initially the mice were caged in groups of 15 but during the study it was noticed that the male mice tended to fight. Lesions of the anogenital area due to biting were frequent and were associated with obstruction of the urinary tract. To avoid further fighting, all the male mice were caged individually from wk 57.

The animals were under continual surveillance for any abnormalities in condition or behaviour and were weighed at monthly intervals. Any animal that appeared to be ill and unlikely to survive was killed.

Blood samples were collected from the tail vein of ten mice of each sex from the control group and the two highest levels of indigo carmine (0·8 and 1·6%) at months 6 and 12 of treatment. These samples were examined for haemoglobin concentration, packed cell volume, total erythrocyte count and total leucocyte count. At autopsy, after 84 wk, blood samples were collected from all surviving animals and the haemoglobin concentration was

measured. Blood smears to demonstrate reticulocytes were prepared from the blood samples taken at months 6 and 12 and preparations to demonstrate the different types of leucocytes were made from all blood samples. However, counts of these cells were confined to the slides prepared from the control mice and those given the highest dose of indigo carmine (1·6%).

An autopsy was conducted on all animals dying or killed during the experiment unless this was precluded by advanced autolysis or cannibalism. After 84 wk all surviving animals were killed by exsanguination from the aorta under barbiturate anaesthesia. At autopsy all organs were examined for gross abnormalities and the heart, liver, spleen and kidneys were weighed. Samples of these organs together with samples of brain, stomach, small intestine, colon, caecum, rectum, pituitary, salivary glands, thyroid, thymus, adrenals, lymph nodes, pancreas, spinal cord, ovaries, uterus, testes, bladder, skeletal muscle and any other tissue that appeared to be abnormal were preserved in buffered formalin. Paraffin-wax sections of the tissues were stained with haematoxylin and eosin for histopathological examination. Sections of all tissues from the control mice and those given dietary levels of 0·2 and 1·6% indigo carmine were examined. At the intermediate levels of 0·4 and 0·8% indigo carmine the histopathological examination was confined to sections of heart, liver, lungs, kidney and spleen, together with any tissue seen to be abnormal at autopsy. In animals where the liver sections stained with haematoxylin and eosin showed foamy macrophages, additional sections of liver were stained to demonstrate lipid using Sudan Black B, Oil Red O, Nile Blue sulphate and the periodic acid–Schiff reaction.

RESULTS

Deaths occurred in all groups of mice (Table 1) but from wk 64 onwards there were no statistically significant increases in the numbers dead in the treated groups compared with controls. In the males there were significantly more animals dead in the group given 0·8% indigo carmine than in the controls during wk 12–16. In the females such differences

Table 1. *Cumulative mortality of mice fed diets containing 0–1·6% indigo carmine for 80 wk*

	Total no. of deaths among									
	Males given dietary levels (%) of					Females given dietary levels (%) of				
Wk on test	0	0·2	0·4	0·8	1·6	0	0·2	0·4	0·8	1·6
8	1	0	0	1	0	0	0	0	0	0
16	3	0	1	6*	1	1	0	0	0	0
24	9	2	1	8	1	1	0	0	0	1
32	9	5	4	9	2	1	2	1	0	4*
40	14	6	4	10	4	1	4*	4*	0	4*
48	17	8	7	12	6	3	8**	5	3	5
56	19	8	10	14	8	4	9**	6	4	5
64	25	11	14	18	11	13	9	7	4	9
72	33	13	15	20	14	20	13	7	10	11
80	45	15*	17	21	18	26	14	11	15	14

Figures represent the number of mice dead or killed *in extremis* from groups of 30 (treated) and 60 (control) mice. Those marked with asterisks differ significantly (Chi-square test) from those of the controls: $*P < 0.05$; $**P < 0.01$.

were found at the lowest level of treatment (0·2%) at wk 40–60, at wk 40 in the group given 0·4% indigo carmine and at wk 32–40 in that given the highest dietary level (1·6%). It was notable that the overall death rate at wk 80 was higher in males (65%) than in females (44%). The deaths also occurred earlier during the experiment in the males so that at wk 28 the proportions of animals dead were 14 and 2% in males and females respectively. The corresponding figures at wk 52 were 31 and 14% and at wk 60 they were 36 and 18%.

Most of the early deaths in the males were in mice that had been fighting. Frequently, bite lesions and abscesses of the ano-genital region were seen, usually accompanied by an ascending urinary tract infection. At autopsy, distended bladders filled with pus were found, together with hydroureter and pale, distended and pitted kidneys. Pus was found in several kidneys, together with hard protein deposits at the neck of the bladder. The frequency of these findings was reduced after the male mice were housed individually, but distended bladders were found throughout the study, although without the signs of infection.

The rate of body-weight gain was not adversely affected by the feeding of indigo carmine, the mean weight gain at wk 81 being 11, 15, 15 and 12 g in males and 15, 10, 11 and 14 g in females fed 0·2, 0·4, 0·8 and 1·6% indigo carmine, respectively, compared with a mean gain of 10 and 12 g in male and female controls. There were no statistically significant differences between the treated and control mice in the organ weights or the organ weights expressed relative to body weight (Table 2). Although there were isolated statistically significant differences in the results of the haematological examinations (Table 3), these were not dose-related nor consistently present at the three examinations.

Table 2. *Relative organ weights of mice fed diets containing 0–1·6% indigo carmine for 80* wk

Dietary level (%)	No. of mice examined	Relative organ weight (g/100 g body weight)				Terminal body weight (g)
		Heart	Liver	Spleen	Kidneys	
		Males				
0	15	0·67	6·31	0·41	2·10	42
0·2	15	0·61	5·57	0·33	1·95	45
0·4	13	0·64	6·20	0·35	1·84	45
0·8	9	0·67	5·75	0·33	1·84	45
1·6	12	0·69	7·79	0·47	1·84	45
		Females				
0	28	0·56	5·87	0·54	1·67	34
0·2	16	0·56	5·70	0·51	1·56	35
0·4	16	0·59	6·07	0·65	1·63	33
0·8	14	0·55	6·41	0·61	1·59	33
1·6	12	0·56	6·38	0·67	1·61	35

The figures are means for the numbers of animals shown.

The incidences of most of the histopathological lesions were similar in treated and control animals (Table 4). There were statistically significant increases in the incidence of areas of hepatic infarction in male mice given 0·4% indigo carmine and of hepatic fatty change in the males given 0·8%. In the female mice the number of animals with chronic renal inflammation was significantly increased among those given 0·4 or 0·8% indigo carmine. There was a markedly lower incidence of chronic bladder inflammation among all the treated males, although the difference from controls was statistically significant only in the case of the lowest level of treatment (0·2%). Foamy macrophages were found in the livers

Table 3. *Mean haematological values in mice given diet containing 0–1·6% indigo carmine for up to 80 wk*

Sex and dietary level (%)	No. of mice examined	Hb (g/100 ml)	PCV (%)	RBC (10^6/mm^3)	Retics (% of RBC)	Leucocytes Total (10^3/mm^3)	Differential (%) N	E	L	M
					Wk 52					
Male										
0	10	12·3	43	8·38	2·4	14·1	52	1	47	0
0·8	10	12·2	42	8·57	—	12·2	—	—	—	—
1·6	10	13·6	43	8·52	1·4	10·7	22*	0	77*	1
Female										
0	10	13·6	42	7·66	2·1	7·5	30	0	69	1
0·8	10	12·8	42	7·77	—	8·1	—	—	—	—
1·6	10	12·9	42	8·17	1·8	8·7	30	1	67	2
					Wk 80					
Male										
0	15	13·2	—	—	—	—	43	0	55	2
0·2	15	13·8	—	—	—	—	—	—	—	—
0·4	13	12·8	—	—	—	—	—	—	—	—
0·8	9	13·1	—	—	—	—	—	—	—	—
1·6	12	13·0	—	—	—	—	36	0	62	2
Female										
0	28	13·3	—	—	—	—	26	1	71	2
0·2	16	12·7	—	—	—	—	—	—	—	—
0·4	16	13·0	—	—	—	—	—	—	—	—
0·8	14	11·6*	—	—	—	—	—	—	—	—
1·6	12	11·8	—	—	—	—	28	1	70	1

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

A dash indicates that the estimation was not carried out.

The figures are means for the numbers of mice shown. Those marked with an asterisk differ significantly (Student's t test) from those of the controls: *$P < 0·05$.

Statistically significant reductions ($P < 0·05$) were recorded at wk 26 in the PCV value in males fed 1·6% indigo carmine and in the erythrocyte count in females fed 0·8%.

of mice at all levels. including controls, and the four lipid stains used demonstrated that both neutral and compound lipids were present in these macrophages. Additional lesions not listed in Table 4 were fibrosis in the hearts of three male control mice and one male fed 0·4% indigo carmine, a skin abscess in one male fed 1·6%, amyloid degeneration of the ovary in one mouse in the 0·2% group, chronic testicular inflammation in one control and chronic inflammation of the seminal vesicles in one mouse fed the 1·6% level.

The most common benign tumour found was pulmonary adenoma, the incidence rates for the groups fed 0, 0·2, 0·4, 0·8 and 1·6% indigo carmine, respectively, being 3/42, 7/23, 0/17, 1/18, and 2/24 for males and 3/50, 0/28, 0/19, 1/18 and 1/25 for females. The incidence in males fed 0·2% (7/23) differed significantly ($P < 0·05$ by the Chi-square test) from that in the control group. The most common malignant tumour was generalized lymphoblastoma. The incidence rates, similar in control and test groups, were 4/42, 0/23, 0/17, 1/18 and 0/24 in males and 8/50, 5/28, 1/19, 0/18 and 4/25 in females for the 0, 0·2, 0·4, 0·8 and 1·6% diets, respectively. Benign cysts of the ovary and a rectal adenocarcinoma occurred only in control mice. Tumours found solely in mice given the highest dietary level (1·6%) were a single case of testicular interstitial-cell tumour, a subcutaneous fibrosarcoma, an ovarian granulosa-cell tumour and a thyroid adenoma. Those found only in intermediate

Table 4. *Incidence of principal histopathological findings (excluding tumours) in mice fed diets containing 0–1·6% indigo carmine for 80 wk*

Organ and finding	No. of mice examined...	No. of mice affected in									
		Males given dietary level (%) of					Females given dietary level (%) of				
		0	0·2	0·4	0·8	1·6	0	0·2	0·4	0·8	1·6
		42	23	17	18	24	50	28	19	18	25
Lung											
Chronic inflammation		1	1	0	0	0	3	1	0	2	0
Liver											
Infarction		0	2	3**	0	1	0	0	0	0	0
Necrotic areas		1	0	0	2	0	1	0	0	0	1
Foamy macrophages		6	5	4	3	8	2	3	1	4*	3
Amyloid		4	2	4	1	0	0	1	0	0	0
Fatty change		1	1	1	3*	0	0	0	0	1	0
Nodular hyperplasia		1	1	1	0	2	0	0	0	0	0
Leucocyte infiltration		2	1	1	1	1	1	0	0	1	0
Extramedullary haematopoiesis		1	0	1	1	0	0	0	0	0	0
Inclusion bodies		1	2	1	0	0	0	0	0	0	0
Spleen											
Amyloid degeneration		7	3	5	2	1	1	1	1	1	0
Kidney											
Degenerative changes		11	7	4	3	7	8	3	0	2	5
Chronic inflammation		6	1	2	1	0	1	2	3*	4**	2
Bladder											
Chronic inflammation		10	1*	2	1	2	0	0	0	0	0

The figures represent the incidence of the finding in the number of mice shown and those marked with asterisks differ significantly (Chi-square test) from those of the controls: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$.

doses without similar control findings were an adrenal cortical adenoma in a female given 0·2% indigo carmine and a subcutaneous fibroma in a male given 0·8%.

DISCUSSION

The overall death rate in the present experiment was not influenced by the feeding of indigo carmine. There were isolated periods during the study when the number of animals dead in treated groups exceeded those in the controls to a statistically significant degree. However, the most marked differences were in groups given low dietary levels of the colouring and there was no indication of any dose-related effect. The isolated findings do not indicate any effect of indigo carmine on the longevity of the mice. The high death rate in both control and treated male mice in the early stages of the experiment was associated with the histological findings of chronic inflammation in the bladder and kidney. This syndrome has been seen previously in these laboratories (Gaunt, Mason, Grasso & Kiss, 1974) and, as in the present study, its severity was reduced by individual caging of the male mice. Ascending urinary-tract infection is not uncommon in males that have been caged together and have been fighting (Dunn, 1967). Bites about the genitalia result in superficial septic lesions with secondary spread to the seminal vesicles and prostate causing urethal obstruc-

tion, bladder dilation, hydronephrosis and ascending pyelonephritis (Tucker & Baker, 1967). Despite this chronic infection of the bladder, the feeding of indigo carmine did not adversely affect the incidence of bladder lesions; indeed these tended to be less in the treated than in the control mice.

The amyloid deposition in the spleen and liver, again predominantly in male mice, was thought to be a secondary effect of the chronic inflammatory lesions of the urinogenital system. This would be in keeping with the observation of Tucker & Baker (1967) that amyloidosis, mainly of the spleen, occurred in 50% of their male mice under 12 months old and was invariably associated with chronic inflammatory lesions. In more advanced cases, liver and thyroid were also affected. At present no satisfactory explanation of the amyloid deposition is available.

The macrophage infiltration of the livers of mice of both sexes was not related to indigo carmine administration. This liver lesion is similar to the pathological appearance of human liver in Gaucher's disease, where there is a disturbance in lipid metabolism (Richter, 1966). The macrophages observed in the mouse have been shown to contain lipid, and their occurrence is said to be especially common in mice fed an Oxoid diet (H. Fraser, unpublished observations 1972). Since Oxoid diet was used in the present experiment and the macrophages were shown to contain lipid, a similar aetiology is indicated.

The most frequent neoplasms found in the present study were generalized lymphoblastomas and pulmonary adenomas. Both tumours were found in control and treated mice of both sexes and in the case of the lymphoblastomas showed no association with the feeding of indigo carmine. Tumours of the reticulo-endothelial system are the commonest type of spontaneous tumour found in mice, and because viruses are implicated in their induction they are not regarded as valid evidence of carcinogenic potential (Grasso & Crampton, 1972). Pulmonary adenomas also have a high rate of spontaneous occurrence in mice (Cloudman, 1941) and it is generally accepted that the induction of pulmonary adenomas in mice has little relevance in terms of human carcinogenic hazard. The finding of an increased incidence of this tumour in the male mice given the lowest level of the colouring with no similar increase in females or in either sex on higher levels of treatment cannot be taken as an indication of any carcinogenic effect of the colouring. The overall incidence, particularly in the male control group was lower than was found in a concurrent experiment in the same strain of mouse (Gaunt *et al.* 1974) and the incidence in males given the low level was comparable with this background for the strain used.

The three benign tumours (an ovarian granulosa-cell tumour, an adrenal cortical tumour and an adenoma of the thyroid) found in treated females without any occurrence of corresponding tumours in the controls of the present experiment cannot be related to treatment. Granulosa-cell tumours of the ovary are not common, but their spontaneous occurrence has been observed in another strain of mouse in these laboratories (Grasso, Hardy, Gaunt, Mason & Lloyd 1974). In a concurrent study using the CD1 strain, this tumour was found in a mouse treated with a non-carcinogenic material (Gaunt *et al.* 1974). The spontaneous occurrence of ovarian granulosa cell tumours has been reported by other workers (Cloudman, 1941; Russfield, 1967). Similarly, the thyroid adenoma is a rare but well-recognized tumour in untreated mice (Russfield, 1967; Tucker & Baker, 1967). The spontaneous incidence of this neoplasm varies in different strains of mouse and has been reported to be as high as 8%. Adrenal cortical adenoma is again an uncommon tumour but has been reported previously in control mice in these laboratories (Brantom, Gaunt & Grasso, 1973). The absence of any corresponding finding with the higher levels of indigo

carmine, together with the known spontaneous occurrence of these tumours, indicates that the finding of these isolated tumours is not related to indigo carmine treatment.

The one malignant tumour (a subcutaneous fibrosarcoma at 1·6%) and the two benign tumours (a subcutaneous fibroma at 0·8% and a testicular interstitial-cell tumour at 1·6%) found in treated males without a corresponding finding in the controls are all tumours known to occur spontaneously and as such are unlikely to be related to treatment. Subcutaneous fibrosarcoma is one of the more common malignant tumours of the subcutaneous tissue and similar neoplasms have been seen in untreated mice in these laboratories (Brantom *et al.* 1973; Grasso *et al.* 1974). Interstitial-cell tumours of the testes, though not common, have been reported in untreated mice on several occasions (Brantom *et al.* 1973; Gaunt *et al.* 1974; Grasso *et al.* 1974). In the same way, subcutaneous fibroma has been recorded in untreated mice with a frequency similar to that seen in the present study (Gaunt, Brantom, Grasso & Kiss, 1973).

On the basis of the above evidence, it is considered that all the tumours found in the present study represented the expected natural incidence rather than evidence of the carcinogenicity of indigo carmine.

The altered ratio of neutrophils to lymphocytes at wk 52 in the blood of males given 1·6% indigo carmine cannot be taken as an adverse effect of the colouring since the values in the treated mice were those expected of normal animals while the control values were abnormal. This may be a reflection of a response to the apparently greater degree of renal tract infection in the control animals. That this change was a response to an infection seemed likely since the total white cell count was higher in the control mice. The slight anaemia seen in isolated groups of mice in the first 26 wk of the study was not apparent when the haematological examinations were repeated at wk 52. Such isolated findings cannot be attributed to the compound and are more likely to be due to chance. This may be the explanation of the low haemoglobin values found in females at the end of the study, but this seems less likely with the larger group sizes used. Therefore, unless evidence to the contrary can be produced, this must be considered as an effect of the material. On this basis, the no-untoward-effect level obtained in this study was 0·4% of the diet. This is equivalent to an intake of approximately 600 mg/kg/day or, allowing a 100-times safety factor, an intake of 360 mg/day for a 60-kg adult and correspondingly less in children.

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SACCHARIN: LACK OF CHROMOSOME-DAMAGING ACTIVITY IN CHINESE HAMSTERS *IN VIVO*

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Abstract—Oral administration of a high dose of saccharin (1·5 g/kg/day for 3 days) to Chinese hamsters followed by chromosome analysis of bone-marrow cultures did not reveal any statistically significant increase in chromosomal abnormalities. The numbers of polyploid and aneuploid cells and the structural chromosomal aberrations in the bone marrow showed no changes in comparison with a control group. An increase in the population of dividing cells was induced in the test animals by an injection of pertussis vaccine prior to the administration of saccharin.

INTRODUCTION

The mutagenic and carcinogenic properties of artificial sweeteners have been studied by various investigators. It is not far-fetched to associate carcinogenic and chromosome-damaging activity. On the one hand, many malignant tumours are known to have an aberrant karyotype, and indeed in some cases, as in chronic myeloid leukaemia, a consistent chromosome abnormality, the Philadelphia or Ph'-chromosome, is present (Baikie, 1964; Moore, Ekert, Fitzgerald & Carmichael, 1974; Nowell & Hungerford, 1960). As we know by the staining pattern of the chromosome banding (Caspersson, Gahrton, Lindsten & Zech, 1970), this chromosome is a deleted no. 22. This was recently described by Rowley (1973) as a translocation of a (9p+, 22q-)-type.

On the other hand, environmental factors can cause leukaemia in man and animals and also give rise to chromosome aberrations. This was found for radiation (Court Brown & Doll, 1957), for benzene (Dean, 1969; Forni, Cappellini, Pacifico & Vigliani, 1971; Hartwich, Schwanitz & Becker, 1969) and for 7,12-dimethylbenz[*a*]anthracene (DMBA) (Kurita, Sugiyama & Nishizuka, 1968 & 1969; Rees, Majumdar & Shuck, 1970). Moreover, certain hereditary diseases, associated with numerical and structural chromosome abnormalities, carry an increased risk of leukaemia. This is the case in Fanconi's anaemia, as described by Bloom, Warner, Gerald & Diamond (1966), Perkins, Timson & Emery (1969) and Swift & Hirschhorn (1966). The same tendency was shown in Bloom's syndrome (German, 1969; Sawitsky, Bloom & German, 1969) and ataxia telangiectasia (Gropp & Flatz, 1967; Hecht, McCaw & Koler, 1973; Lampert, 1969).

According to our experience, *in vitro* experiments are not sufficient to prove or exclude chromosomal damage by drugs. We found an increase in aneuploid cells and structural abnormalities of the chromosomes in *in vitro* experiments with nitrogen mustard. These findings could not be confirmed *in vivo*. Our theory was that chromosomal aberrations could not be demonstrated *in vivo* because the population of cells in mitosis was too small.

To exclude the possibility that the number of bone-marrow cells in the phase of the mitotic cycle sensitive for chromosome damage was too small at the time of treatment with

a mutagen, the population of dividing cells was increased beforehand. This was done by inducing an immunological reaction in the test animal with pertussis vaccine (De Vries, 1971). This antigen caused in the animal a lymphocytosis, which was for the most part due to a production of new cells. In our project on chromosomal damage by artificial sweeteners, the first agent tested was saccharin, Chinese hamsters being used as the test animals. Their small number of chromosomes ($2n = 22$) is a great advantage in chromosome analysis. Before the saccharin treatment, our pertussis technique was applied.

EXPERIMENTAL

Material. Sodium saccharinate was obtained from Farbenfabriken Bayer AG and was administered in aqueous solution. The saccharin contained six or seven impurities, the most important of which (comprising 0·5% of the material) was identified as *o*-toluenesulphonamide. The other impurities have not yet been identified.

Animals. Chinese hamsters, aged 2–3 months and weighing about 20 g, were divided into two groups each of 20 animals, a control group being necessary because it was impossible to analyse chromosomes from bone-marrow cells from the same animal before and after treatment. For the estimation of the minimum number of animals in each group, it was assumed that the incidence of chromosome abnormalities in control animals would be between 5 and 10%; for an increase in the percentage of aberrations to 50% to be demonstrated with a probability of 0·95, an experimental and control group each of 20 animals was necessary (Rumke, 1969).

Treatment. Animals in both groups were given an ip injection of pertussis vaccine (prepared in our Institute), each animal receiving 0·25 ml of a suspension containing 16×10^9 bacteria/ml. On days 2, 3 and 4 after the pertussis injection, water or saccharin dissolved in water was administered by gastric intubation to animals of the control and test group, respectively. The scheme of injections is illustrated in Fig. 1. Saccharin was given in a dose of 1·5 g/kg body weight/day. In the absence of data on the acute toxicity of saccharin in the Chinese hamster, an LD_{50} equal to that in the mouse was assumed, and on this basis the dose level chosen was relatively high, being 10% of the LD_{50} for the mouse.

Chromosome analysis. Bone-marrow cultures were taken on day 8 after the pertussis injection, a PHA injection having been given on day 6. In every culture, 50 metaphases were analysed. Selection of mitoses occurred with a low-power objective ($\times$ 10). No numerical or structural abnormalities can be seen at this magnification. The total numbers of aneuploid cells, polyploid cells and structural abnormalities were noted in every culture with a high-power oil-immersion objective (Zeiss Planapo, $\times$ 100). The number of polyploid cells was related to the total number of mitoses observed in the course of selection of the 50

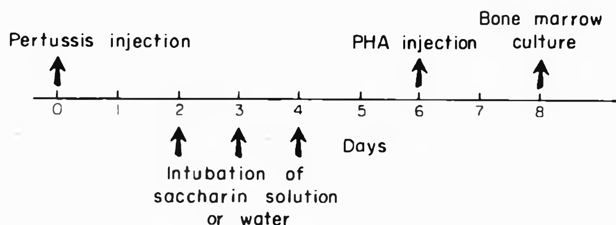

Fig. 1. Scheme of treatment of Chinese hamsters in the control group and in a test group given doses of 1·5 g saccharin/kg body weight/day for 3 days.

metaphases for analysis. The total number of breaks was expressed in terms of the minimal number of breaks necessary for the formation of the total number of structural abnormalities in 50 metaphases.

Statistical analysis. It was assumed that the observed frequencies would follow approximately a Poisson distribution. In order to obtain a normal distribution, the data were transformed according to the transformation of Freeman and Tukey: $y = \sqrt{x} + \sqrt{x + 1}$ (De Jonge, 1964). A student's t test was applied to the transformed data. Because we were not interested in the possible decrease in chromosome aberrations by saccharin, only the probability of an increase in abnormalities by chance was tested. For this reason a one-tailed test was used.

RESULTS

The results of the chromosome analyses are given in Tables 1, 2 and 3. A tendency towards an increase in the number of polyploid cells was found following saccharin treat-

Table 1. *Number of polyploid cells on 50 metaphases in the bone marrow of control and saccharin-treated Chinese hamsters*

Polyploid cells*		No. of hamsters	
x	y	Controls	Saccharin-treated
0	1	4	5
1	2·41	4	2
2	3·15	4	3
3	3·73	4	3
4	4·24	4	1
5	4·69	0	2
6	5·10	0	1
7	5·47	0	1
8	5·83	0	1
12	7·07	0	1

*x = number found; y = transformed values.
The differences between the numbers of control and treated hamsters with any given number of polyploid cells were not significant: $t = 0·9830$ (38 df); $0·10 < P_R < 0·25$.

Table 2. *Number of aneuploid cells on 50 metaphases in the bone marrow of control and saccharin-treated Chinese hamsters*

Aneuploid cells*		No. of hamsters	
x	y	Controls	Saccharin-treated
0	1	7	6
1	2·41	4	1
2	3·15	4	3
3	3·73	2	3
4	4·24	3	3
5	4·69	0	4

* x = number found; y = transformed values.
The differences between the numbers of control and treated hamsters with any given number of aneuploid cells were not significant: $t = 1·2797$ (38 df); $0·10 < P_R < 0·25$.

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Table 3. *Number of structural chromosome abnormalities in 50 metaphases in the bone marrow of control and saccharin-treated Chinese hamsters*

Structural abnormalities*		No. of hamsters	
x	y	Controls	Saccharin-treated
0	1	6	7
1	2·41	3	7
2	3·15	8	3
3	3·73	0	2
4	4·24	1	1
5	4·69	2	0

* x = number found; y = transformed values.

The differences between the numbers of control and treated hamsters with any given number of structural chromosome abnormalities were not significant: $t = -0{\cdot}9576$ (38 df); $P_R > 0{\cdot}75$.

ment, but this was not statistically significant according to the traditional standards. Even with this high dose level of 1·5 g saccharin/kg body weight, no statistically significant differences were found between the control and treated groups.

DISCUSSION

The alleged carcinogenic potential of cyclamate and saccharin came into the news through experiments on rats and mice. Cytogenetic investigations led to conflicting results. In *in vitro* experiments with cyclamate, no increase in chromosome breaks was found in human lymphocytes (Shamberger, Baughman, Kalchert, Willis & Hoffman, 1973), in rat kangaroo cells (Green, Palmer & Legator, 1970) or in plant cells (Majumdar & Lane, 1970; Majumdar & Schlosser, 1972).

It is known, however, that many chemicals that cause chromosome damage in animal cells do not induce the same aberrations in plants (Majumdar & Newton, 1972). Different plant species may even react differently to the same chemical, as was found by Riley & Neuroth (1970), who studied the effect of LSD on plant chromosomes.

An *in vitro* chromosome-damaging effect of cyclamate was found in human lymphocyte cultures and monolayers (Stoltz, Khera, Bendall & Gunner, 1970; Stone, Lamson, Chang & Pickering, 1969) and in Chinese hamster cells (Dixon, 1973; Kristoffersson, 1971). Careful examination of the doses used in the *in vitro* experiments revealed an increase in chromosome abnormalities at doses higher than 200 μg/ml. If the *in vivo* dose of 2–5 g taken daily by man (Bauchinger, Schmid, Pieper & Zöllner, 1970) is used for calculating the level of cyclamate in tissue fluid, a value of 55–140 μg/ml tissue fluid is obtained, which is lower than the *in vitro* doses associated with chromosome damage. In *in vivo* experiments no chromosome aberrations were found in Drosophila (Rotter & Mittler, 1973), mouse spermatocytes (Léonard & Linden, 1972) and in rat bone-marrow and spermatogonial cells (Dick, Schniepp, Sonders & Wiegand, 1974; Friedman, Richardson, Richardson, Lethco, Wallace & Sauro, 1972). In all these cases, cyclamate was given in food or drinking-water.

Dick *et al.* (1974) did not find any effect of cyclamate on human chromosomes *in vivo*. However, the use of only four subjects in a group was, in our opinion, not sufficient to

exclude a chromosome-damaging effect. In studies involving large groups of human subjects (Bauchinger *et al.* 1970) and gerbils (Majumdar & Solomon, 1971a, b), however, an *in vivo* chromosome-damaging effect was described. Perhaps extrapolation of the cytogenetic activity of cyclamates from lower animals like Drosophila is not possible. In the dominant lethal test, a decrease in fertility was found with a 2% level of cyclamate in the food.

The number of publications concerned with the chromosome-breaking activity of saccharin is much smaller. *In vitro* chromosome damage was found with relatively high concentrations in onion-root tips (Sax & Sax, 1968) and human lymphocytes (Chang & Stacey, 1973) and in Chinese hamster cells (Kristoffersson, 1971) with doses of 2 mg/ml and higher. This *in vitro* dose does not correlate with the human daily intake of 1 g (*Nature, London*, 1972). A dose of 1 g/day corresponds to about 30 μg/ml tissue fluid, so 2 mg/ml is 66 times higher than the level corresponding to the normal human intake.

Concerning the *in vivo* experiments, it is probably more correct in small animals to calculate the dose from the basal metabolism instead of on a weight basis (Van Noordwijk, 1964). Because the basal metabolism of small animals is relatively high compared with that of man, the dose for the Chinese hamster can be calculated by multiplying the average human dose by the ratio of the basal metabolism for the animal to that of an adult man. Using Brody's formula for this purpose [$y = \log$ (basal metabolism) $= 0{\cdot}734 \log$ (body weight) + (a constant)], the hamster dose of 1·5 g/kg is equal to about ten times a human dose of 1 g daily (*Nature, London*, 1972), which corresponds to about 17 mg/kg body weight for man.

In our *in vivo* experiments with Chinese hamsters, no chromosomal aberrations were found even with this high dose of saccharin. Hicks, Wakefield & Chowaniec (1973) mentioned the probability that impurities in the saccharin were responsible for the carcinogenic effect found earlier in rats (*Science, New York*, 1972). These impurities apparently had no effect in our experiments on the possible genetic damage effected by saccharin in hamsters.

In conclusion, cytogenetic studies on cyclamate have demonstrated chromosomal damage *in vitro* with a dose above 200 μg/ml as well as *in vivo* in man and gerbils. *In vitro* experiments with saccharin led to chromosomal aberrations at doses 66 times higher than those ingested by man. No aberrations were found *in vivo*. It is more accurate, in our opinion, to extrapolate cytogenetic damage from animals to man from *in vivo* experiments. Even with a very high dose, no chromosomal abnormalities were found in Chinese hamster bone-marrow cells, so we think the statement is justified that saccharin has no *in vivo* chromosome-damaging effect on mitotic cells.

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SHORT-TERM TOXICITY OF ISOAMYL SALICYLATE IN RATS

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Abstract—Isoamyl salicylate was given to groups of 15 male and 15 female rats at dietary concentrations of 0 (control), 50, 500 or 5000 ppm for 13 wk. There was a decrease in weight gain at the highest dietary level accompanied by a reduced food intake, but a paired-feeding study showed that this was due to the diet's unpalatability. The females given the highest dietary level drank more water than the controls and produced slightly greater volumes of more dilute urine. The relative kidney weight was increased in rats on the 500 and 5000 ppm levels without any histopathological changes. It is concluded that isoamyl salicylate was exerting a mildly nephrotoxic effect. The relative liver weight was increased at the highest level of feeding but there were no other effects attributable to treatment. The no-untoward-effect level from this study was 50 ppm of the diet, providing a mean intake of approximately 4·7 mg/kg/day.

INTRODUCTION

Isoamyl salicylate, also known as isopentyl-2-hydroxyphenyl methanoate or 3-methylbutyl 2-hydroxybenzoate ($C_6H_4(OH)\text{-}CO_2\text{-}[CH_2]_2\text{-}CH(CH_3)_2$), is currently used in the UK as a constituent of flavourings. Although at present there are no specific regulations governing the use of flavourings in the UK, the Food Standards Committee (1965), in its Report on Flavouring Agents, considered isoamyl salicylate but did not include it among the seventeen materials recommended to be prohibited for use in food. It was included (as FEMA no. 2084) in the list of flavourings 'generally recognized as safe' by the Flavoring Extract Manufacturers' Association (1965) and is permitted by the US FDA for use as a synthetic food flavouring (Sec. 121.1164 of the Code of Federal Regulations). The Council of Europe (1974) suggests 3 ppm as an acceptable use level in food.

There are no published data specifically concerned with the metabolism of isoamyl salicylate, although Williams (1959) quotes data for an unspecified amyl salicylate, suggesting that there was little hydrolysis of the ester in man. Some other esters of salicylic acid and short-chain alcohols have been studied. Of these, methyl salicylate has been shown to be readily hydrolysed in rats, dogs and man (Davison, Zimmerman & Smith, 1961). These authors showed that the acute toxicity of this ester was similar to that of sodium salicylate, indicating a rapid conversion to the acid. They found that the ester was most readily metabolized *in vitro* by the liver although the kidney, intestinal mucosa, spleen and pancreas were also active in this respect. It is likely that in the intact animal the intestine may be important in the hydrolysis, since Williams (1959) showed that a greater proportion of a dose of methyl salicylate was excreted unchanged when injected than when administered orally. As well as the unchanged ester, the excretory products included the glucuronide and sulphate derivatives and other metabolites of salicylic acid (Williams, 1959).

If hydrolysis of the isoamyl ester does occur, the toxicity may be due to the unchanged ester or to either of the hydrolysis products and there is information concerning the toxicity of both of these products. Carpanini, Gaunt, Kiss, Grasso & Gangolli (1973) reviewed the data concerning the metabolism of isoamyl alcohol, showing that it was mainly oxidized in the liver. In addition, small quantities were excreted unchanged in the air and either unchanged or as a glucuronide in the urine. These authors also found a no-untoward-effect level of 1000 mg/kg/day for isoamyl alcohol in a short-term study in rats. The Sixth Report of the Joint FAO/WHO Expert Committee on Food Additives (1962) reviewed the data on salicylic acid and showed that the main organs affected were the liver and kidney. Hypoprothrombinaemia and gastric irritation have also been reported, and the Joint FAO/WHO Expert Committee on Food Additives (1962) considered this acid to be unsuitable for use as a food additive. Since this review, it has been shown that salicylic acid can induce foetal abnormalities (Larsson, Boström & Ericson, 1963).

There are no reports of toxicological studies with isoamyl salicylate, so that although a use level in food has been recommended for the ester (Council of Europe, 1974), this does not appear to be based on toxicological studies. The present paper describes a short-term toxicity study on this flavouring in rats, carried out as part of the BIBRA safety evaluation programme.

EXPERIMENTAL

Materials. Isoamyl salicylate was supplied by Bush Boake Allen Ltd., London, and complied with the following specification: Specific gravity (at 20°C), 1·050–1·052; refractive index (20°C), 1·507–1·509; free acid, max 0·1%; ester content, min. 99%; dryness to toluene, no turbidity. It was stated that the composition of the alcohols in the sample used was: Isobutyl, 3·5%; *n*-butyl, 0·3%; isoamyl, 95·1%; optically active amyl, 1·1%.

Animals and diet. Rats of the Wistar strain obtained from a specified-pathogen-free colony were given ground Spillers' Laboratory Small Animal Diet and tap water *ad lib.* The animal room was maintained at 20 ± 1°C with a relative humidity of 50–70%.

Experimental design and conduct

Loss of isoamyl salicylate from the animal diet. Diets containing 1 and 2% isoamyl salicylate were prepared. Samples of these were placed immediately in a sealed container while other samples were placed in animal-feeding pots and exposed to the atmosphere in an animal room for 48 hr. The samples were extracted with methanol and the isoamyl salicylate content of the extract was estimated using a Pye 104 dual flame gas chromatogram fitted with a 5 ft glass column packed with 10% Carbowax 20M on Celite. It was found that there was an 8% loss of the ester from the 2% diet over a period of 48 hr but no loss from the 1% diet. As the highest dietary concentration of the ester to be used in the feeding study was 0·5%, it was considered that administration in the diet was appropriate. Fresh diet was prepared twice weekly.

Short-term feeding study. Groups of 15 males (body weight 51–86 g) and 15 females (body weight 58–84 g), housed five to a cage, were given diets containing 0 (control), 50, 500 or 5000 ppm isoamyl salicylate for 13 wk. Additional groups of five rats of each sex of similar body weight were given diet containing 0, 500 or 5000 ppm for 2 or 6 wk. The animals were observed frequently for abnormalities of condition or behaviour and were weighed initially, at days 1, 2, 6, 9 and 13 and then at weekly intervals up to day 91. The consumption of food and water was measured over a 24-hr period preceding the day of weighing.

Urine was collected during the last 2 days of treatment and examined for appearance, microscopic constituents and content of glucose, ketones, bile salts and blood. A renal concentration test was carried out at the same time. This consisted of measurements of the specific gravity and volume of urine produced during a 6-hr period of water deprivation and of that produced during a 2-hr period following a water load of 25 ml/kg. At wk 6 and 13 the same measurements were made on the urine produced from 16–20 hr after the water load. A count of the number of cells in the urine was carried out on the 2-hr sample.

At the end of the appropriate period of feeding, the animals were killed by exsanguination from the aorta under barbiturate anaesthesia, following a 24-hr period without food. An autopsy was conducted, during which any macroscopic abnormalities were noted, and the brain, heart, liver, stomach, small intestine, caecum, spleen, kidneys, adrenal glands, gonads, pituitary and thyroid were weighed. Samples of these organs and of lung, lymph nodes, salivary gland, trachea, oesophagus, aorta, thymus, urinary bladder, colon, rectum, pancreas, uterus and skeletal muscle were preserved in 10% buffered formalin. Paraffin-wax sections of these tissues were stained with haematoxylin and eosin for microscopic examination. The histopathological examination was carried out on the tissues from all rats given 5000 ppm isoamyl salicylate, from half the control animals and from half of those given diet containing 50 ppm of the ester. The examination was confined to the liver, kidneys, spleen and heart in the rats given the intermediate level (500 ppm).

Blood taken at autopsy was examined for haemoglobin concentration, packed cell volume and counts of erythrocytes and leucocytes. Slides were prepared from all blood samples to demonstrate reticulocytes and the different types of leucocytes but counts of these were confined to the samples from the control rats and those given 5000 ppm isoamyl salicylate. Serum was analysed for the content of urea, glucose, total protein and albumin and for the activities of glutamic–oxalacetic transaminase, glutamic–pyruvic transaminase and lactic dehydrogenase.

Paired-feeding study. Two groups of ten rats, caged individually, were given either a diet containing 5000 ppm isoamyl salicylate or the control diet for 98 days. The groups consisted of rats from the same litters and each treated rat was paired with a litter-mate control. Each day each control rat was given a quantity of food equal to that consumed during the previous day by its treated litter-mate. The rats were weighed at intervals.

RESULTS

Short-term feeding study

The rats given 5000 ppm isoamyl salicylate were visibly smaller than the controls and approximately half of them showed signs indicative of a respiratory infection from wk 3. These consisted of pilo-erection, a tendency to adopt a hunched sitting position and noisy, croaking respiration. In most animals these signs regressed, but in one female they continued and the rat was killed during wk 6. It was lethargic, hypothermic and showed rapid shallow respiration. At autopsy, the stomach and upper small intestine were devoid of food, indicating that the rat had not been eating normally. Histological examination revealed mucus and pus cells in the bronchioles together with renal tubular dilatation.

Throughout the experiment, the rate of body-weight gain in both sexes of rats given 5000 ppm isoamyl salicylate showed a statistically significant reduction compared with the controls (Table 1). This was evident after 1 day of treatment and by the end of the study the

weight of the males and females at this dietary level was lower than the controls by 15 and 9%, respectively. In addition the food intake was reduced throughout the study at the highest level of feeding (5000 ppm) so that the mean intake over the experimental period was less than the control values to a statistically significant degree (Table 1). The overall reductions were 20 and 10% in males and females respectively. The most marked reductions in food intake were in the first few days of treatment, when the differences from controls were statistically significant despite being based on only three observations for each group.

On the first day of treatment, the water intake was reduced in rats of all treatment groups compared with that of the controls, the differences being statistically significant at the highest dietary level in both sexes. For the remainder of the study the water intake of the male rats given any level of the ester was similar to that of the controls, whereas the females given the highest dietary level showed an increase in water intake. This increase was statistically significant on day 2 of treatment and on occasions during the rest of the test, and the overall mean water intake by this group was significantly increased ($P < 0{\cdot}01$; ranking method of White, 1952) compared with that of the controls.

Mean intakes of the flavouring over the entire test period, calculated from body-weight and food-intake data, were 4·7, 46·0 and 415 mg/kg/day for males and 4·8, 46·9 and 475 mg/kg/day for females fed dietary levels of 50, 500 and 5000 ppm, respectively.

There were isolated statistically significant differences between treated and control animals in the results of the haematological examinations (Table 2). The only reduced values were in the erythrocyte counts of the females given 500 and 5000 ppm isoamyl salicylate for 2 wk. There were no comparable findings in males at the same time or in either sex at subsequent examination. There were no differences between the treated and control

Table 1. *Mean body weight and food intake of rats fed diets containing 0–5000 ppm isoamyl salicylate for 13 wk*

		Values for							
		Males fed dietary levels (ppm) of				Females fed dietary levels (ppm) of			
Determination	Duration of feeding (days)	0	50	500	5000	0	50	500	5000
Body weight (g)	0†	76	75	74	76	74	73	73	74
	1	83	82	81	76**	81	80	79	75**
	20	212	210	209	179***	150	169	149	138*
	41	318	316	321	260***	196	204	190	177*
	62	378	368	380	320***	219	223	211	200*
	88	442	409	440	378***	240	246	229	218*
Food intake (g/rat/day)	0†	10·8	11·1	11·5	11·3	11·2	10·7	10·7	11·3
	1	13·1	11·9	12·3	8·0**	11·0	11·3	10·9	6·9**
	20	21·5	23·1	21·8	17·3**	17·8	16·3	15·2	14·2*
	41	20·8	23·8	24·1	19·5	15·1	16·0	15·3	14·3
	62	25·3	23·4	25·1	22·3	17·4	18·6	17·0	16·8
	88	22·4	19·3	20·5	18·3*	15·1	15·7	14·4	15·6
	Mean‡...	20·8	20·6	20·9	17·4**	15·8	15·9	14·8	14·2*

†First day of feeding.
‡Excluding pre-treatment value.
The figures for body weight are means for groups of 15 rats and those for food intake are means for three cages of five rats and were measured over the 24-hr period preceding the day shown. The figures marked with asterisks differ significantly from those of controls: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$; $***P < 0{\cdot}001$. The methods used were Student's *t*-test for body weight, the ranking method of White (1952) for overall mean food intake and Lord's range test (Langley, 1968) for individual means.

Table 2. *Mean haematological values of rats fed on diets containing 0–5000 ppm isoamyl salicylate for 2 and 13 wk*

Sex and dietary level (ppm)	No. of rats examined	Hb (g/100 ml)	PCV (%)	RBC (10^6/mm^3)	Retics (% of RBC)	Leucocytes Total (10^3/mm^3)	Differential (%) N	E	L	M
				Wk 2						
Male										
0	5	14·2	42	6·43	2·3	5·6	11	1	87	1
500	5	14·0	43	6·61	—	5·7	—	—	—	—
5000	5	13·7	41	6·52	2·1	4·3	19	0	79	2
Female										
0	5	13·6	41	6·27	1·4	2·7	11	1	88	0
500	5	13·6	40	5·84*	—	2·5	—	—	—	—
5000	5	14·2	43	5·48*	1·5	3·6	21	1	77	1
				Wk 13						
Male										
0	15	17·3	45	8·21	1·0	5·8	19	1	79	1
50	15	17·3	45	8·30	—	5·4	—	—	—	—
500	15	16·6	44	7·96	—	5·7	—	—	—	—
5000	15	17·3	46	8·23	0·6	5·9	17	0	82	1
Female										
0	15	15·4	44	7·44	1·2	3·5	18	0	81	1
50	15	15·4	44	7·33	—	3·2	—	—	—	—
500	15	15·9*	45	7·44	—	3·2	—	—	—	—
5000	14	15·9*	44	7·20	1·3	4·0	19	0	81	0

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

A dash indicates that the count was not carried out.

The figures are the means for the numbers of rats shown and those marked with an asterisk differ significantly (Student's *t* test) from the control value: $*P < 0.05$.

Determinations at wk 6 were free from any indication of adverse effects.

groups in the serum analyses. No abnormal constituents were found in the urine, but the rats given the highest dietary level tended to produce urine of a lower specific gravity following prolonged dehydration. The difference from controls was statistically significant ($P < 0.05$; White, 1952) in both sexes at wk 6 but only in females at wk 13. During wk 2 the females given this dietary level produced an increased volume of urine in the 6-hr collection.

There were scattered statistically significant changes in organ weights and relative organ weights (Table 3). The relative brain weight was increased at the highest dietary level in males at wk 6 and 13, while in the same animals the actual liver weight was lower than that of the controls. However, because of lower body weights, this latter difference was not evident when the weights were expressed relative to body weight. Conversely, in the females given 5000 ppm the relative liver weight was increased at all examinations. An increase in relative liver weight was also found at wk 2 in males given the highest dietary level. The relative spleen weights were increased compared with the controls in male rats given 5000 ppm isoamyl salicylate for 6 wk and in females on the same diet for 13 wk, whilst the spleen weight and relative spleen weight were increased in males given 500 ppm for 6 wk. Statistically significant changes in kidney weight were confined to rats treated

Table 3. *Mean relative organ weights of rats fed diets containing 0–5000 ppm isoamyl salicylate for 6 or 13 wk*

Sex and dietary level (%)	No. of rats examined	Relative organ weight (g/100 g body weight)												Terminal body weight (g)
		Brain	Heart	Liver	Spleen	Kidneys	Stomach	Small intestine	Caecum	Adrenals†	Gonads‡	Pituitary†	Thyroid†	
Wk 6														
Male														
0	5	0·62	0·30	3·07	0·22	0·71	0·47	2·34	0·27	24·6	1·01	3·3	8·0	296
500	5	0·61	0·31	3·07	0·26***	0·71	0·48	2·58	0·29	25·3	1·08	3·2	7·2	297
5000	5	0·69*	0·33	3·12	0·27*	0·77	0·50	2·64	0·34*	21·3	1·25***	3·2	9·0	245**
Female														
0	5	0·83	0·38	2·74	0·27	0·70	0·58	2·99	0·37	44·1	70·2	3·6	10·8	185
500	5	0·84	0·37	2·83	0·28	0·72	0·63	3·16	0·40	44·4	73·0	4·0	10·5	187
5000	5	0·95	0·46	3·43*	0·27	0·74	0·64	3·34	0·48	43·8	82·7	3·1	12·4	163
Wk 13														
Male														
0	15	0·43	0·27	2·50	0·16	0·56	0·39	1·83	0·28	17·8	0·81	2·3	4·2	422
50	15	0·46	0·27	2·57	0·18	0·57	0·40	1·89	0·28	19·8	0·85	2·5	4·2	406
500	15	0·43	0·28	2·51	0·17	0·61**	0·41	1·87	0·27	18·6	0·82	2·6	3·9	427
5000	15	0·48*	0·28	2·45	0·19	0·61	0·40	1·96	0·30	20·1	0·95*	2·6	4·5	386
Female														
0	15	0·73	0·31	2·44	0·24	0·63	0·52	2·56	0·35	37·8	65·8	4·9	6·8	240
50	15	0·72	0·32	2·45	0·26	0·63	0·53	2·64	0·35	41·7	70·3	6·0	6·5	235
500	15	0·75	0·32	2·47	0·25	0·64	0·54	2·47	0·37	41·0	65·3	5·5	6·6	228
5000	14	0·77	0·33	2·73***	0·28*	0·68*	0·56	2·80*	0·37	41·4	63·4	5·3	7·5	216*

†Relative weights of these organs are expressed in mg/100 g body weight.
‡Relative weights of the female gonads are expressed in mg/100 g body weight.
Values are means for the numbers of animals shown and those marked with asterisks differ significantly (Student's t test) from those of controls: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$; $***P < 0{\cdot}001$.

for 13 wk, when there were increases in relative weight in both sexes given the highest level of flavouring and in the kidney weight and relative kidney weight of males on the intermediate level (500 ppm). There was an isolated increase in the small-intestine weight in males given 500 ppm for 6 wk but the remainder of the changes in organ weight were confined to rats given the highest level. These consisted of increases in the relative small-intestine weight in females at wk 13 and in the relative caecum weight in males at wk 6, a decrease in adrenal-gland weight in males at wk 6, an increase in relative testis weight at wk 6 and 13, an increase in pituitary and relative pituitary weight in males at wk 2 and a decrease in pituitary weight in males at wk 6.

The histopathological examination revealed lung changes consisting of thickening of the alveolar walls together with lymphocyte cuffing of the blood vessels and bronchi. Also there were changes in the kidney consisting of lymphocyte infiltration and a protein exudate into the renal tubules. However, the distribution of these findings was similar in treated and control rats.

Paired-feeding study

The rate of body-weight gain was similar in the two groups throughout the 98 days of the study. There were no statistically significant differences at any time.

DISCUSSION

The reduced rate of body-weight gain seen in rats given 5000 ppm isoamyl salicylate was associated with a reduced food intake. In addition, it was noticed that the greatest reduction in food intake was in the early stages of the experiment, suggesting that the reduced growth rate was due to a failure to eat a distasteful diet. This suggestion was confirmed by the paired-feeding study in which the differences in weight gain were eliminated by restricting the food intake of the controls to that of the treated rats. Such a reduced weight gain, attributable to an unpalatable diet, cannot be taken to indicate a toxic effect.

The majority of the differences between the treated and control rats in the haematological examination did not indicate any adverse effect. The isolated decreases in the erythrocyte count at wk 2 in females given 500 and 5000 ppm isoamyl salicylate were small, being approximately 10% of the control value, and there were no comparable decreases in the haemoglobin concentration or packed cell volume. In addition the absence of any change in the number of reticulocytes indicated that the rate of erythropoiesis was not altered. It is concluded therefore that these decreases in erythrocyte count did not reflect any adverse effect of isoamyl salicylate. The increases in relative spleen weight could have resulted from an increased erythrophagocytosis in the spleen. However, the degree of splenic haemosiderosis detected in the histopathological examination did not differ in the control and treated animals. Moreover, under conditions of increased splenic weight due to an increased erythrophagocytosis there is usually a rapid compensatory reticulocytosis (Gaunt, 1973), but this did not occur in these animals.

The finding that the female rats given 5000 ppm isoamyl salicylate excreted increased volumes of urine with a lower specific gravity than that produced by the controls indicates a functional injury to the kidney. This change was not seen in the male rats, but in both sexes there was an increased relative kidney weight without any histopathological change. In addition, increased relative kidney weights were also evident in males given 500 ppm. These observations were taken, in the absence of any contrary evidence, to indicate some toxic effect of the ester on the kidney, particularly since one of the possible metabolites,

salicylic acid, is known to be nephrotoxic (Joint FAO/WHO Expert Committee on Food Additives, 1962).

Since functional changes of the kidney were detected in females alone, it is possible that the increased water intake by the females given 5000 ppm, without similar changes in the males, may have been a result of the renal changes. Hatton & Bennett (1970) consider that the urge to drink in rats is controlled by plasma osmolality and, if this is so, the excretion of a more dilute urine could result in an increased water intake. Alternatively, it has been suggested (Maller & Wank, 1971) that the total volume of water plus solid diet remains constant in the rat. On this basis, the increased water intake could have resulted from the decreased food intake but this fails to account for the restriction of the increased water intake to females, since the amount of food consumed was more markedly reduced in the males.

Apart from the increased kidney weight there were increases in the relative liver weight, again with no indication of any histopathological changes. The liver is known to be involved in the metabolism of some salicylate esters (Davison *et al.* 1961) as well as in the metabolism of the acid, and it is possible that the increased relative liver weight may have reflected an increased metabolic demand. Such increases in relative liver weight have been shown to occur with other materials and are thought to be of little toxicological significance (Golberg, 1967).

The finding of an increase in relative brain weight in the animals with lower body weight was not surprising, since Peters & Boyd (1966) showed that in partially starved animals there was no loss of brain weight. Under such conditions of normal organ weight but reduced body weight, the calculation of relative organ weights will be expected to give increased ratios. In a number of studies in these laboratories this situation has been found to apply in the case of the testis and it may well account for other increases in relative organ weights at the highest level of treatment. The remainder of the changes in organ weight were scattered and cannot be said to represent any effect of the test material.

On the basis of the results of this study it is concluded that the no-untoward-effect level for isoamyl salicylate is 50 ppm of the diet. The average intake of the compound at this level was 4·7 mg/kg/day and allowing a 100-fold safety factor this would suggest a safe daily intake of 0·047 mg/kg or 2·8 mg/day for a 60-kg adult and correspondingly less in children. From data supplied by seven of the leading flavouring manufacturers, it was calculated that the maximum likely intake of isoamyl salicylate in man was 1·7 mg/day, so the no-untoward-effect level in this study is at least 150 times the likely intake in man. As the lowest effect level in the present study was ten times the no-untoward-effect level and the effects seen were very slight, it seems probable that the margin of safety may be even greater.

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STUDIES ON DEGRADED CARRAGEENAN IN RATS AND GUINEA-PIGS

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Abstract—Degraded carrageenan (DC) was administered to rats as a 5% solution in the drinking-water, and to both rats and guinea-pigs as 0·5 and 0·25% solutions. Rats given the 5% concentration developed severe diarrhoea. When they were killed, after treatment for 3 wk, no DC was demonstrable histochemically in the small or large intestine, but analytically it could be demonstrated in both these organs and in the spleen, liver and kidney. In rats treated with the 0·5 or 0·25% solutions, no DC could be detected either histochemically or analytically. DC was demonstrated by both techniques in the intestine, spleen, liver and kidney during the treatment of guinea-pigs with the 0·5% solution. In guinea-pigs given the lower concentration, DC was shown by both histochemistry and chemical analysis to be present in the caecum from wk 2 of treatment, and analysis of the internal organs provided some evidence of absorption. In all the organs in which it was found, DC was contained within the macrophages. Ulceration was observed at wk 4 in guinea-pigs treated with 0·5% DC, but not in those given the lower concentration of DC nor after shorter exposure. Reversibility studies in guinea-pigs demonstrated that no DC was found in the intestine or any of the internal organs 8 wk after cessation of treatment at the 0·25% level, and only small amounts remained at this stage in animals given 0·5% DC.

INTRODUCTION

It has been consistently reported (Grasso, Sharratt, Carpanini & Gangolli, 1973; Maillet, Bonfils & Lister, 1970; Marcus & Watt, 1969; Watt & Marcus, 1969 & 1970) that guinea-pigs and rabbits develop caecal and colonic ulcerations when degraded carrageenan (DC) is administered to them in the drinking-water, in the diet or by gavage. Reports about other species are less consistent. Some workers (Grasso *et al.* 1973; Poulsen, 1973; Sharratt, Grasso, Carpanini & Gangolli, 1971) failed to find any intestinal lesions in the rat, hamster, squirrel monkey and pig; others (Benitz, Golberg & Coulston, 1973; Fabian, Abraham, Coulston & Golberg, 1973; Marcus & Watt, 1969) have reported ulcerations in the rhesus monkey, mouse and rat.

The results of our earlier investigations in guinea-pigs (Grasso *et al.* 1973) suggested that ulceration developed as a consequence of the formation of granulomas in the lamina propria of the caecum and proximal colon. These granulomas appeared to result from the trans-epithelial passage of DC, which seemed to occur when guinea-pigs were given drinking-water containing DC in concentrations of 1% or more.

On the other hand, studies in rats maintained on 1% DC clearly indicated that the colon and caecum were free from either granulomas or ulcerations (Grasso *et al.* 1973). Rats given DC at a concentration of 5% developed diarrhoea within a few days, and this persisted throughout the 8 wk that treatment was continued. At the end of this period, no changes were observed macroscopically or microscopically in the intestine. However, according to Fabian *et al.* (1973), rats treated for 25 wk or longer with 5% DC developed

squamous metaplasia in the rectum and distal colon following an infiltration of macrophages (containing DC) and polymorphs, and ulceration.

It was not clear from the experiments of Fabian *et al.* (1973) to what extent the presence of DC within the lamina propria could be accounted for by damage to the intestinal epithelium. In order to provide some clarification of the problem, the present investigation was undertaken both at levels which, in our experience, produced adverse effects and at lower levels which failed to do so.

EXPERIMENTAL

Animals and diet. Guinea-pigs were of the Duncan Hartley strain and weighed between 300 and 350 g. They were allowed unlimited access to drinking-water and to SGI pelleted diet fortified with ascorbic acid. The rats were of the Wistar strain and specified-pathogen-free and weighed 150–200 g. They were allowed unlimited access to Spillers' Laboratory Small Animal Diet and to drinking-water.

Material and treatment. The DC employed in this study was from the same batch as that used in our previous experiments (Grasso *et al.* 1973) and was administered in the drinking-water to both rats and guinea-pigs. Appropriate control groups were maintained on a diet and drinking-water free from DC. Three levels of treatment were used for the rats, one group of two males and two females being given a 5% solution of DC in the drinking-water, 18 males and 18 females being given a 0·5% solution and another 18 males and 18 females being given a 0·25% solution. Guinea-pigs were given two levels of treatment, a 0·5% solution of DC in the drinking-water being given to a group of 14 males and 14 females and a 0·25% solution being given to 18 males and 18 females.

Conduct of experiment. Rats maintained on a 5% solution of DC were all killed after 3 wk, but sequential studies were carried out on guinea-pigs and rats maintained on the 0·5 and 0·25% solutions of DC. Two males and two females of each species at each dosage level were taken off treatment at weekly intervals, up to wk 4 in the case of guinea-pigs treated with 0·5% DC solution and up to wk 12 in the case of the rats and the other group of guinea-pigs. In order to allow as much DC as possible to be cleared from the intestinal lumen, its administration was stopped 48 hr before the animals were killed by exsanguination under sodium pentobarbitone anaesthesia. During this 48-hr period the animals were allowed fresh tap-water *ad lib.*

Specimens of liver, spleen, kidney, caecum, small intestine, colon, rectum, lungs, brain, stomach, lymph nodes and gonads were fixed in formalin and processed in the usual way for the preparation of paraffin sections for histological examination. The first six organs were examined histochemically and analytically for carrageenan by the techniques described by Gangolli, Wright & Grasso (1973). The analytical method was capable of detecting 0·25 μg DC in a total sample.

Reversibility studies were carried out only in guinea-pigs. Six males and six females were fed 0·5% DC for 4 wk and killed in three batches of four animals 2, 5 and 8 wk after cessation of treatment. Another eight guinea-pigs were treated with 0·25% DC for 12 wk and then killed 4 or 8 wk after cessation of treatment.

RESULTS

Studies in rats

Within 3 days of the administration of 5% DC in the drinking-water, rats developed severe diarrhoea. The diarrhoea persisted throughout the experiment, but the faecal con-

sistency improved, changing from watery to semi-solid. The animals gained less weight than the controls. Macroscopically no changes were evident in the caecum, colon or rectum; microscopically there was oedema in the distal part of the rectum but no clear evidence of macrophage infiltration was present. Histochemically no DC was demonstrable in the small or large intestine or rectum. The other organs examined were macroscopically and microscopically normal. Analytically, DC was identified in both the intestinal wall and in internal organs. The mean values (in μg/g wet tissue), based on analysis of organs from the four animals, were colon 229, caecum 141, ileum 108, spleen 95, liver 84 and kidney 72.

The rats treated with 0·5 or 0·25% DC appeared to be healthy throughout the experiment and gained weight. The faeces were well formed but softer than normal. No DC was detected either histochemically or analytically in the intestine or internal organs of rats maintained on these levels of DC for 12 wk.

Studies in guinea-pigs

There was no obvious impairment of health in the guinea-pigs treated with 0·5 or 0·25% DC. A mild diarrhoea developed during wk 4 in the animals maintained on the higher concentration, but those on the lower concentration were entirely free from diarrhoea, although faecal consistency was softer than that in the controls.

Animals treated with 0·5% DC developed caecal and colonic ulceration by the end of wk 4. The lesions were confirmed histologically. In appearance they resembled the ulcers seen in previous experiments (Grasso *et al.* 1973). No ulcers were observed in the large intestine of guinea-pigs killed earlier, but macrophage infiltration was detectable at wk 2 and 3. Histochemically DC was demonstrable in macrophages in the large intestine at wk 1–4. No abnormality was detected in other organs either at autopsy or in histological

Table 1. *Histochemical demonstration of carrageenan in tissues of guinea-pigs given drinking-water containing 0·5 or 0·25% carrageenan for 4 or 12 wk respectively*

Wk no.	Total no. of animals	No. of animals showing positive demonstration of carrageenan in: Lymph nodes	Liver	Spleen	Caecum	Colon
		0·5% Carrageenan				
1	4	0	0	0	2	0
2	4	0	0	0	4	0
3	4	1	2	0	4	1
4	5	4	5	5	5	3
6	3	1	3	1	2	0
9	3	2	2	1	3	0
12	3	0	1	0	0	0
		0·25% Carrageenan				
1	4	0	0	0	0	0
2	4	0	0	0	2	0
3	4	0	0	0	4	2
4	3	0	0	0	1	1
8	3	0	0	0	1	0
12	4	0	0	0	1	1
16	3	0	0	0	1	1
20	3	0	0	0	0	0

No carrageenan was demonstrated in the kidney or small intestine of any of the animals.

Table 2. *Concentrations of carrageenan in tissues of guinea-pigs given 0·5% carrageenan in the drinking-water for up to 4 wk*

Wk no.†	No. of animals	Carrageenan concn* (μg/g) in Small intestine	Caecum	Colon	Spleen	Liver	Kidney
1	4	112 (0–127)	50 (0–119)	125 (0–278)	243 (0–651)	155 (37–385)	164 (91–309)
2	4	46 (0–93)	72 (0–164)	77 (27–108)	10 (0–30)	103 (30–233)	21 (0–53)
3	3	29 (0–78)	150 (0–440)	60 (7–132)	0	90 (22–145)	0
4	4	184 (123–260)	156 (0–251)	234 (187–305)	96 (30–205)	660 (131–775)	99 (40–200)
6	3	62 (0–125)	154 (76–243)	170 (71–162)	86 (0–213)	648 (344–1103)	142 (44–229)
9	3	0	55 (0–165)	11 (3–30)	17 (0–52)	347 (123–695)	4 (0–13)
12	3	0	0	7 (0–21)	0	67 (43–85)	6 (0–18)

*Values are means for the numbers of animals indicated, with the ranges in parentheses.
†Surviving animals received untreated drinking-water after wk 4.

examinations, but DC was demonstrable in lymph nodes and the liver at wk 3 and 4 and in the spleen at wk 4 (Table 1).

When the guinea-pigs were returned to DC-free drinking-water, the pathological lesions gradually resolved and DC became more difficult to demonstrate. After treatment had been discontinued for 8 wk, DC could be demonstrated only in the Kupffer cells of the liver of one of three guinea-pigs. The other organs were entirely free from DC when examined either histochemically or analytically.

Table 3. *Concentrations of carrageenan in tissues of guinea-pigs given 0·25% carrageenan in the drinking-water for up to 12 wk*

Wk no.†	No. of animals	Carrageenan concn* (μg/g) in Small intestine	Caecum	Colon	Spleen	Liver	Kidney
1	4	0	0	0	0	0	0
2	3	0	<2	<2	28 (0–86)	26 (0–40)	25 (0–46)
3	2	<2	4 (2–6)	0	0	0	2
4	2	0	<2	0	0	0	0
8	2	33 (16–51)	34 (30–38)	<2	<2	7 (2–12)	2
12	3	<2	18 (0–56)	<2	<2	0	0
16	2	<2	13 (0–26)	29 (0–58)	0	6 (0–7)	0
20	2	0	0	0	0	0	0

*Values are means for the numbers of animals indicated, with the ranges in parentheses.
†Surviving animals received untreated drinking-water after wk 12.

DC was found analytically in samples of the intestine and internal organs from wk 1 of treatment with 0·5% DC (Table 2) and from wk 2 with the lower dose (Table 3). The amount present varied considerably both between individual animals and between the different organs. In most organs, more DC was found at the end of wk 4 of treatment with 0·5% DC than in any of the preceding 3 wk, a finding which coincided with the development of ulcers in the large intestine.

The amount of DC in the intestine decreased rapidly soon after treated guinea-pigs were returned to DC-free drinking-water, but the internal organs, with the exception of the spleen, lost their DC much less rapidly. However, the amounts found at wk 12, 8 wk after cessation of treatment, were only approximately 10% of those found at the end of wk 4 of treatment (Table 2).

Administration of 0·25% DC in the drinking-water for 12 wk did not lead either to a macrophage infiltration in the lamina propria or to the development of colonic or caecal ulceration. However, DC could be identified histochemically from wk 2 to wk 12 of treatment in some of the few macrophages that are normally found in the lamina propria of the large intestine of guinea-pigs. No DC could be identified in the caecum or colon of guinea-pigs killed after only 1 wk of treatment. Other organs studied were free of any pathological changes and no DC was found histochemically throughout the experiment (Table 1). Small amounts of DC were detected by analytical techniques, however, in the samples from the intestine and internal organs examined from wk 2 to wk 12 of treatment.

In guinea-pigs given ordinary drinking-water after a 12-wk period of treatment with 0·25% DC, the DC appeared to be gradually eliminated and none could be demonstrated by analytical or histochemical techniques 8 wk after cessation of treatment (Table 3). A similar trend was seen in animals originally given 0·5% DC for 4 wk (Table 2).

DISCUSSION

The results of this experiment indicate, in agreement with our previous results, that within a period of observation limited to 3–12 wk, guinea-pigs develop colonic and caecal ulceration when treated with DC while rats do not. Apart from this striking difference in the pathological response between these two species, there is also a marked difference in the amount of DC found histochemically and analytically in the intestinal wall, particularly in the large intestine, and in the internal organs. Not only was DC demonstrable in substantial amounts in guinea-pigs after treatment with 0·5% for only 2 wk and in the absence of any clinical or pathological evidence of intestinal damage, but it was also demonstrable in guinea-pigs given 0·25% DC, a concentration that produced no adverse effects even after administration for 12 wk. Within the limitations of our observations, it appears that DC absorption in the guinea-pig and its deposition in the organs studied precedes any detectable damage to the intestinal mucosa.

In our rat experiments, DC was found only in the tissues of those animals maintained on a 5% solution. This concentration produced severe diarrhoea within a few days of the start of treatment and this persisted, with some amelioration, as long as the treatment was continued. No overt pathological changes were found, however. The cause of the diarrhoea is not known for certain but it is probable, in view of earlier results (Grasso *et al.* 1973), that carrageenan concentrations of this order exert an osmotic effect, which enhances intestinal motility and which may have an adverse effect upon the intestinal epithelium. An early consequence of this adverse effect may be an alteration in the permeability of the

mucosal epithelium resulting in carrageenan absorption. It is conceivable that if the diarrhoea is allowed to persist for several months, it may lead to overt morphological changes and eventually to the formation of granulomas containing metachromatic material (presumed to be carrageenan) and to ulceration and metaplasia in the mucosal epithelium of the distal colon, particularly at the anorectal junction, all of which were described by Fabian *et al.* (1973).

At lower concentrations, DC does not appear to have this effect in rats. In our experiments there was no evidence of diarrhoea, nor were any lesions detected in the distal colon, in rats treated with 0·5 or 0·25% DC. Furthermore, no DC was detected analytically or histochemically in these rats. However, Fabian *et al.* (1973) reported the presence of macrophages containing metachromatic material in the distal colon of rats given 0·5% carrageenan for at least 6 months, but no adverse findings were reported in these animals. If one accepts this observation as indicating the presence of carrageenan, one must conclude that the intestinal barrier against the absorption of carrageenan in the rat is incomplete. It is difficult to account fully for the discrepancy between our histochemical and analytical results and the histochemical findings of Fabian *et al.* (1973). Since our analytical method employs the metachromatic reaction to toluidine blue, this discrepancy is unlikely to be due to a difference in sensitivity. On the other hand, our studies were much shorter than those of Fabian *et al.* (1973), so that there may well have been a difference in the amount of locally deposited DC.

The observations suggest that DC can pass across an apparently healthy intestinal barrier, particularly that of the large bowel, in both guinea-pigs and rats. At the appropriate dose level this may lead within a comparatively brief period to granulomas and ulcerations in the caecum and colon of the guinea-pig, without any sign of an alteration in faecal consistency. In the rat, pathological changes appear to develop only after a prolonged episode of diarrhoea, as demonstrated by soft or semi-fluid faeces. The diarrhoea appears to lead to a considerable reduction in the efficiency of the intestinal barrier, since carrageenan is found in the internal organs at an early stage. The extent to which the development of subsequent pathological processes in the rat intestine is dependent on the presence of carrageenan rather than on the chronic diarrhoea can only be elucidated by further experiments.

An important feature of our reversibility studies in the guinea-pig was the rapid disappearance of DC from the small intestine within a few days of the cessation of treatment. However, in other organs, 2 wk elapsed before this reduction was apparent. The reason for this time-lag is uncertain, but it could reflect the absorption of residues of carrageenan from the intestinal wall and perhaps also from the intestinal lumen after the termination of treatment. These results suggest that a mechanism exists in the rat and guinea-pig for the adequate disposal of the bulk of the carrageenan absorbed under the experimental conditions of this study.

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STUDIES ON THE METABOLISM OF DIMETHYLNITROSAMINE IN THE RAT. I. EFFECT OF DOSE, ROUTE OF ADMINISTRATION AND SEX

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Abstract—A study has been made of factors associated with the metabolism of dimethylnitrosamine (DMN) in the rat. Particular attention has been given to the oral route of administration, and existing data on the dose-dependent rate of metabolism of DMN have been extended to concentrations of DMN down to 0·1 mg/kg. Considerable inter-animal variation in the rate of DMN metabolism was observed. However, the rate of metabolism appeared to be related to the dose over the entire range studied (0·1–50 mg/kg). Immature animals metabolized DMN at a significantly higher rate than mature animals of the same sex. The rate of metabolism was similar for all three parenteral routes of injection, but was significantly lower after oral administration. Studies on the disappearance of [^{14}C]DMN from the stomach and small intestine of rats showed that the lower rate of metabolism after oral administration was probably due to slower absorption of DMN from the stomach. The kinetics of the disappearance of radioactivity from the small intestine were shown to vary with the intraluminal concentration of [^{14}C]DMN, following a monoexponential curve at very low concentration (0·001 mg/ml) and a biexponential curve at higher concentrations (up to 5 mg/ml).

INTRODUCTION

Nitrosamines have been shown to be potent carcinogens in a wide variety of animal species including the rat, and the available evidence suggests that the development of the biological effect requires metabolic activation to toxic intermediates (Magee & Barnes, 1967). The metabolism of nitrosamines has been studied in several laboratories following the original investigations of Magee (1956), who showed that dimethylnitrosamine (DMN) was rapidly degraded in the intact animal. Subsequently, Heath (1962) established that following the ip administration of ^{14}C-labelled DMN to the mature female rat, $^{14}CO_2$ was exhaled at a rate corresponding to the disappearance of [^{14}C]DMN from the blood. However, the lowest dose investigated was 16 mg/kg, which is high in relation to the LD_{50} value of 35·9 mg/kg reported in the same study.

We are currently undertaking a detailed investigation of the metabolism of nitrosamines, and have therefore begun by re-examining and extending earlier work on DMN metabolism *in vivo* with particular reference to low concentrations of the compound. We have also included an investigation of the effect of route of administration on metabolism. Since man is known to ingest small quantities of nitrosamines in food (Magee, 1971), we have examined in more detail the fate of DMN administered by the oral route. A preliminary communication of this work has appeared (Heading, Phillips, Lake, Gangolli & Lloyd, 1974).

EXPERIMENTAL

Materials. DMN was obtained from Eastman Kodak Ltd., Liverpool. [^{14}C]DMN was synthesized from [^{14}C]dimethylamine hydrochloride (Radiochemical Centre, Amersham, Bucks.) by the method of Dutton & Heath (1956). The purity of the DMN was 99% by gas–liquid chromatography (Palframan, McNab & Crosby, 1973).

Animals. The animals used were male and female specified-pathogen-free Wistar albino rats. Immature animals of both sexes were used when they weighed between 80 and 120 g, while the body-weight ranges for mature female and male rats were 180–220 g and 260–300 g, respectively. All animals were maintained on Spratts Laboratory Diet No. 1 and housed at 20 ± 2°C.

Metabolic studies. [^{14}C]DMN in 0·9% (w/v) NaCl solution was administered at appropriate dose levels to rats (0·2 ml/100 g body weight) by the ip, sc and iv routes and by oral intubation. Following the DMN administration, rats were caged individually in 'Metabowls' (Jencons Scientific Ltd., Hemel Hempstead, Herts.) and allowed free access to food and water. Air was drawn through the system at a constant flow-rate of 250 ml/min, and exhaled $^{14}CO_2$ was trapped in ethanolamine–2-ethoxyethanol (1:4, v/v), after being passed through an acid wash (1 M-HCl) to remove any unchanged nitrosamine. The ethanolamine solutions were changed at intervals during a period of at least 7 hr after dosing, and samples were taken for scintillation counting. Urine, separated from faeces, was also collected as required and aliquots (0·5 ml) were counted.

Additionally, experiments were carried out on the significance of stomach absorption on DMN metabolism. The rats were anaesthetized with sodium pentobarbitone (40 mg/kg) and a small incision was made in the abdominal wall. Following ligation of the pylorus, [^{14}C]DMN was injected into the centre of the stomach, the abdominal wall was stitched and the animal was placed in a 'Metabowl'. Control rats were treated in the same manner, but [^{14}C]DMN was injected sc instead of into the stomach. A loss of righting reflex persisted in these rats for approximately 5 hr.

Gastro-intestinal absorption studies

Stomach. Absorption of [^{14}C]DMN from the stomach was studied using a method based on that described by Casper, Pekas & Dinusson (1973). Rats were anaesthetized with sodium pentobarbitone, the abdomen was opened, and the stomach was ligated at both ends, care being taken to avoid occlusion of blood vessels. An appropriate dose of [^{14}C]DMN in 0·5 ml 0·9% (w/v) NaCl solution was injected into the centre of the stomach, and after an absorptive period ranging from 6 to 30 min, the stomach was excised, cut open and washed out with 0·9% NaCl solution. Portions of the combined contents and washings were assayed for radioactivity, and following deproteinization with sulphosalicylic acid (Heath & Jarvis, 1955); the concentrations of DMN were determined by gas–liquid chromatography.

Small intestine. Absorption from the small intestine was studied using the operative technique described by Matthews, Craft, Geddes, Wise & Hyde (1968). An appropriate dose of [^{14}C]DMN in 0·25 ml 0·9% NaCl solution was injected into one intestinal loop and 0·25 ml 0·9% NaCl solution into an adjacent loop, each loop being approximately 5 cm long and isolated between ligatures in the length of small intestine between 10 and 30 cm from the pylorus. The saline loop was used as a blank. After an absorptive period ranging from 0 to 30 min, the loops were excised and washed out with 0·9% NaCl solution. Portions of the combined contents and washings were assayed for radioactivity. Recovery ex-

periments with solutions of [^{14}C]inulin, as described by Matthews *et al.* (1968), gave a mean recovery of 101·9 ± 2·3% (n = 5). For comparative purposes the disappearance of radioactivity from the stomach and small intestine after the introduction of [^{14}C]methanol was also studied.

Radioactivity measurements

Samples were counted in a scintillation fluid of toluene–2-ethoxyethanol (1:1, v/v) containing PPO (0·3%, w/v) and POPOP (0·03%, w/v). Radioactivity was measured in a Nuclear Chicago Mk 1 Scintillation Counter and the efficiency was determined by the external channels-ratio method (Baillie, 1959).

RESULTS

Dose-related excretion of $^{14}CO_2$

Figure 1 illustrates the $^{14}CO_2$ excretion rate over a 7-hr period, following the administration of [^{14}C]DMN to female rats by the ip route at a dose level of 5 mg/kg. The maximum rate of $^{14}CO_2$ production occurred between hr 1 and 4, and less than 3% of the injected dose was exhaled between 6 and 24 hr. Exhalation of unchanged [^{14}C]DMN accounted for less than 0·4% of the injected dose during the experimental period. Table 1 shows the effect on the metabolic rate of a range of dose levels of [^{14}C]DMN administered ip to mature male and female rats. The maximum rate of $^{14}CO_2$ production was related to the dose in both males and females, although at some dose levels the rate differed in the two sexes. The percentage of the dose converted to $^{14}CO_2$ in 7 hr was similar for all but the highest dose regardless of the sex of the animals.

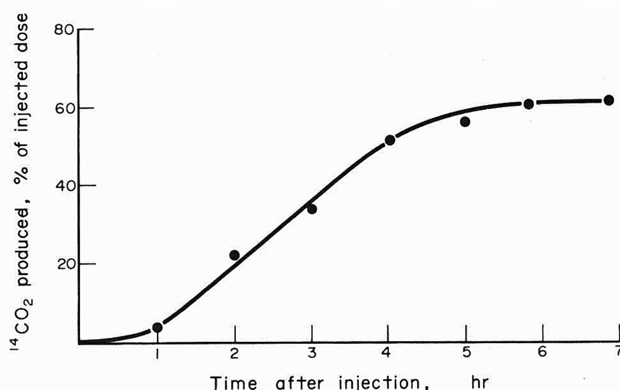

Fig. 1. Metabolism of [^{14}C]DMN in the mature female rat: percentage of a dose of 5 mg [^{14}C]DMN/kg converted to $^{14}CO_2$ in the 7 hr immediately following ip injection. Each point is the mean for four animals.

Effect of sex and age

The effect of age on the rate of $^{14}CO_2$ production is seen in Table 2, in which results for young males and females are compared with those for mature animals. For both male and female rats the maximum rate of $^{14}CO_2$ production was significantly lower in the mature animals. There was no difference, however, in the percentage of the dose converted to $^{14}CO_2$ in the 7 hr following injection.

Table 1. *Effect of dose on the rate of [^{14}C]DMN metabolism in mature male and female rats after ip injection*

	Males		Females	
Dose (mg/kg)	Max rate of [^{14}C]DMN metabolism (mg/kg/hr)*	$^{14}CO_2$ production in 7 hr (% dose)†	Max rate of [^{14}C]DMN metabolism (mg/kg/hr)	$^{14}CO_2$ production in 7 hr (% dose)
50	2·80–3·15 (2)	19–27 (2)	4·02–8·75 (3)	25–32 (3)
5	1·31–2·01 (4)	55–66 (4)	1·49–1·93 (6)	45–55 (6)
1	0·40–0·44 (2)	74–62 (2)	0·26–0·28 (3)	52–55 (3)
0·1	0·023–0·033 (2)	58–72 (2)	0·031–0·047 (2)	62–73 (2)

*Calculated as in Heath (1962).
†Percentage of the dose of injected ^{14}C exhaled at $^{14}CO_2$ in 7 hr.
Figures represent the range of values obtained for the number of animals stated in parentheses.

Table 2. *Effect of age on the rate of [^{14}C]DMN metabolism after an ip injection of 5 mg [^{14}C]DMN/kg*

Production of $^{14}CO_2$†	Males		Females	
	Young	Mature	Young	Mature
Max rate (%/hr)	29·96 ± 3·67	19·68 ± 1·77**	33·1 ± 3·52	19·43 ± 0·83**
In 7 hr (%)	57·08 ± 2·44	61·88 ± 2·40	57·5 ± 2·0	52·67 ± 2·10

†Maximum rate of $^{14}CO_2$ production is expressed as a percentage of the dose of injected ^{14}C exhaled hourly as $^{14}CO_2$, while $^{14}CO_2$ produced in 7 hr is the percentage of the dose of injected ^{14}C exhaled as $^{14}CO_2$ in that time.
Values are means ± SEM for groups of seven young male rats, of four mature males, of four young females and of six mature females. Those marked with asterisks are significantly lower (**$P < 0·01$) than those for the young rats of the same sex.

Table 3. *Effect of route of administration on the metabolism of a dose of 5 mg [^{14}C]DMN/kg to mature female rats*

Route of injection	Max rate of $^{14}CO_2$ production (% of dose/hr)	$^{14}CO_2$ exhaled (% of dose/7 hr)	^{14}C in 24-hr urine (% of dose)
	Untreated rats		
Intravenous	18·9 ± 2·5	54·0 ± 3·5	7·7 ± 0·55
Intraperitoneal	19·6 ± 1·6	54·0 – 3·0	7·0 ± 0·30
Subcutaneous	20·9 ± 0·8	55·0 ± 3·5	8·9 ± 1·13
Oral	12·4 ± 1·1*	48·0 ± 1·5	5·7 ± 0·66
	Rats with ligated stomach		
Subcutaneous	13·5 ± 0·2	64·0 ± 4·5	—
Intragastric	6·1 ± 1·0**	28·5 ± 4·5**	—

Values are means ± SEM for groups of four rats, except for the ip-treated group, for which the first two columns give means for groups of five. The values marked ** are significantly lower ($P < 0·01$) than those for the sham-operated controls, while that marked * is significantly lower ($P < 0·05$) than the corresponding value for each of the parenteral routes.

Effect of route of administration on DMN metabolism

Following the administration of [^{14}C]DMN at a dose level of 5 mg/kg to mature female rats, the maximum rate of $^{14}CO_2$ production was similar for the three parenteral routes investigated, but slower for the oral route (Table 3). The $^{14}CO_2$ produced within 7 hr, however, was similar for all routes of administration, as was the ^{14}C present in urine collected during the 24 hr following dosing. The role of the stomach as a site of absorption of DMN following oral administration was investigated in animals in which the stomach was ligated at the pylorus (Table 3). Following intubation of [^{14}C]DMN the maximum rate of $^{14}CO_2$ production was only 45% of that of sham-operated control animals, and the percentage $^{14}CO_2$ produced in 7 hr was also only 45% of the control value. The percentage of administered [^{14}C]DMN metabolized to $^{14}CO_2$ in 7 hr was similar in sham-operated and intact animals, although the maximum rate was lower in the former.

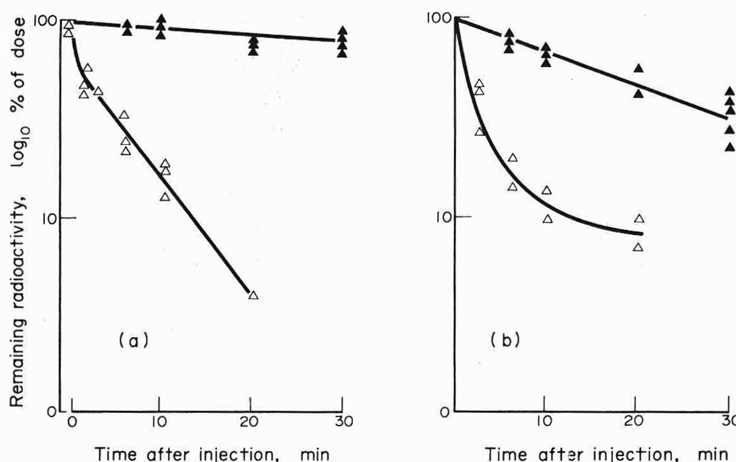

Fig. 2. Disappearance of radioactivity injected as [^{14}C]DMN (a) or [^{14}C]methanol (b) into the lumen of loops of small intestine (△) or into the stomach (▲) of female rats, during 30 min following injection of the labelled compound into the lumen in a concentration of 0·01 mg/ml saline. Each point represents a separate experiment.

Gastro-intestinal absorption of DMN

The rates of disappearance of ^{14}C from loops of small intestine and from the stomach after injection of 0·01 mg [^{14}C]DMN/ml saline solution are shown in Fig. 2a. For absorption from the lumen of the small intestine, the disappearance curve for a concentration of 0·001 mg/ml saline solution was monoexponential, but for higher concentrations the curve was biexponential, as might be expected for a highly lipid-soluble compound (Doluisio, Crouthamel, Tan, Swintosky & Dittert, 1970). The rate of disappearance was the same for fed and starved animals. The disappearance of [^{14}C]DMN from the stomach over the concentration range 0·01–5·0 mg/ml was considerably slower than that from the small intestine. The disappearance curve was monoexponential over the 30 min period studied. After 30 min, the fall in the concentration of DMN in the stomach corresponded to the fall in the concentration of radioactivity. Thus, from an initial concentration of [^{14}C]DMN of 5 mg/ml, the ^{14}C concentration fell to 60·5 ± 4·9%, and the DMN concentration to 58·0 ± 4·0%. Absorption from starved rats was consistently more rapid than from the corresponding fed rats (Table 4). The differences between fed and starved animals

Table 4. *Disappearance of radioactivity from the ligated stomach of fed and starved rats given an intragastric injection of [^{14}C]DMN or [^{14}C]methanol*

Administered concn (mg/ml saline)	Radioactivity remaining at 30 min (% of administered dose)		
	[^{14}C]DMN		[^{14}C]methanol
	Fed	Starved	Starved
5·0	80·4 ± 6·5*	60·5 ± 4·9	71·2 ± 7·30
1·0	96·4 ± 3·41*	65·3 ± 3·3	—
0·1	—	—	48·6 ± 3·2†
0·01	79·1 ± 5·16	70·5 ± 4·9	44·2 ± 5·9†

Values are means ± SEM for groups of five or more animals. Those marked with an asterisk differ significantly (*$P < 0·05$) from the corresponding values for starved rats, while those arrowed differ significantly (†$P < 0·05$) from the value for an initial concentration of 5·0 mg/ml.

were statistically different ($P < 0·05$) in two out of three concentrations of DMN studied. There was no evidence that the percentage of ^{14}C remaining in the stomach was influenced by the initial concentration of DMN over the range studied.

The kinetics of absorption of radioactivity with [^{14}C]methanol as a model amphipathic compound are more complex than with [^{14}C]DMN. Disappearance from the small intestine followed a biexponential time course at all concentrations investigated (0·1 to 0·001 mg/ml), although the initial phase became less rapid with increasing concentration. Like [^{14}C]DMN, however, more than 50% was removed from the lumen in 3 min and more than 89% in 20 min (Fig. 2b). The rate of disappearance of radioactivity from the stomach of starved animals was inversely related to the initial concentration (Table 4), although at an initial concentration of 0·1 mg/ml, the disappearance was monoexponential over the first 30 min.

DISCUSSION

The metabolic conversion rate of DMN to CO_2 in the intact animal has been shown to be dose-dependent over the range of 0·1–50 mg/kg. These findings are in general agreement with those obtained by Heath (1962) over a more restricted dose range. The considerable inter-animal variation prevents the demonstration of a precise Michaelis–Menten relationship. However, if we assume the same quantitative relationship between the rate of disappearance of DMN from the blood and the rate of appearance of $^{14}CO_2$ in the expired air as derived by Heath (1962), the approximate values of K_m and V of 14·5 mg/kg and 5·5 mg/kg/hr for our female rats are of the same order as those for the MRC Porton–Wistar strain. Our colony differed from that used by Heath (1967) in showing no greater variability in the metabolic rate for male animals than for females, although the coefficient of variation of metabolic rate was lowest for mature females.

Following ip administration, DMN is rapidly distributed in body-water space prior to being metabolized to CO_2 (Magee, 1956). The observed similarity in the $^{14}CO_2$ production rates with all the three parenteral routes investigated suggests the likelihood of a common mechanism for the disposition and metabolism of DMN in the body. The relatively slow initial rate of $^{14}CO_2$ excretion and the subsequent attainment of the expected overall conversion by 7 hr following oral administration of DMN is consistent with the finding that

disappearance from the stomach is slow but that, following gastric emptying, DMN and/or its metabolites are rapidly absorbed from the small intestine.

The observed similarities in the kinetics of the disappearance of radioactivity following the administration of [^{14}C]methanol and [^{14}C]DMN from the gastro-intestinal tract clearly demonstrate that DMN has absorption characteristics analogous to those of other compounds freely soluble in both aqueous and lipid phases (Doluisio *et al.* 1970; Gibaldi & Grundhofer, 1972; Schanker, Shore, Brodie & Hogben, 1957). Further, the reduced rate of disappearance of DMN from the stomach of fed rats is similar to the situation observed with ethanol, a model amphipathic compound (Wallgren & Barry, 1970).

In the human context, where exposure to nitrosamines results from ingestion of foods containing trace amounts of these compounds, the observed preferential absorption from the small intestine raises the question of possible metabolic transformation, mediated by the mucosal cells, during absorption. Although the available evidence on isolated small-intestinal preparations indicates a very low capability for metabolism of DMN (Magee, 1971), the role of the small-intestinal epithelial cells in transforming DMN, at levels present in food, cannot be ignored. Our own preliminary observations suggest that a significant proportion of DMN administered at low levels is metabolized during absorption. A study of the nature of this metabolic transformation is currently in progress.

The interrelationship between age, sex, rate of metabolism and the toxicity and carcinogenicity of DMN remains to be elucidated, and studies directed towards this end are actively being pursued.

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AFLATOXIN B_1 HYDROXYLATION BY HEPATIC MICROSOMAL PREPARATIONS FROM THE RHESUS MONKEY

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Abstract—Aflatoxin B_1 hydroxylation to aflatoxin Q_1 and aflatoxin M_1 is catalysed by hepatic microsomal mixed-function oxidases from the rhesus monkey and is accompanied by the formation of three unidentified organosoluble metabolites. The enzyme systems involved require NADPH and molecular oxygen for maximum catalytic activity and both are inhibited by carbon monoxide and by SKF 525-A. The optimum pH for aflatoxin Q_1 formation is 7·4 and that for aflatoxin M_1 is 8·4. The apparent K_m and V for aflatoxin Q_1 production are 0·166 mM and 7·7 nmol/mg microsomal protein/min, respectively, and for aflatoxin M_1 production 0·012 mM and 0·44 nmol/mg microsomal protein/min. The results are discussed in relation to the hepatocarcinogenic potency of aflatoxin B_1 in the rhesus monkey and man.

INTRODUCTION

The high acute toxicity and the extremely potent hepatocarcinogenic activity of aflatoxin B_1 (AFB_1; Fig. 1) in certain animals have prompted world-wide attention to the mode of action and the control of this mycotoxin, which is known to be a contaminant of animal feeds and human foodstuffs. Whether AFB_1 is carcinogenic in man is uncertain,

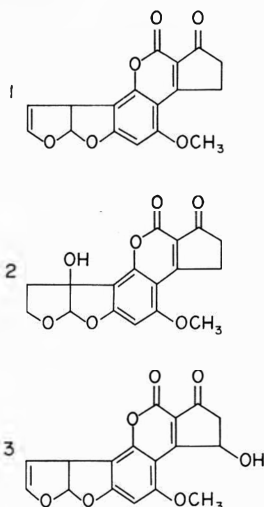

Fig. 1. Structures of aflatoxin B_1 (AFB_1; 1) and its microsomal metabolites, aflatoxin M_1 (AFM_1; 2) and aflatoxin Q_1 (AFQ_1; 3).

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although recent epidemiological studies have demonstrated an association between AFB_1-contaminated foods and an increased incidence of liver cancer in certain human populations (Alpert, Hutt, Wogan & Davidson, 1971; Keen & Martin, 1971; Shank, Bhamarapravati, Gordon & Wogan, 1972).

Available evidence indicates that AFB_1 requires metabolic activation to elicit its carcinogenic activity (Garner 1973; Garner, Miller & Miller, 1972; Schoental, 1970) and that variation in susceptibility of animals to aflatoxin-induced cancer is associated with differences in the pattern of disposition and metabolism of AFB_1 (Patterson, 1973).

Recently, monkey-liver microsomal preparations have been used in this laboratory for the production of aflatoxin Q_1 (AFQ_1; Fig. 1). This metabolite is the prominent biotransformation product of AFB_1 formed *in vitro* in the presence of cell-free monkey-liver preparations. During the writing of this paper, it has also been found that AFQ_1 is an important AFB_1 metabolite in human-liver preparations (A. S. Salhab and D. P. H. Hsieh, unpublished data 1974). This paper reports the basic biochemical properties of the monkey-liver enzyme systems that catalyse the biotransformation of AFB_1 to aflatoxin M_1 (AFM_1) and AFQ_1. These results may be particularly important for future comparative metabolic studies.

EXPERIMENTAL

Animals. Male rhesus monkeys, *Macaca mulatta* (3–4 kg) were quartered at the National Center for Primate Biology, Davis, Calif. They were fed a diet of Purina monkey chow supplemented with fresh fruit.

Chemicals. Ring-labelled [^{14}C]AFB_1 was prepared from cultures of *Aspergillus parasiticus* ATCC 15517 supplemented with [1-^{14}C]acetate according to the procedure of Hsieh & Mateles (1971). Radiochemical purity was indicated by the constant specific activity of the compound upon further thin-layer chromatographic purification. Autoradiography also showed a concentration of radioactivity in a single compact spot corresponding to AFB_1. Non-radioactive AFB_1 was purchased from Calbiochem, LaJolla, Cal., and was used after recrystallization from benzene. AFM_1 was a gift from Dr. M. S. Masri, Western Regional Research Laboratory, Berkeley, Cal., and AFQ_1 was produced in previous investigations in this laboratory (Hsieh, Dalezios, Krieger, Masri & Haddon, 1974a). Other biochemicals purchased from Calbiochem included glucose 6-phosphate (G6P), G6P dehydrogenase, NADP and NADH. SKF 525-A (2-diethylaminoethyl 2,2-diphenylvalerate) and sesamex (acetaldehyde 2-(2-ethoxyethoxy)ethyl 3,4-methylenedioxyphenylacetal) were gifts from Smith, Kline and French, Philadelphia, Pa, and from Shulton, Inc., Clifton, N.J., respectively. All other chemicals were analytical reagent grade.

Enzyme preparations. Monkeys were killed by Surital® (sodium thiamylal) overdose and livers (120–160 g) were removed without delay. Following perfusion with saline, the livers were cut into small pieces and homogenized in 1·15% (w/v) KCl at high speed in a Waring Blendor. The resulting suspension was filtered through fine-mesh nylon organdy or cheese cloth to remove fibrous debris. Microsomal fractions were isolated from the post-mitochondrial supernatant (10,000 g_{max} × 30 min) by centrifugation at 105,000 g_{max} × 60 min using a Beckman Type 50 fixed-angle rotor in a Beckman L-2 ultracentrifuge. The microsomal pellets were resuspended in phosphate buffer, pH 7·4 (50 mM) and the final suspensions contained 1–3 mg protein/ml, estimated by the method of Lowry, Rosebrough, Farr & Randall (1951).

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Metabolism of aflatoxin B_1. Microsomal suspensions containing 1–3 mg protein were incubated with labelled AFB_1 in a 5 ml reaction mixture containing phosphate buffer, pH 7·4 (50 mM), KCl (2·7 mM), NADP (10 mM), G6P (2·4 mM) and G6P dehydrogenase (5 EU). The AFB_1 was added in methanol (770 μg/50 μl). The reaction was initiated by enzyme addition and during the 30-min incubation period the open 25 ml Erlenmeyer flasks were shaken in a water-bath maintained at 37°C. The reactions were terminated by addition of 5 ml ice-cold methanol. The mixtures were extracted four times with 10 ml chloroform, and the resulting extracts were concentrated to dryness using a rotary evaporator and a 37°C water-bath. The residues were each redissolved in 5 ml of 2% (v/v) acetonitrile in benzene.

Identification and quantitation of metabolites. Identification and quantitation of metabolites were based upon thin-layer chromatography with authentic compounds, followed by densitometric measurement of fluorescent spots and scintillation counting of the radioactivity in the spots. Metabolites were also confirmed by autoradiography. The three thin-layer chromatography systems used were Adsorbosil-1 (Applied Science Laboratory, State College, Pa) plates (250 μ in thickness) developed with chloroform–acetone–*n*-hexane, 85:15:20, by vol. (Teng & Hanzas, 1969), chloroform–acetone–water, 88:12:1·5, by vol. (Stubblefield, Shannon & Shotwell, 1969) and chloroform–methanol, 97·3, v/v (Pons, Cucullu, Lee, Robertson, Franz & Goldblatt, 1966). The Schoeffel double-beam spectrodensitometer (Model 3000-2, Westwood, N.J.) was set at excitation wavelength 360 nm and interference monochromator 430 nm. On chromatograms developed in the first solvent system, the relative densitometric responses of AFB_1, AFQ_1 and AFM_1 were 1·0, 0·7 and 1·0 respectively (Hsieh *et al.* 1974*a*). The cofactor requirements of the hydroxylase systems concerned and the effect of inhibitors, pH, incubation time and substrate concentration on the enzymatic hydroxylation of AFB_1 were determined.

RESULTS

Biotransformation of aflatoxin B_1

During a 30-min incubation period in an NADPH-fortified reaction mixture, AFB_1 was metabolized by monkey-liver microsomes to AFQ_1, AFM_1 and three additional unidentified organosoluble derivatives also detected on thin-layer chromatograms. The remaining water-soluble fraction was not characterized further. Each of the five organosoluble derivatives was more polar than AFB_1. The primary metabolite was AFQ_1, which accounted for as much as 52·9% of the initial radioactivity when high enzyme levels were used. Under the experimental conditions described, approximately 30% of the applied radioactivity was retained at the origin. Characterization of these polar metabolites is in progress.

Intracellular localization of oxidases

The intracellular localization of the enzyme systems affecting AFB_1 metabolism was established by assaying the various homogenate fractions obtained by differential centrifugation. The concentration of each preparation was adjusted to be equivalent to 0·2 g liver. The results of the experiment are summarized in Table 1. In each fraction, AFQ_1 was the primary metabolite, and smaller amounts of AFM_1 and water-soluble derivatives were formed. In the 10,000 **g** × 30 min sediment, which contained cellular debris, nuclei and mitochondria, comparatively little AFB_1 metabolism occurred. The centrifugation of the resulting supernatant separated the active microsomal fraction from the relatively inactive soluble fraction.

Table 1. *Intracellular localization of AFB_1 hydroxylases*

Liver fraction*	% of initial radioactivity†			
	Substrate	Metabolites		
	AFB_1	AFQ_1	AFM_1	W
Whole homogenate	26·2	29·5	7·2	16·9
10,000 g × 30 min sediment	65·5	10·1	2·4	3·2
105,000 g × 60 min sediment	13·2	52·9	6·1	5·5
105,000 g × 60 min supernatant	78·5	4·0	1·0	3·0

AFB_1 = aflatoxin B_1 AFQ_1 = aflatoxin Q_1
AFM_1 = aflatoxin M_1 W = water-soluble compounds
*Each incubation contained the equivalent of 0·2 g liver from a male rhesus monkey, the incubation time being 30 min at 37°C.
†Values are means of triplicate incubations; % initial radioactivity for each sample was obtained from analysis of four portions of each extract. Approximately 30% of the radioactivity in the extracts of reaction mixtures was retained near the point of origin after development of the thin-layer chromatographic plates.

Following incubation of AFB_1 with the microsomal fraction, 52·9% was recovered as AFQ_1, 6·1% as AFM_1 and 5·5% as water-soluble derivatives. These experiments clearly justify the classification of the enzyme systems that catalyse the two AFB_1 hydroxylations as microsomal enzymes. The rapid turnover of AFB_1 to yield AFQ_1 may deplete substrate levels for the enzyme system catalysing AFM_1 formation.

In agreement with the findings of earlier workers, the hepatic microsomal fraction is most active in oxidative AFB_1 metabolism. The monkey-liver system is extremely active in the formation of AFQ_1, a fact that has subsequently permitted its use for the synthesis of AFQ_1 (Hsieh *et al.* 1974*a*) for toxicological investigations (Hsieh, Salhab, Yang & Wong, 1974*b*).

Requirements of the hydroxylase systems and effects of inhibitors

Data presented in Table 2 clearly indicate the enzymatic nature of AFB_1 metabolism and give further support to the classification of the enzyme systems involved in the forma-

Table 2. *Cofactor requirements of the microsomal metabolism of AFB_1*

Incubation condition*	% of initial radioactivity†			
	Substrate	Metabolites		
	AFB_1	AFQ_1	AFM_1	W
Control	13·0	48·4	4·3	5·3
Plus heat-treated enzyme	92·0	0·0	0·0	0·3
Minus NADP	74·7	0·5	0·5	1·2
Minus NADP plus NADH	60·0	8·0	0·5	1·8
Plus NADH	8·0	51·8	5·1	6·0
Plus nicotinamide and $MgCl_2$	8·3	50·0	6·8	6·4

AFB_1 = aflatoxin B_1 AFQ_1 = aflatoxin Q_1
AFM_1 = aflatoxin M_1 W = water-soluble compounds
*Monkey-liver microsomes were incubated in all cases for 30 min at 37°C.
†Values are means from triplicate determinations.

tion of AFQ_1 and AFM_1 as typical microsomal oxidases. Heat-treatment destroyed the metabolic activity of microsomal suspensions and incidentally also resulted in an improvement in the efficiency of AFB_1 extraction from the incubation media. A contributory factor may have been the alteration of an aflatoxin-binding factor in the microsomes. The formation of AFQ_1, AFM_1 and the water-soluble fraction were each NADPH-dependent and could not be increased significantly by addition of NADH to the incubation medium. Nicotinamide and $MgCl_2$ (both in mM concentration), increased AFM_1 formation by approximately 50% compared with the control incubation but had a negligible effect on AFQ_1 production.

Microsomal oxidation requires the participation of cytochrome *P*-450, the haemoprotein terminal oxidase which binds carbon monoxide. When AFB_1 was incubated in a CO–air atmosphere ($CO:O_2$, 5:1), the rates of formation of AFQ_1 and AFM_1 were reduced by 66 and 33% respectively. The hydroxylase system catalysing the formation of AFQ_1 was also strikingly more sensitive than the AFM_1-forming system to 5×10^{-4} M SKF 525-A (82·2 *v.* 37·2% inhibition) and to the methylenedioxyphenyl compound, sesamex, in a concentration of 5×10^{-4} M (94 *v.* 0% inhibition). This differential sensitivity to these inhibitors may represent a fundamental difference between the two microsomal AFB_1-hydroxylase systems.

Effect of pH on AFB_1 hydroxylations

Figure 2 shows the effect of pH on the rates of hydroxylation of AFB_1 to AFQ_1 and AFM_1. The highest rates of conversion were obtained at pH 7·4 for AFQ_1 formation and at pH 8·4 for AFM_1 formation using monkey-liver microsomes and an incubation medium containing 0·05 M-tris-HCl. These results contrast with those of Schabort & Steyn (1969), who reported an optimum pH of 7·4 for AFB_1 4-hydroxylase using rat-liver microsomes.

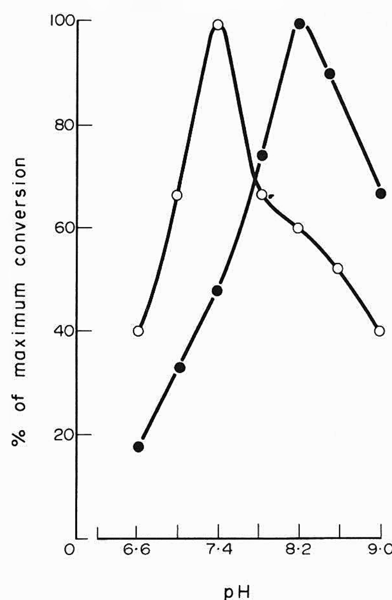

Fig. 2. Effects of pH on the rate of formation of aflatoxin Q_1 (○) and aflatoxin M_1 (●). Tris-HCl buffer was used in the incubation medium and values are means from triplicate incubations.

Time course of AFB_1 metabolism

The relative rates of transformation of AFB_1 to AFQ_1, AFM_1 and water-soluble derivatives (W) are shown in Fig. 3. The rapid formation of AFQ_1 is the most outstanding characteristic of the rate profiles presented. Perhaps because of cofactor or substrate depletion, each of the rate profiles breaks sharply at about 20 min. No secondary precursor–product relationships are evident among the transformations studied, since the amounts of the various products increased steadily from zero time. The rapid rate of conversion of AFB_1 to AFQ_1 necessitates the use of low enzyme levels and short incubation periods in studies of the kinetics of AFB_1 hydroxylation.

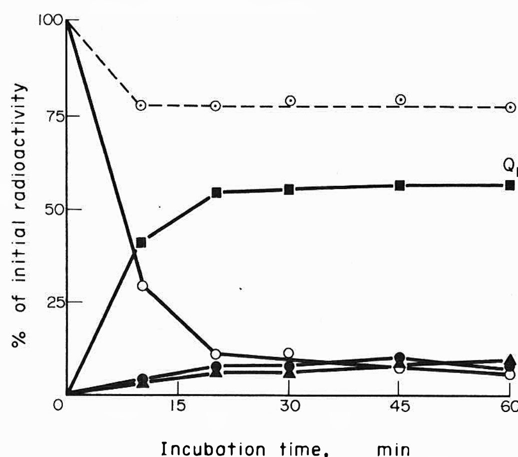

Fig. 3. Time course of aflatoxin B_1 metabolism catalysed by rhesus monkey-liver microsomes. Profiles represent residual aflatoxin B_1 (○), aflatoxins Q_1 (■) and M_1 (●) and the uncharacterized water-soluble fraction (▲). The upper curve (⊙) represents the sum of the four factors measured. Values are means from triplicate determinations.

Effect of substrate level on AFB_1 hydroxylations

Five different AFB_1 levels were used to establish the maximum velocity (V) and the apparent Michaelis–Menten constant (K_m). The solution of AFB_1 in methanol was added to the substrate in 25 μl aliquots and the reaction period was reduced to 10 min to ensure zero-order kinetics. Double-reciprocal plots of velocity as a function of substrate concentration are given in Fig. 4. The contrast between the two curves is striking. The appar-

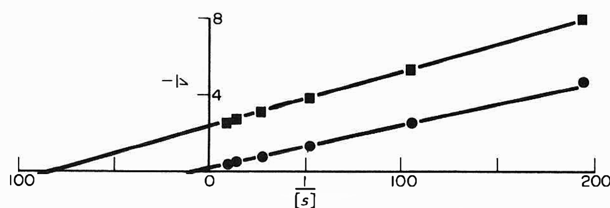

Fig. 4. Double-reciprocal plots for the formation of aflatoxins Q_1 (●) and M_1 (■). Values are means from triplicate determinations. The apparent Michaelis constants, K_m and V, are 0·166 mM and 7·70 nmol/mg protein/min, respectively, for aflatoxin Q_1, and 0·012 mM and 0·44 nmol/mg protein/min for aflatoxin M_1.

ent K_m and V for AFQ_1 formation are 0·166 mM and 7·7 nmol/mg microsomal protein/min, and the constants for AFM_1 formation are 0·012 mM and 0·44 nmol/mg microsomal protein/min. The substrate concentration required for half-maximum velocity of AFQ_1 formation is ten times greater than that for AFM_1 formation under the incubation conditions used in this study.

DISCUSSION

Cell-free preparations of rhesus monkey liver fortified with NADPH and incubated in an air atmosphere were outstanding in their ability to metabolize AFB_1 to more polar metabolites. The major metabolite was AFQ_1, which was only recently identified as the major product of the metabolism of AFB_1 by monkey and human liver *in vitro* (Hsieh *et al.* 1974a; Masri, Haddon, Lundin & Hsieh, 1974; A. S. Salhab and D. P. H. Hsieh, unpublished data 1974). The well-known AFM_1 was formed in smaller amounts and chloroform extracts of incubation media also contained detectable amounts of three unknown metabolites.

The AFB_1-hydroxylase systems were localized in the microsomal fraction of cell-free homogenates and exhibited a stringent requirement for NADPH. The observed inhibition by carbon monoxide indicated involvement of the microsomal electron-transport system, including cytochrome *P*-450. Similarly, the hydroxylase systems were sensitive to inhibition by SKF 525-A and sesamex, except that sesamex in 5×10^{-4} M concentration failed to block AFM_1 production. On the basis of these biochemical properties, the AFB_1 hydroxylases of monkey liver can be classified as typical microsomal mixed-function oxidases.

Experimental animals vary considerably in their toxic carcinogenic responses to AFB_1 as well as in their abilities to metabolize the toxin. This diversity of response suggests that metabolism may be an important factor in determining the toxic action of AFB_1 in different animal species. Patterson (1973) used the time required to metabolize an LD_{50} dose of the toxin, calculated from the *in vitro* metabolic capacity of livers, to correlate the overall metabolic rate with the hepatotoxic effects among eight animal species. He put forward the theory that the "fast metabolizing" animals (e.g. ducklings), which are capable of handling an LD_{50} dose in under 12 min, are more vulnerable to acute effects, whereas the "slow metabolizing" animals (e.g. rats) are more susceptible to chronic or carcinogenic effects. From the maximum velocity, V, for AFQ_1 formation (7·7 nmol/mg protein/min) determined in this study and the LD_{50} of 2·2 mg/kg determined for monkeys by Rao & Gehring (1971), it can be calculated that the time required for the monkey to metabolize one LD_{50} dose is approximately 10 sec. Therefore, the rhesus monkey belongs to the "fast metabolizing" group and is probably vulnerable only to the acute toxicity of AFB_1 and relatively resistant to its carcinogenic effects. Moreover, it has recently been established that AFQ_1 was only 5·5% as toxic as AFB_1 and was not mutagenic to *Salmonella typhimurium* auxotrophs (Ames, Durston, Yamasaki & Lee, 1973; Hsieh *et al.* 1974b). It is therefore reasonable to assume that the rhesus monkey, which possesses a high potential for hydroxylating AFB_1 to AFQ_1, is a relatively resistant species to carcinogenic effects.

Results of comparative biochemical studies indicate that the rhesus monkey may be more similar to man than are other experimental animals (Smith, 1967). It was found (A. S. Salhab and D. P. H. Hsieh, unpublished data 1974) that AFQ_1 was the prominent *in vitro* metabolite of AFB_1 in human liver preparations treated in the same manner as the monkey-liver preparations in the present investigation. If man responds to AFB_1 exposure

as does the rhesus monkey, he would be relatively resistant to the chronic, carcinogenic effects which are a major concern in the aflatoxin problem. Additional comparative studies are in progress.

Since the rhesus monkey may be a good metabolic model for man, results obtained in monkeys are of particular interest. In male rhesus monkeys dosed by the ip route, AFP_1 (demethylated AFB_1) excreted as the glucuronide and sulphate was the most prominent metabolite, accounting for 22% of the administered dose (Dalezios, Wogan & Weinreb, 1971). In a more recent study (Dalezios, Hsieh & Wogan, 1973), in which the oral route was used, the primary metabolite was AFM_1, which accounted for approximately 20% of the administered dose.

These two *in vivo* studies of AFB_1 disposition in rhesus monkeys have yielded results which indicate that the route of administration has a profound effect on AFB_1 metabolism. In neither study was AFQ_1 observed. This surprising result warrants further investigation in view of the rapid conversion of AFB_1 to AFQ_1 under *in vitro* conditions.

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A PROCEDURE FOR THE EXTRACTION AND ESTIMATION OF RUBRATOXIN B FROM CORN

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Abstract—Ingestion by farm animals of food contaminated by toxigenic fungi has been shown to be responsible for a number of epizootes. Species of Penicillia and Aspergilli have been identified as the dominant flora of some mould-contaminated animal feeds responsible for outbreaks of disease among farm animals. In the absence of any detection or assay procedure for rubratoxin B, a hepatotoxin elaborated by *Penicillium rubrum*, it was only possible to infer the presence of this mycotoxin in agricultural commodities from the isolation of the mould. In a simple method developed for the detection of rubratoxin B in corn, the toxin was extracted with ethyl acetate. The ethyl acetate extract was concentrated and aliquots were applied to silica-gel thin-layer plates, which were developed in unlined tanks with ascending methanol–chloroform–glacial acetic acid–water (20:80:1:1, by vol.). With this developing system and the spotting of samples under nitrogen, the low-R_F 'artefact' observed in earlier work was eliminated. The chromatography plates were heated at 200°C for 10 min, and a distinctive fluorescent derivative of rubratoxin B (R_F = 0·6) was then observed under long-wave ultraviolet light. Under the conditions used, the lower limit of detection for rubratoxin was 0·5 μg.

INTRODUCTION

In early 1952, Sippel, Burnside & Atwood (1953) described an epizootic of pigs and cattle that had consumed mouldy corn. Of the fungi isolated from the incriminated corn by Burnside, Sippel, Forgacs, Carll, Atwood & Doll (1957), only two, *Aspergillus flavus* and *Penicillium rubrum*, caused illness and death when fed to experimental animals. Fresh corn contaminated with *P. rubrum* was more toxic than corn contaminated with *A. flavus*. Feeds artificially contaminated with *P. rubrum* were toxic to pigs, horses, goats and mice. *P. rubrum* has been isolated, often as the dominant organism, from other field intoxications involving dogs (Newberne, Bailey & Seibold, 1955) and pigs (Blevins, Glenn, Hamdy, Brodasky & Evans, 1969).

A disease of poultry associated with the contamination of their feed by fungi has been reported by Forgacs & Carll (1962). In fact, Forgacs, Koch, Carll & White-Stevens (1958) showed that *P. rubrum* and the closely related species, *P. purpurogenum*, caused good grain to become toxic to chicks. Chicks that died in these controlled laboratory experiments were found to have haemorrhage and congestion of various tissues.

Rubratoxin B, the major toxic component produced by *P. rubrum*, is a potent hepatotoxic agent but is not a carcinogen. Evidence of potentiation of the lethal action of rubratoxin B, but not of the carcinogenic action of aflatoxin B_1, was found in rats treated with rubratoxin and fed a diet containing aflatoxin B_1 (Wogan, Edwards & Newberne 1971). The clinical signs of toxicity induced by rubratoxin B and the broad range and species susceptibility of this toxin are similar to those caused by aflatoxin (Hayes, Neville & Hollingsworth, 1973). Rubratoxin is also a growth retardant, a potent embryocide and a teratogen (Hood, Innes & Hayes, 1973).

Although *P. rubrum* has been isolated from the diets involved in field outbreaks, no information is available concerning the natural distribution of rubratoxin B in animal feeds or human foodstuffs. It is possible that a natural disease may result from rubratoxin alone or from the interaction of rubratoxin with another toxin. However, this hypothesis cannot be confirmed until a method is developed for isolating and estimating rubratoxin B in agricultural products. This paper reports such a method for rubratoxin B in corn. The method is simple and sufficiently sensitive for field use.

EXPERIMENTAL

Preparation of rubratoxin B. Crystalline rubratoxin B was produced by *P. rubrum* Stoll in surface cultures and purified by the method of Hayes & Wilson (1968). Purity was established by melting point, infra-red and mass spectra, thin-layer chromatography and UV molar extinction coefficient. The mycotoxin was stored in the dark until used.

Preparation of mouldy corn samples. Low-form culture flasks (no. 4422, 2500 ml; Corning Glass Works, Corning, N.Y.) containing 100 g cracked yellow corn and 300 ml of a 1% sucrose solution were sterilized at 121 lb/in.^{2} for 15 min. The corn was inoculated with a heavy conidial suspension from a corn slant of a known rubratoxin B-producing strain of *P. rubrum* and incubated for 21 days at ambient temperature under normal fluorescent lighting in a stationary environment. The mouldy corn was removed from the flask and air-dried at 30°C until brittle. Additional corn samples containing known concentrations of rubratoxin B were prepared by adding rubratoxin to the corn. Individual 100 g samples of either moulded or spiked corn were extracted and analysed for rubratoxin B.

Extraction procedure. The efficiency of organic solvents to extract rubratoxin selectively from corn without also removing compounds that interfered with thin-layer separation of the mycotoxin was evaluated. Corn samples (100 g) were mixed with a volume of solvent equal to three times the sample weight (Fig. 1). The mixture was blended in an explosive-proof Waring Blendor for 5 min and quantitatively transferred to a covered, stoppered 500 ml Erlenmeyer flask. The contents of the flask were agitated overnight on a magnetic stirrer. After removal of solids by gravity filtration, the solvent volume was reduced to one-tenth of the original volume in a rotary evaporator. The flask containing the reduced extract was tightly stoppered, wrapped in aluminium foil and stored at 4°C until the material therein was chromatographed.

Thin-layer chromatography. Standard 20 × 20 cm glass plates coated with a 0·250 mm layer of silica gel (Brinkman Instruments, Inc., Westbury, N.Y.) were used for thin-layer chromatography. The plates were air-dried for 30 min, activated at 100°C for 1 hr and stored over calcium sulphate until used. Immediately before use, plates were predeveloped with chloroform–methanol–glacial acetic acid (80:20:1, by vol.). A plate was removed from the elution tank and placed directly into an antioxidation spotting chamber, which

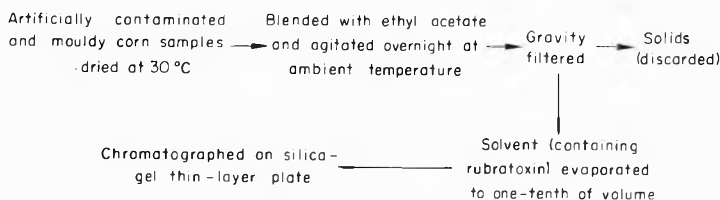

Fig. 1. Outline of procedure for extraction and purification of rubratoxin B from corn.

was purged with nitrogen at the rate of 500 cm^3/min. The plate was dried and a 10 μl sample was applied 1·5 cm from the bottom of the plate. When the solvent had evaporated, the chromatography plate was removed from the spotting cabinet and placed immediately in an unlined, unequilibrated glass chromatography tank containing 100 ml chloroform–methanol–glacial acetic acid–water (80:20:1:1, by vol.). After the solvent front had migrated approximately 16 cm from the origin, the plate was removed from the elution tank, marked and air-dried.

Detection of rubratoxin B. Rubratoxin B (5 μg) was spotted on to silica gel plates but not developed. A number of chemicals applied with an aerosol reagent sprayer (Sprayon Products, Cleveland, Ohio) were tested as potential developers of the nondescript rubratoxin. The mycotoxin was also subjected to pyrolysis. Before charring but after sample spotting (standards and corn extracts) the plate was viewed under long- and short-wave ultraviolet light in a Chromato-vue model CC-20 cabinet (Ultra Violet Products, Inc., San Gabriel, Cal.). All fluorescent areas were marked. Charring was accomplished by placing the chromatography plate on a preheated aluminium plate in a gravity convection oven equilibrated at the desired temperature ($\pm$3·0°C) for 10–15 min. The plate was removed, cooled and again viewed under ultraviolet light and the newly formed fluorescent area was marked.

The fluorescent derivative of rubratoxin which resulted from this heating was transferred with warm glass-distilled water from the thin-layer plate into a 25 ml volumetric flask using a vacuum zone collector. The sample was analysed in a Turner Model 110 Fluorometer (Turner Instruments, Inc., New York) using a 360 nm primary filter for excitation and a 460 nm secondary filter for fluorescence.

Optimization of the procedure. An atmosphere of nitrogen, hydrogen or oxygen (500 cm^3/min) was used instead of the normal room atmosphere during application of rubratoxin B and during evaporation of the sample-spotting solvent, to eliminate the low R_F 'artefact' of rubratoxin B previously reported (Hayes & Wilson, 1968). Predeveloped plates were placed in the antioxidation spotting chamber and dried before application of 5 μg rubratoxin. The gas purge continued until the solvent evaporated. Control plates containing 5 μg rubratoxin were spotted and exposed to the normal atmospheric conditions of the room for predetermined time intervals to permit increasing exposure of the toxin to this atmosphere. Plates were chromatographed, charred and visualized as previously described.

The optimal temperature for development of the rubratoxin derivative was determined by heating 15 μg of toxin for 10 min at 25°C intervals between 50 and 250°C. The thin-layer plates were developed and extracted and the fluorescent derivative was estimated as described earlier.

Optimal charring time was ascertained by varying the exposure time of samples at 200 $\pm$ 3·0°C. Time of exposure was from 2 to 16 min at 2-min intervals. After charring, the rubratoxin derivative was extracted and estimated spectrophotofluorometrically.

The lower limit of detection of the fluorescent derivative of rubratoxin B by the charring-visualization procedure was determined by spotting a series of rubratoxin B samples (0·26–5·25 μg) on silica-gel thin-layer plates. Samples were applied in a nitrogen atmosphere. The intensity of fluorescence of the derivative was estimated directly on the thin-layer plate and reported as: intense (+ + +), moderate (+ +), weak (+) or nil (−).

Evaluation of the procedure. Rubratoxin B was added to 100 g cracked yellow corn at concentrations of 101, 200 and 599 ppm. Triplicate samples of each toxin concentration

were extracted with ethyl acetate and processed as previously described. The percentage recovery of mycotoxin from spiked-corn samples was determined by comparing their fluorescence with that of standard rubratoxin B which was extracted with ethyl acetate and treated in all respects like the spiked-corn samples. Fluorescence was reported as $\bar{X} \pm SD$.

The lower limit of rubratoxin B detection in cracked yellow corn was determined by artificially contaminating triplicate 100 g samples with 54, 70, 98, 226 and 400 ppm. The corn was extracted, the extracts were reduced, 10 μl aliquots were chromatographed and charred and the concentration of the fluorescent derivative was determined.

Twelve corn cultures contaminated by a known rubratoxin-producing mould were examined for the presence of rubratoxin B. The samples were dried at 30°C and homogenized and 100 g portions were extracted with 300 ml ethyl acetate. Aliquots of the reduced extract along with known concentrations of toxin were processed as previously described. All unknowns and standards were in triplicate.

The destruction by heat of rubratoxin in mouldy corn samples was investigated. Cultures were grown as described and 100 g portions from 50 mouldy corn samples were mixed to form a composite. This composite was dried and a homogeneous mixture was prepared. Triplicate 100 g portions were heated at 10°C intervals between 25 and 100°C for 2 hr. Three additional samples were autoclaved at 121 lb/in.^{2} for 15 min. Extraction and estimation of the toxin was as previously described. Rubratoxin B (150 and 600 ppm) and the reduced extracts were spotted on silica-gel plates in a nitrogen atmosphere. The plates were developed, dried and charred at 200°C for 10 min and fluorescence was determined.

RESULTS

Of several organic solvents tested for their ability to extract rubratoxin B selectively from corn, ethyl acetate and acetonitrile were the only solvents that extracted the toxin. Since ethyl acetate removed fewer interfering substances and was less hazardous, it was used in the remaining experiments.

The stability of rubratoxin B on silica-gel adsorbent layers was examined because it has been our experience as well as that of others (Moss, 1971) that when the adsorbed mycotoxin was exposed to room atmosphere for periods longer than 3–4 min, a decomposition product formed on the thin-layer plate. The decomposition product migrated more slowly ($R_F = 0{\cdot}1$) than freshly applied or residual rubratoxin B ($R_F = 0{\cdot}6$). When application of the toxin and evaporation of the spotting solvent were carried out in an atmosphere of nitrogen, hydrogen or oxygen, the low-R_F derivative was eliminated.

Bromocresol green was the only tested spray reagent that formed a colour complex with rubratoxin B, but it was not sensitive enough to detect 5 μg of the toxin. Pyrolysis of the toxin resulted in the production of a fluorescent derivative of rubratoxin B that allowed detection of 5 μg rubratoxin B on a thin-layer plate. Amounts of rubratoxin B as low as 0·5 μg fluoresced strongly enough after charring to allow visualization (Table 1).

The detection procedure for the fluorescent derivative of rubratoxin B was optimized since the development of a sensitive assay was the objective of these experiments. The fluorescent derivative was not produced at ambient temperature (Fig. 2). Only minimal production occurred below 100°C but larger quantities were produced at temperatures between 150 and 175°C with peak production at 200°C. Temperatures in excess of 200°C resulted in decreased production of the fluorescent derivative. The optimal charring time

Table 1. *The sensitivity of rubratoxin B visualization on thin-layer plates as determined by visual observation during irradiation with ultraviolet light*

Rubratoxin B solution applied*			
Concn ($\times 10^{-1}$ mg/ml)	Volume (µl)	Weight of toxin (µg)	FDRB†
1·05	50	5·25	+ + +
	40	4·20	+ + +
	30	3·15	+ + +
	20	2·10	+ + +
	10	1·05	+ +
	5	0·53	+
0·53	10	0·53	+
0·26	10	0·26	−

* All samples were spotted on silica-gel plates in ethyl acetate, in triplicate.
†Fluorescent derivative of rubratoxin B. Degrees of fluorescence: + + +, strong; + +, moderate; +, weak; −, none.

at 200°C was 8–10 min (Fig. 3). Substantial amounts of the fluorescent derivative of rubratoxin B were also produced at 12 and 14 min with a sharp decline thereafter. In all remaining experiments, 10 min at 200°C was used.

At least 91% of the rubratoxin added to corn samples was recovered by our procedure (Table 2). Little variation was observed in the recovery of rubratoxin B in the corn samples artificially contaminated with different concentrations of toxin. Rubratoxin B was detected in corn samples containing 70 ppm rubratoxin B but not in samples containing 54 ppm (Table 3). The present lower limit of detection of rubratoxin B in contaminated corn samples is, therefore, in the range 70–100 ppm, although less than 1 µg rubratoxin B can be detected on thin-layer plates.

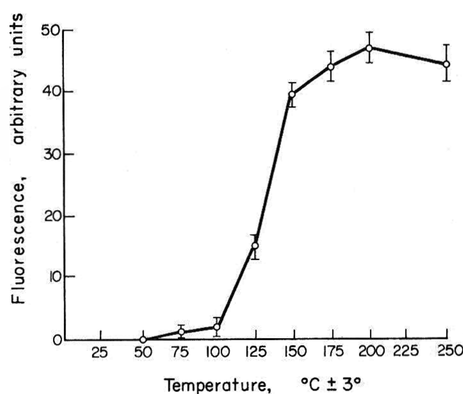

Fig. 2. Effect of temperature on production of the rubratoxin B fluorescent derivative. Rubratoxin (15 µg in ethyl acetate) was spotted on silica-gel plates and heated for 10 min and the fluorescent derivative was extracted. Each value represents a minimum of four samples run in duplicate, the bars indicating the range.

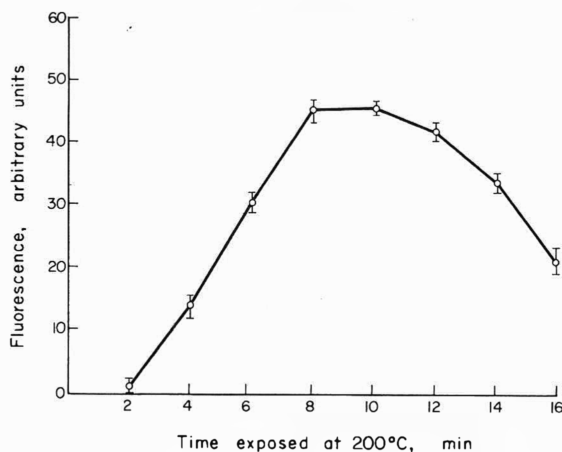

Fig. 3. Effect of heating time on production of the rubratoxin B fluorescent derivative. Rubratoxin (15 μg in ethyl acetate) was spotted on silica-gel plates and heated at 200°C and the fluorescent derivative was extracted. Each value represents a minimum of four samples run in duplicate, the bars indicating the range.

Cracked yellow corn contaminated with *P. rubrum* was incubated for 14 days at ambient temperature. The corn was dried, portions were extracted with ethyl acetate, the extract was processed under optimal conditions and the fluorescent area was removed from the silica-gel plate. The fluorescent derivative of rubratoxin B was estimated. The concentrations of rubratoxin B extracted from the twelve cultures of mould-contaminated corn were 0, 95, 157, 190, 316, 354, 365, 424, 498, 512 and 585 ppm, each value representing a minimum of four 100 g samples run in duplicate.

An important consideration in the food industry is the stability of a toxin in foodstuffs that have undergone heat-processing. The amount of rubratoxin B recovered from mouldy

Table 2. *Percentage recovery of rubratoxin B from corn samples artificially contaminated with known concentrations of the mycotoxin*

Sample*	Rubratoxin B added (ppm)	Fluorescence† ($\bar{X} \pm$ SD)	Rubratoxin B recovered (ppm)	Recovery (%)
Standards				
1	101	4·3 ± 0·8	101	100
2	150	5·6 ± 1·1	150	100
3	200	10·7 ± 0·6	200	100
4	400	21·1 ± 0·8	400	100
5	620	33·3 ± 2·1	620	100
Test samples				
1	101	3·3 ± 0·8	92	91·1
2	200	9·1 ± 0·8	185	92·5
3	599	29·6 ± 1·3	553	92·3

*Corn samples (100 g) were extracted with 300 ml ethyl acetate. Standards contained known concentrations of rubratoxin B and were subjected to the same extraction and concentration procedures, with 100% recovery. Standards were run in triplicate and were used to establish a concentration curve of fluorescence versus concentration.

†Fluorescence reported in machine units; the value given represents a minimum of four samples run in duplicate.

Table 3. *Estimation of the lower limit of detection for rubratoxin B in corn*

Rubratoxin B concn (ppm)	FDRB*
400	+
226	+
98	+
70	+
54	–

*Fluorescent derivative of rubratoxin B. fluorescence being reported as present (+) or absent (–).

corn samples heated at predetermined temperatures for 2 hr is shown in Fig. 4. The mycotoxin, or the characteristic that promotes formation of the fluorescent derivative, is rapidly destroyed by temperatures above 65°C. Some fluorescent derivative was still detectable after heat-treatment at 85°C. Heating at 100°C for 2 hr or exposure of the contaminated corn sample to 121 lb/in.^{2} for 15 min eliminated the production of the fluorescent derivative of rubratoxin B.

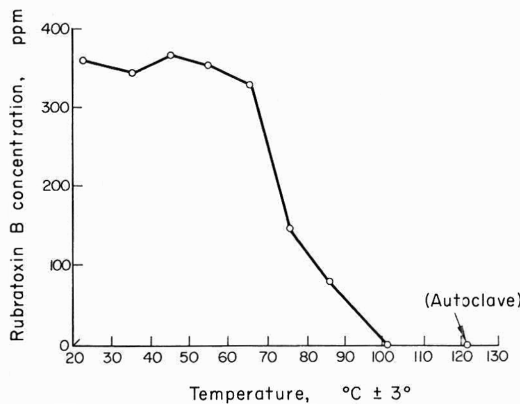

Fig. 4. Destruction by heat of rubratoxin B in mouldy corn samples. Samples were subjected to varying temperatures for 2 hr (or autoclaved for 15 min) and analysed for the presence of rubratoxin B. Each value is the mean of two samples run in duplicate.

DISCUSSION

Most research with rubratoxin B has been aimed at determining the mechanism of toxicity (Hayes & Hannan, 1973) or bioproduction (Hayes, Wyatt & King, 1970). No field assay for this toxin is available even though *P. rubrum* has been reported in a number of field outbreaks. Hayes & Wilson (1968) have reported a procedure for extraction of the toxin from a liquid medium and others (Townsend, Moss & Peck, 1966) have employed similar procedures. A bioassay employing *Tetrahymena pyriformis* has been developed for rubratoxin B (Hayes, Melton & Smith, 1974; Wyatt & Townsend, 1974). Neither procedure, however, is suitable for the detection of this mycotoxin in agricultural commodities.

A colorimetric method has been developed to estimate the presence of rubratoxin A in mixtures of crystalline rubratoxins A and B (Moss & Hill, 1970), but no method for the estimation of rubratoxin B has been reported. A procedure for rubratoxin B analysis was therefore developed, and following the discovery that a fluorescent derivative was produced on silica-gel plates after heating, a spectrophotofluorometric assay was chosen.

Although most investigators use a modification of the Hayes & Wilson (1968) method, which uses ethyl ether to extract the toxin, this solvent was ineffective for selective extraction of rubratoxin B from corn. Ethyl acetate, which previously had been used by Townsend *et al.* (1966), was the best of the solvents tested for this purpose. Because of the importance of the charring phenomenon in the development of a product for visualization, charring time and temperature were examined to determine the optimal conditions for maximum conversion of rubratoxin B to its fluorescent derivative. The optimum temperature was 200°C and the optimum time for heating at this temperature was 10 min.

The percentage recovery and sensitivity of the procedure were also determined. At least 90% of the rubratoxin added to corn samples was recovered. Since the fluorescent derivative of rubratoxin B can be detected on thin-layer plates in amounts as low as 0·5 μg but the lowest level of detection in corn samples is currently 70 ppm, it appears that extraction is the limiting factor in our procedure.

Burnside *et al.* (1957) and Wilson & Wilson (1962), while characterizing the toxin obtained from cultures of *P. rubrum*, observed that heating crude preparations of the toxin to 60–90°C had no effect upon the toxicity of their extracts. Our results show, however, that the toxin was partially destroyed or at least the formation of the fluorescent derivative was reduced after corn samples containing rubratoxin B were exposed to 65°C for 2 hr. Heating in excess of 85°C, however, was necessary to eliminate the fluorescent derivative.

The loss of mycotoxin by the formation of a low-R_F artefact on the chromatography plate has been attributed to atmospheric exposure and/or hydrolysis of the anhydride groups of the mycotoxin (Hayes & Wilson, 1968; Moss & Hill, 1970). The formation of this artefact and subsequent loss of the sample was eliminated by carrying out sample application to the adsorbent layer and evaporation of the spotting solvent in an atmosphere of nitrogen, oxygen or hydrogen.

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ABSORPTION, DISTRIBUTION AND EXCRETION OF [^{14}C]CTAB, A QUATERNARY AMMONIUM SURFACTANT, IN THE RAT

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Abstract—The absorption, distribution and excretion of orally administered [^{14}C]CTAB, a quaternary ammonium surfactant, was studied in female rats. About 80% of the dose of radioactivity was found in the gastro-intestinal tract 8 hr after administration, only small amounts were found in the blood plasma and about 2% of the administered radioactivity was excreted in the bile during the first 12 hr after treatment. The low levels of radioactivity in the serum and bile, together with the large amounts of radioactivity found in the gastro-intestinal tract, indicated poor intestinal absorption of CTAB. Only small amounts of radioactivity were found in the liver, kidneys, spleen, heart, lungs and skeletal muscle, and the tissue radioactivity declined rapidly, only traces being found in the examined tissues 4 days after [^{14}C]CTAB administration. Within 3 days of ingestion, 92% of the administered radioactivity had been excreted in the faeces and 1% in the urine. No radioactivity was found in the expired CO_2 collected during day 1 after administration of [^{14}C]CTAB. Thin-layer chromatography of bile and urine samples indicated that CTAB was metabolized to some extent in the rat.

INTRODUCTION

Surface-active quaternary ammonium compounds have a high antimicrobial activity and are widely used as skin disinfectants, surgical antiseptics, laundry additives and sanitizing agents for eating- and drinking-utensils and in milk- and other food-processing plants. Numerous reports in the literature have dealt with the toxicity of quaternary ammonium surfactants; an extensive review of reports on this subject is given by Cutler & Drobeck (1970). Although the oral toxicity of quaternary ammonium surfactants has been thoroughly studied, only a few studies of the intestinal absorption, distribution within the body and metabolism of quaternary ammonium surfactants have been reported.

Adelson & Sunshine (1952) reported the death of an adult woman who died about 25 min after accidentally ingesting an alkylbenzyltrimethylammonium surfactant; the authors claimed to have found quaternary ammonium compounds when analysing the liver. The rapid death in this case seems to imply a rapid absorption of the surfactant. Bogs & Lohse (1971) studied the distribution of an alkylbenzyldimethylammonium surfactant in dogs, cats and rabbits after oral, rectal and intramuscular administration of a tenfold lethal dose, and found only small amounts of the compound away from the site of administration. Some clinically used monoquaternary ammonium compounds have been found to be relatively stable in animals (Levine & Clark, 1955) and several were shown by Hughes, Millburn & Williams (1973) to be excreted mainly unchanged in the bile and urine after ip injection.

The study reported here was concerned with the absorption. distribution and excretion of radioactivity in rats given a single sublethal oral dose of the monoquaternary ammonium surfactant, cetyltrimethylammonium bromide (CTAB).

EXPERIMENTAL

Test material. Trimethyl([1-^{14}C]cetyl)ammonium bromide ([^{14}C]CTAB) was purchased from the Radiochemical Centre, Amersham, Bucks. The specific activity of the compound was 17·6 μCi/mg and the radiochemical purity was 99%.

Experimental procedures

Distribution studies. Female Sprague-Dawley rats (210–240 g) were starved for 24 hr and then given [^{14}C]CTAB (0·8 mg/kg) as an aqueous solution by gastric intubation. The administered volume was 4·0 ml/kg body weight. Blood samples were taken from the tail under mild ether anaesthesia. The animals were killed 2, 4, 8, 24, 48, 72 and 96 hr after intubation. In experiments lasting for more than 24 hr the animals were provided with food and water *ad lib.* 8 hr after intubation. Tissue samples weighing 100–200 mg were cut in duplicate from the liver, kidneys, spleen, heart, lungs and hind leg (gastrocnemius muscle) for radioassay. In animals killed 8 hr after gastric intubation, the gastro-intestinal tract was removed and divided into four parts, namely the stomach, the proximal and distal halves of the small intestine and the caecum together with the colon. The contents of the different parts of the gastro-intestinal tract were collected by rinsing with saline.

Excretion studies. For the study of the excretion of ^{14}C-labelled compounds, similarly treated animals were kept in metabolism cages permitting the separate collection of urine and faeces, which were removed at 4-hr intervals for a period of 3 days and kept refrigerated until examined. Faeces were homogenized in ethanol and aliquots were taken for radioassay. Expired CO_2 was trapped in 10% NaOH at 4-hr intervals during day 1 after intubation. Saturated $BaCl_2$ was added and the precipitated $BaCO_3$ was filtered off and dried, aliquots being taken for radioactivity determinations.

Bile collections. Biliary excretion was studied by means of a polyethylene cannula inserted into the common bile duct of rats anaesthetized with sodium pentobarbitone (40 mg/kg, ip). When bile flowed freely, the abdominal incision was closed and an aqueous solution of [^{14}C]CTAB (0·8 mg/kg) was administered by gastric intubation. The bile was collected for 12 hr at 2 hr intervals and the samples were refrigerated until examined.

Analytical methods

Chromatography. Some of the faeces, bile and urine samples were subjected to thin-layer chromatography on silica gel F-254 (Merck AG), using chloroform–methanol–water (65:25:4, by vol.). Autoradiograms were prepared by exposing the chromatograms to X-ray film (Structurix D-7, Agfa-Gevaert). Aqueous solutions of [^{14}C]CTAB were incubated for 30 min with bile, urine and faeces (as aqueous homogenates), collected from the animals before the administration of [^{14}C]CTAB. These samples and [^{14}C]CTAB dissolved in ethanol were run as references. No further attempts were made to identify the metabolites observed.

Measurement of radioactivity. Aliquots of bile, urine and blood plasma were pipetted into counting vials and a scintillation fluid (Aquasol, NEN Chemicals, Dreieichenhain bei Frankfurt/M., Germany) was added. Tissues and 0·2–0·3 g aliquots of the faecal homogenates were solubilized in Protosol (NEN Chemicals) by incubation overnight at 55°C and were then counted in a toluene-based scintillation fluid (4·0 g Omnifluor/litre toluene, from NEN Chemicals). The different parts of the gastro-intestinal tract and their contents were placed in a solution containing equal quantities of 40% NaOH and ethanol and incubated overnight at 55°C. Aliquots (0·2–0·3 g) of the solubilized

samples were pipetted into counting vials and a toluene–ethanol-based scintillation fluid was added. This scintillation fluid had the following composition: 680 ml toluene, 320 ml ethanol, 5·88 g 2,5-diphenyloxazole (PPO) and 0·12 g *p*-bis-(*o*-methylstyryl)benzene. The $BaCO_3$ precipitates were thoroughly ground and 0·3–0·4 g samples were counted in Aquasol with the scintillation fluid gelled by addition of water. Thin-layer chromatograms were quantified by counting scrapings in the toluene–ethanol-based scintillation fluid. All samples were counted in a liquid scintillation spectrometer (LKB-Wallace 81000) and the values obtained were corrected for quenching using [^{14}C]toluene as an internal standard.

RESULTS

Distribution of radioactivity

The distribution of radioactivity in the gastro-intestinal tract 8 hr after oral administration of [^{14}C]CTAB is shown in Table 1. A total of about 80% of the administered radioactivity was found in the gastro-intestinal tract, about 87% of this amount being in the gastro-intestinal contents. About 90% of the administered dose had left the stomach within 8 hr.

Table 1. *Distribution of radioactivity in the gastro-intestinal tract of rats 8 hr after oral intubation of 0·8 mg [^{14}C]CTAB/kg*

Region of gut		Radioactivity (% of administered dose*)
Stomach		10·0 ± 3·1†
Small intestine, proximal half		2·5 ± 0·3†
Small intestine, distal half		13·5 ± 1·5†
Caecum and colon		53·8 ± 2·4†
Complete gastro-intestinal tract:	Total	79·9 ± 1·3†
	Contents	69·8 ± 2·4
	Wall	10·1 ± 2·3
Gastric emptying		90·0 ± 3·1

*Values represent means ± SEM for five rats.
†Wall plus contents.

Only small amounts of radioactivity were found in tissues other than the gastro-intestinal tract. As is apparent from Fig. 1, the level of radioactivity in all the organs examined exceeded that of the blood plasma, the peak level in which occurred 2–4 hr after administration of the dose. The liver and kidneys showed the highest levels of radioactivity, the peak in these two organs occurring approximately 8 hr after dosing. At that time, assuming an even distribution of radioactivity in the liver tissues, the liver contained about 0·8% of the administered radioactivity, but 4 days after the administration of [^{14}C]CTAB only traces of radioactivity remained in the liver and kidneys. The amount of radioactivity found in the skeletal muscle and spleen was 5–10% of that found in the liver. In the heart and the lungs (not shown in the figure) the levels of radioactivity were about the same as those in skeletal muscle.

Excretion of radioactivity

The excretion of radioactivity in bile is shown in Fig. 2. About 2% of the administered dose was excreted in the bile during the first 12 hr after administration of [^{14}C]CTAB. There was thus no appreciable enterohepatic circulation of radioactivity. The low levels

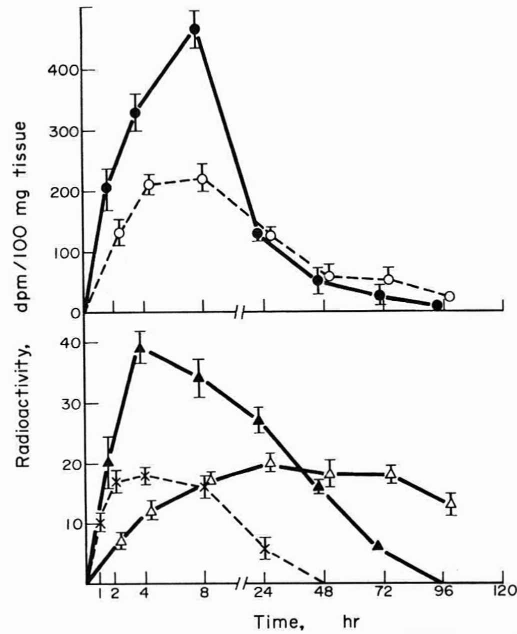

Fig. 1. The amount of radioactivity (dpm/100 mg) in the liver (●), kidney (○), spleen (▲), skeletal muscle (△) and blood plasma (×) of rats killed at various times after receiving an oral dose of [^{14}C]CTAB (0·8 mg/kg). The results are given as the means ± SEM for three or four determinations.

of radioactivity in the serum and bile, together with the large amounts of radioactivity found in the gastro-intestinal tract, indicated poor intestinal absorption of CTAB. Thin-layer chromatography of bile revealed five spots of radioactivity all with an R_F value smaller than that of the [^{14}C]CTAB standard. Cationic surfactants can form ion-pairs with organic anions and it has been suggested that quaternary ammonium compounds are

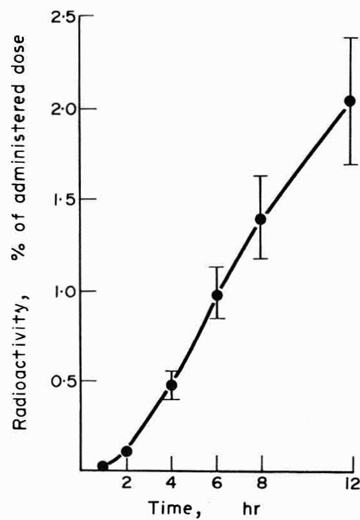

Fig. 2. Cumulative excretion of radioactivity in the bile after oral intubation of 0·8 mg [^{14}C]CTAB/kg. The results are given as the means ± SEM for three determinations.

transferred across the gut wall as neutral complexes by virtue of their combination with endogenous anions (Levine & Pelikan, 1961). To test whether such a complex could explain the spots found on the chromatograms, [^{14}C]CTAB was incubated with bile collected from the animals before treatment. However, chromatography of these incubates showed only one radioactive spot, with an R_F value similar to that of the [^{14}C]CTAB standard.

The urinary and faecal excretion of radioactivity over a 3-day period following the administration of [^{14}C]CTAB is summarized in Table 2. After 3 days, 92% of the administered dose of radioactivity had been excreted in the faeces and only 1% in the urine. Thin-layer chromatography of ethanolic faecal extracts revealed three radioactive spots. That with the same R_F value as the [^{14}C]CTAB standard accounted for about 85% of the radioactivity in the faeces collected during day 1, while the other two spots had R_F values smaller than that of the [^{14}C]CTAB standard. In the urine, the peak levels of radioactivity were found in samples collected 4–8 hr after dosing. Thin-layer chromatograms of urine samples showed four radioactive spots, the R_F values of which were in one case similar to and in the others smaller than the [^{14}C]CTAB standard. Faecal homogenates and urine incubated with [^{14}C]CTAB showed only one radioactive spot, with the same R_F value as the standard. No radioactivity was found in the expired CO_2 collected during day 1 after administration of [^{14}C]CTAB.

Table 2. *Distribution of radioactivity in urine and faeces after oral intubation of 0·8 mg [^{14}C]CTAB/kg*

	Radioactivity (% of administered dose*)		
Day	Faeces	Urine	Faeces + Urine
1	89·10 ± 2·23	1·06 ± 0·11	90·16 ± 1·98
2	2·83 ± 1·20	0·13 ± 0·02	2·96 ± 1·06
3	0·56 ± 0·17	0·03 ± 0·02	0·59 ± 0·15
Total	92·49 ± 2·06	1·22 ± 0·10	93·71 ± 2·15

*Values represent means ± SEM for four rats.

DISCUSSION

Acute toxicity studies with quaternary ammonium surfactants have shown that the surfactants are 10–100 times more toxic when administered by the iv than by the oral route (Finnegan, Larson, Smith, Haag, Reid & Dreyfuss, 1953), a finding which implies poor intestinal absorption of the surfactants. In this study, about 80% of the dose of radioactivity was found in the gastro-intestinal tract 8 hr after oral administration of [^{14}C]CTAB, only small amounts of radioactivity were found in the blood plasma and there was no appreciable enterohepatic circulation of radioactivity. These findings indicate that CTAB is poorly absorbed in the gastro-intestinal tract. Incomplete absorption of monoquaternary ammonium compounds has previously been reported by Levine, Blair & Clark (1955), who studied the intestinal absorption of certain anticholinergic monoquaternary ammonium salts using *in vivo* loops and found that the total amount absorbed ranged from 10 to 20% at the end of 3–4 hr, the major part of the absorption occurring within about 30 min.

Only small amounts of radioactivity were found in tissues other than the gastro-intestinal tract. The surfactant is apparently not preferentially distributed to any single target

organ. The highest concentration of radioactivity was found in the liver, which contained about 0·8% of the administered radioactivity 8 hr after gastric intubation. Part of this radioactivity probably represented unchanged [^{14}C]CTAB; column chromatography of ethanolic liver extracts from rats given an oral dose of [^{14}C]CTAB have shown that about 40% of the radioactivity in the liver probably corresponds to [^{14}C]CTAB (H. Sarelin and B. Nygård, unpublished data 1974). The tissue radioactivity declined rapidly, only traces being found 4 days after dosing.

The administered radioactivity was almost totally excreted in the faeces within 3 days. Since bile-duct cannulation showed that only a small percentage of the radioactivity was excreted via the bile and since only small amounts entered the blood, it was apparent that most of the radioactivity present in the faeces following oral administration represented unabsorbed material.

Highly polar compounds are not readily metabolized in the body. The surfactant studied is strongly polar in that it contains a quaternary ammonium group and thus exists as a cation at physiological pH. While it was found that CTAB passed largely unabsorbed and unchanged through the gastro-intestinal tract, thin-layer chromatography of urine and bile samples showed that most of that absorbed was probably subjected to metabolic transformation. Sufficient material for identification of the metabolites was not present in the biliary and urinary excretions. It has been found that CTAB in low concentrations (10^{-4}–10^{-3} M) can damage the intestinal mucosa (Moore, Zatzman & Overack, 1971; Nissim, 1960; Taylor, 1963), and the amount of CTAB administered was therefore kept to a minimum. No radioactivity was found in the expired CO_2, so no complete oxidation of the cetyl group occurred. *N*-Dealkylation of the surfactant therefore seems unlikely. Hughes *et al.* (1973) studied the excretion of ip injected CTAB and certain other monoquaternary ammonium compounds and found that cations with a molecular weight exceeding 250 were excreted mainly unchanged in the bile. Some of the compounds were found to be excreted in the bile as glucuronic acid conjugates, and it is possible that the metabolites demonstrated in the bile in this study were conjugates of this type.

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SHORT PAPERS

EFFECT ON GUINEA-PIGS OF FEEDING NITROSOMORPHOLINE AND ITS PRECURSORS IN COMBINATION WITH ASCORBIC ACID

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Summary—Nitrosomorpholine (70 mg/litre drinking-water) or morpholine (6·33 g/kg food) plus sodium nitrite (1·0 g/litre drinking-water) was administered to guinea-pigs, which were fed a diet containing either a high or a low level of ascorbic acid or no ascorbic acid at all. Animals on the ascorbic acid-deficient diet developed scurvy before toxic effects of either the nitrosamine or the nitrosamine precursors were evident. Precancerous liver changes occurred in guinea-pigs receiving nitrosomorpholine together with the high or low levels of ascorbic acid. Animals receiving the nitrosamine precursors together with ascorbic acid were not affected. Guinea-pigs and other species that require exogenous sources of ascorbic acid may be protected from the hazard of *in vivo* nitrosation by the presence of ascorbic acid in the stomach.

Introduction

Nitrosamines have been detected in a wide variety of consumer products, including grains, meats, alcohol and tobacco (Lijinsky & Epstein, 1970; Magee & Barnes, 1967; Rhoades & Johnson, 1972). Several recent studies indicate that, under suitable conditions, secondary amines can react with nitrites to form nitrosamines *in vivo* (Greenblatt, Mirvish & So, 1971; Lijinsky & Greenblatt, 1972; Wolff, 1972), suggesting that the potential carcinogenic hazard of nitrosamines may be even greater than was originally suspected. Experiments with mice (Greenblatt, 1973) and rats (Kamm, Dashman, Conney & Burns, 1973) have shown that the presence of ascorbic acid or ascorbate in the stomach prevents the hepatotoxicity resulting from the ingestion of secondary amines plus nitrite, apparently by blocking the nitrosation reaction. These animals, however, normally synthesize ascorbic acid rather than depend on a dietary source. The risk of nitrosamine formation from environmental precursors in species, such as man, that require a daily intake of ascorbic acid has been difficult to evaluate.

The present report describes an attempt to study nitrosamine toxicity and the nitrosation process in guinea-pigs, which resemble man in requiring an exogenous source of ascorbic acid (Chatterjee, 1973). Nitrosomorpholine was selected as the test nitrosamine in these preliminary studies because of its demonstrated capacity to induce hepatotoxicity and liver tumours in rodents within a short period of time (Druckrey, Preussmann, Ivankovic & Schmähl, 1967).

Experimental

Animals and materials. Male and female guinea-pigs of the Duncan Hartly strain, weighing 200–250 g, were obtained from Carworth Laboratories, New York*. The basic diet (ground oats, 40·0%; ground bran, 15·0%; alfalfa, 8·0%; sodium chloride, 0·5%; calcium carbonate, 1·0%; magnesium sulphate, 0·5%) was purchased from Nutritional Biochemicals, Cleveland, Ohio. L-Ascorbic acid, certified morpholine and sodium nitrite (crystalline) were obtained from Fisher Scientific Co., Atlanta, Ga. Nitrosomorpholine was prepared from morpholine and sodium nitrite in an acid solution (Pensabene, Fiddler, Dooley, Doerr & Wasserman, 1972). Ascorbic acid (at a high level of 6·7 g/kg food or a low level of 150 mg/kg) and/or morpholine (6·33 g/kg) were blended into the basic diet. Nitrosomorpholine (70 mg/litre) or $NaNO_2$ (1·0 g/litre) was added to the drinking-water, which was supplied to each cage in amber bottles. The guinea-pigs were allowed free access to the food and water supply.

Treatment. Guinea-pigs were randomly divided into nine groups, each consisting of three males and three females. Each group was fed one of the levels of ascorbic acid or an ascorbic acid-free diet and one of the three treatment regimens, as shown in Table 1. All possible combinations of ascorbic acid and nitrosamine or precursors were administered. Animals were killed after the feeding periods listed. Sections of liver and lung and occasional samples from other organs were fixed in buffered formalin. Histopathological examination was performed by Dr. T. E. Murchison, Dawson Research Corp., Orlando, Fla.

Table 1. *Liver and lung alterations in guinea-pigs fed nitrosomorpholine or precursors in combination with ascorbic acid*

Dietary level of ascorbic acid*	Treatment†	Duration of feeding (wk)	Type of tissue alteration‡	
			Liver	Lung
High	None (control)	25		
High	Nitrosomorpholine	25	Focal giant liver cells (3)	
High	Morpholine + $NaNO_2$	25		
Low	None (control)	9		
Low	Nitrosomorpholine	9	Occasional giant cells (3) and liver-cell hyperplasia (2)	Lymphoid hyperplasia (1)
Low	Morpholine + $NaNO_2$	9		
None	None (control)	3		
None	Nitrosomorpholine	3	Liver-cell hyperplasia (1)	
None	Morpholine + $NaNO_2$	3		

*High, 6·7 g/kg; low, 150 mg/kg.
†Nitrosomorpholine, 70 mg/litre drinking-water; $NaNO_2$, 1·0 g/litre drinking-water; morpholine, 6·33 g/kg diet.
‡Numbers in parentheses indicate the numbers of animals affected/group of six.

Results and Discussion

Guinea-pigs in the three groups fed the high level of ascorbic acid remained healthy throughout the 25 wk of trials. A routine analysis (Schaffert & Kingsley, 1954) during wk

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

24 confirmed the presence of ascorbic acid in the urine of these animals, indicating tissue saturation with ascorbic acid. All the guinea-pigs fed diets devoid of ascorbic acid exhibited classical symptoms of vitamin C deficiency (including weight loss, diarrhoea and loss of leg mobility) after 2 wk. Survivors in these groups were killed during wk 3. Several guinea-pigs on the low-level ascorbic acid diet developed deficiency symptoms after 6–8 wk and the survivors were killed at 9 wk.

Pathological findings in the liver and lung tissue from guinea-pigs receiving the various treatment and diet combinations are summarized in Table 1. Lesions indicative of a precancerous condition were observed in livers from about half of the animals receiving nitrosomorpholine. Concomitant feeding of ascorbic acid did not prevent the hepatotoxicity, which occurred in both sexes. The most pronounced hepatotoxicity developed in guinea-pigs that ingested the nitrosamine for the full 25 wk. However, those receiving nitrosomorpholine for only 9 wk showed moderate liver alterations. Thus, in guinea-pigs, nitrosomorpholine, like diethylnitrosamine (Argus & Hoch-Ligeti, 1963), appears to have an effect primarily on the liver. The occurrence of lung hyperplasia in a single animal receiving treatment for 9 wk and the liver-cell hyperplasia seen in one animal that survived for only 3 wk cannot definitely be attributed to the nitrosamine treatments since the incidence was too small.

Carcinogenic nitrosamines and nitrosoureas have been recovered from the stomach contents of rats that received, by intubation, the precursors of these *N*-nitroso compounds (Lijinsky & Greenblatt, 1972; Mirvish & Chu, 1973). Mice that ingested morpholine together with $NaNO_2$ have been shown to develop a high incidence of lung tumours, presumably as a result of nitrosamine formation (Greenblatt *et al.* 1971). Morpholine has been shown to react with nitrite *in vitro* to form nitrosomorpholine when pH and other conditions simulated those of mammalian stomach contents; nitrosation did not occur in the presence of ascorbic acid (Mirvish, Wallcave, Eagen & Shubik, 1972).

In the present study, the feeding of morpholine and $NaNO_2$ together with ascorbic acid, in concentrations that caused tumours in mice, did not cause any of the toxic responses seen with nitrosomorpholine. Although we speculate that the presence of ingested ascorbic acid prevented nitrosamine formation, this fact could not be proved unequivocally, since removal of ascorbic acid from the diet of guinea-pigs resulted in their early death. Nevertheless, man and other species requiring dietary sources of ascorbic acid may have a built-in protection against *in vivo* nitrosation. We are currently investigating other methods of measuring nitrosamine formation in guinea-pigs.

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MUTAGENIC EVALUATION OF AN ALCOHOLIC EXTRACT FROM γ-IRRADIATED POTATOES*

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Summary—A mutagenic study was carried out on extracts of irradiated and non-irradiated potatoes using the dominant lethal test in the mouse. Potatoes were irradiated with approximately 12 krad of γ-radiation and an alcoholic extract prepared from them was given to a group of five male mice by gavage for 7 days. For comparison, two additional groups, each of five male mice, were given an extract of non-irradiated potatoes and physiological saline, respectively. Each male was then mated sequentially with three female mice for each of 5 successive weeks. The females were killed on day 18 after their caging with a male. Corpora lutea were counted and examinations were made for live, dead and resorbed embryos. The feeding of extracts of irradiated or non-irradiated potatoes had no effect on pregnancy rates, total numbers of implantation sites or early and late foetal deaths. No mutagenic effect was observed following these treatments.

Introduction

The dominant lethal assay is a convenient test for major genetic damage in mammals and has been used for measuring the mutagenic effects of a wide range of pharmaceuticals, environmental contaminants, pesticides and food additives (Epstein, Arnold, Andrea, Bass & Bishop, 1972).

Russian workers have claimed recently that dominant lethal mutations were induced in mice after oral administration of an alcoholic extract of irradiated potatoes (Kopylov, Osipova & Kuzin, 1972). Such effects were not observed in mice given a similar extract prepared from unirradiated potatoes. The extracts used were prepared 24 hr after irradiation using procedures that are standard for the extraction of *o*-polyphenols and *o*-polyquinones. It was previously reported that similar extracts were toxic to plants and caused weight loss and a decrease in leucocyte counts in rats (Kuzin, Plyshevskaya, Kopylov, Ivanitskaya, Lebedeva, Kolomijtseva, Tokarskaya & Melnikova, 1965).

Potatoes irradiated with γ-radiation at dose levels sufficient to inhibit their sprouting have been cleared for human consumption by health authorities in many countries, and the irradiation dose of 10 krad used in the Russian work mentioned above is within the range normally used for the inhibition of sprouting. Because of the serious implications of the Russian findings, the present study was undertaken in an attempt to duplicate and verify those results.

Experimental

Materials. The potatoes used in this study were of the Katahdin variety, which is widely grown in the eastern regions of the United States and Canada They were supplied by the

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Canada Department of Agriculture Research Station in Fredericton, New Brunswick, from a crop planted at the Potato Breeding Substation, Alma, New Brunswick, during the first week of June, top-killed during the second week of September and harvested during the final week of September, 1972. The potatoes had been stored at approximately 5·5°C and 90% relative humidity for about 30 wk before being shipped to Bio-Research Laboratories where they were stored for 2 wk at approximately 4°C before being transferred to the irradiation facility during the second week of May 1973. They received no chemical treatment of any kind.

Irradiation. One 1-kg sample of potatoes was irradiated in the pneumatic cobalt-60 irradiation facility of Atomic Energy of Canada Ltd., at Ottawa. The potatoes were irradiated in a single layer at a dose rate of 55 rad/min for 221 min. Readings on four dosimeters irradiated simultaneously showed a mean irradiation dose of 12·331 krad. A second 1-kg sample of potatoes was put into the irradiation facility under identical conditions but was not irradiated.

Preparation of potato extracts. Following their removal from the irradiation facility, both batches of potatoes (irradiated and non-irradiated) were cooled by storage at 0–4°C overnight. Within 24 hr of irradiation or sham irradiation, the unpeeled potatoes were homogenized at −5°C with 95% ethanol adjusted to pH 2·0 with HCl. The homogenate was mixed thoroughly for 2 hr, the solids were separated in a Buchner filter and the alcohol was removed using a rotary evaporator in an argon stream at 20°C. The ratio of tissue: alcohol was 1:5. The concentrated aqueous solution was adjusted to pH 2·0 with NaOH and filtered into a dark flask. Oxygen was removed by flushing with argon and the flask was sealed. In the dominant lethal test these extracts were given to male mice by gavage for 7 days, treatment starting the day following the preparation of the extracts.

Dominant lethal test. Mature male and female ICR Swiss albino mice were obtained from Bio-Breeding Laboratories, Ottawa. These mice were derived from a nucleus of animals originally obtained from the Charles River Laboratories. They were therefore essentially identical with the ICR/HA Swiss mice commonly used in the dominant lethal assay (Epstein *et al.* 1972). During the entire study all animals were given a standard mouse laboratory diet and water *ad lib.* After a 1-wk period of acclimatization, 15 male mice were assigned at random to three experimental groups. These groups of five male mice* were given physiological saline (control, group I), the extract of non-irradiated potatoes (group II) or the extract of irradiated potatoes (group III), each mouse being treated twice daily by gavage with 0·4 ml of the respective solution (saline or extract) for 7 consecutive days. The total daily dose of 0·8 ml of extract was equivalent to a daily intake of 3 g potatoes (fresh weight). Treatments were started within 48 hr of the irradiation of the potatoes and within 24 hr of the preparation of the extracts. A minimum time lapse of 3 hr was observed between the two treatments on any given day.

Immediately after the last treatment each male was mated with three virgin females* for 1 wk. Upon completion of wk 1 of mating, all females were removed from the cage of each male and replaced in each case with a new batch of three virgin females for wk 2 of mating. The same procedure was followed for wk 3, 4 and 5. Females were killed on day 18 after their initial day of caging with the male; this was 14 days after the mid-week of their presumptive mating and was therefore between day 11 and 17 of their respective

*When this study was instituted the male and female group size used was generally considered acceptable, although the resulting implantation rate of 150–195/wk was somewhat less than the minimum of 200 currently recommended.

pregnancies. At autopsy each female was examined for evidence of pregnancy, corpora lutea were counted and examinations were made for deciduomas and live and dead foetuses.

Results

Pregnancy rates were consistently high in all three groups during the 5 wk of mating (Table 1). The numbers of total implantations/pregnancy, also shown in Table 1, were within the normal limits established by Epstein *et al.* (1972) in a large number of control studies. Since late foetal deaths were rarely observed in this study, both early and late deaths were pooled and the total numbers of deaths/pregnancy are presented in Table 1, which shows that extracts of non-irradiated or irradiated potatoes caused no increase in the number of deaths/pregnancy in this study. Only four females in the entire study showed more than three deaths/pregnancy. One of these, which was mated 3 wk after treatment of the male with the extract of non-irradiated potatoes, showed ten early deaths and no live foetuses and was therefore eliminated from the calculations as being atypical.

Statistical evaluation using Student's t test revealed no differences in numbers of implantations, foetal deaths or live pups between the controls and either group given a potato extract.

Table 1. *Pregnancy rate, total implantations and foetal deaths following treatment of male mice with extracts of non-irradiated and irradiated potatoes*

Parameter	Group no.†	Values resulting from mating during wk*				
		1	2	3	4	5
Pregnancy rate (%)‡	I	93	93	100	80	87
	II	80	93	80	87	93
	III	100	10C	93	93	87
Mean total implantations/pregnancy	I	11·9	10·9	11·2	11·2	12·4
	II	12·0	11·9	10·8	10·4	12·2
	III	11·4	12·6	12·5	11·3	12·0
Mean foetal deaths/pregnancy	I	1·1	1·0	1·0	1·3	1·1
	II	0·9	1·0	1·1	1·1	1·3
	III	0·4	1·3	1·1	0·5	0·8

*Wk numbered from end of treatment.
†Treatments: group I—control; group II—extract of non-irradiated potatoes; group III—extract of irradiated potatoes.
‡(Females pregnant/females mated) × 100.

Discussion

The work of Kopylov *et al.* (1972) was repeated as closely as possible, on the basis of their descriptions, and the extraction procedures used were identical with those described in the Russian paper, but alcoholic extracts prepared from irradiated potatoes were not found to be mutagenic in this study. The reason for this discrepancy between the Russian results and our own is not clear. One possible explanation would be that the effect reported by the Russian workers is dependent upon the strain of mouse used, but their report does not identify the strain. ICR Swiss mice, which have been used most widely in dominant lethal mutation studies, were used in our trials. These mice, from Bio-Breeding Laboratories, Ottawa, have been used in teratology studies of various drugs and chemicals during recent years and the results obtained have been identical with those recorded in mice of

the same strain shipped directly from Charles River Laboratories. Therefore, although no positive control group was included in this study, there was no reason to suspect that the reaction of the Ottawa-bred rats in this assay would differ from that of the Charles River rats used by Epstein *et al.* (1972).

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INDUCTION OF CHOLANGIOCARCINOMA FOLLOWING TREATMENT OF A RHESUS MONKEY WITH AFLATOXIN

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Summary—A female rhesus monkey, which had received aflatoxin for a period of over 5 yr, partly by intramuscular injection but principally by oral intubation, developed a metastasizing intrahepatic bile-duct carcinoma, which was detected at autopsy 5·25 yr after toxin administration was discontinued.

Introduction

Aflatoxins are known to be highly potent hepatocarcinogens in several species of experimental animal (Newberne & Butler, 1969). So far, there have been only two instances in which hepatocellular carcinoma was found in a rhesus monkey following the feeding of aflatoxin (Adamson, Correa & Dalgard, 1973; Gopalan, Tulpule & Krishnamurthi, 1972). The present communication reports the induction of an intrahepatic bile-duct carcinoma in a rhesus monkey treated with aflatoxin.

Experimental

The experimental details of this study were reported in an earlier communication (Gopalan *et al.* 1972). In summary, a female rhesus monkey, weighing 2·0 kg, was treated with a crystalline preparation of mixed aflatoxins (B_1 44%, G_1 44%, and B_2 and G_2 2%) for 5·5 yr from October 1963. For the first 12 months, the preparation was given intramuscularly on 5 days/wk in a dose of 50 μg/day for 1 month and 100 μg/day thereafter, while for the remaining 54 months of treatment a dose of 100 μg/day was given orally on 5 days/wk.

After treatment was discontinued, the animal was kept under observation. Liver histology was studied by needle biopsy at regular intervals and an open-wedge biopsy was done in October 1971. Liver morphology was unremarkable at all times, including the time of explorative laparotomy. During the last two months of observation the monkey became severely anorexic and inactive. It lost weight progressively and finally became moribund and was killed on 20 July 1974.

Results and Discussion

The animal weighed 4·5 kg at autopsy. A massive globular greyish-white hard mass occupied the lateral portion of the right lobe of the liver and extended from the superior to the inferior surface. The growth was adherent to the diaphragm. On sectioning, necrotic material exuded, leaving behind irregular cavities and a few greyish-brown haemorrhagic areas. The cut surface over wide areas was greyish-white, however (Fig. 1). A few satellite

nodules, about 1 cm in diameter, were found in association with the tumour. Three lymph-nodes in the porta hepatis were enlarged and two were greyish-white and hard. The liver with the tumour weighed 465 g.

Microscopic examination of the tumour revealed duct-like neoplastic structures in a stroma of predominantly fibrous tissue (Fig. 2). These structures were lined with a single layer of cuboidal epithelium with eosinophilic cytoplasm and round vesicular nuclei. In some of these structures the lumen was distended with large squamous cells (Figs 2 & 3). The neoplasm also included extensive areas of squamous-cell carcinoma exhibiting keratinization and tonofibrils between the cells (Fig. 4). Giant nuclei and mitoses were common, but the characteristic epithelial pearls were seen only rarely. In a few areas the tumour presented a picture of papillary adenocarcinoma with tall columnar cells and mucous secretion (Fig. 5). There were extensive areas in which the tumour tissue was necrotic. The stroma in general was moderately fibrous and showed minimal infiltration with round cells. Neoplastic invasion of the blood vessels and tumour emboli were identified in a careful search. The remaining liver parenchyma of the right lobe showed centrilobular necrosis of recent origin. The lymph nodes in the porta hepatis presented a picture of metastatic squamous-cell carcinoma (Fig. 6) and the diaphragm was found to be infiltrated by tumour tissue. The only site of remote metastasis demonstrated in this animal was a lymph node in the hilum of the right lung. On the basis of the morphology, the tumour was designated an adenoacanthoma of the intrahepatic bile ducts. Alternatively it might be called an adenosquamous cholangiocarcinoma.

It is considered, for the following reasons, that this tumour was induced by aflatoxin. First, the animal received aflatoxin for a prolonged period and this toxin is, in several species of animals, one of the most potent hepatocarcinogens known (Newberne & Butler, 1969). Secondly, cholangiocarcinomas constitute one of the known sequelae of chronic aflatoxicosis in lower animals (Butler & Barnes, 1964; Butler, Greenblatt & Lijinsky, 1969; Carnaghan, 1965; Grice, Moodie & Smith, 1973), but the frequency with which these tumours arise is much lower than that of the hepatocellular carcinomas. Thirdly, this type of tumour does not seem to arise spontaneously in monkeys (Gopalan *et al.* 1972; O'Gara & Adamson, 1972).

Of the two aflatoxin-treated monkeys that have been studied at this Institute, one developed a hepatocellular carcinoma while the other developed a neoplasm of the intrahepatic bile ducts. The monkey that developed hepatocellular carcinoma (Gopalan *et al.* 1972) was a male, while the one considered here was a female. Also, these two animals received different amounts of aflatoxin, the male receiving twice the quantity of toxin given to the female. There is, however, no way of finding out whether the difference in the times taken by these animals to develop the cancer and the variation in their morphology could be accounted for by the difference in their sex and/or in the total dosage of the toxin received by each animal.

These observations in non-human primates lend increasing support to the concept that aflatoxin consumption is possibly related to human primary liver cancer in some areas like Swaziland (Keen & Martin, 1971) and Thailand (Shank, Gordan, Wogan, Nondasuta and Subhamani, 1972). They also underline the need for a constant review of the so-called 'safe' limits of these toxins in human diets.

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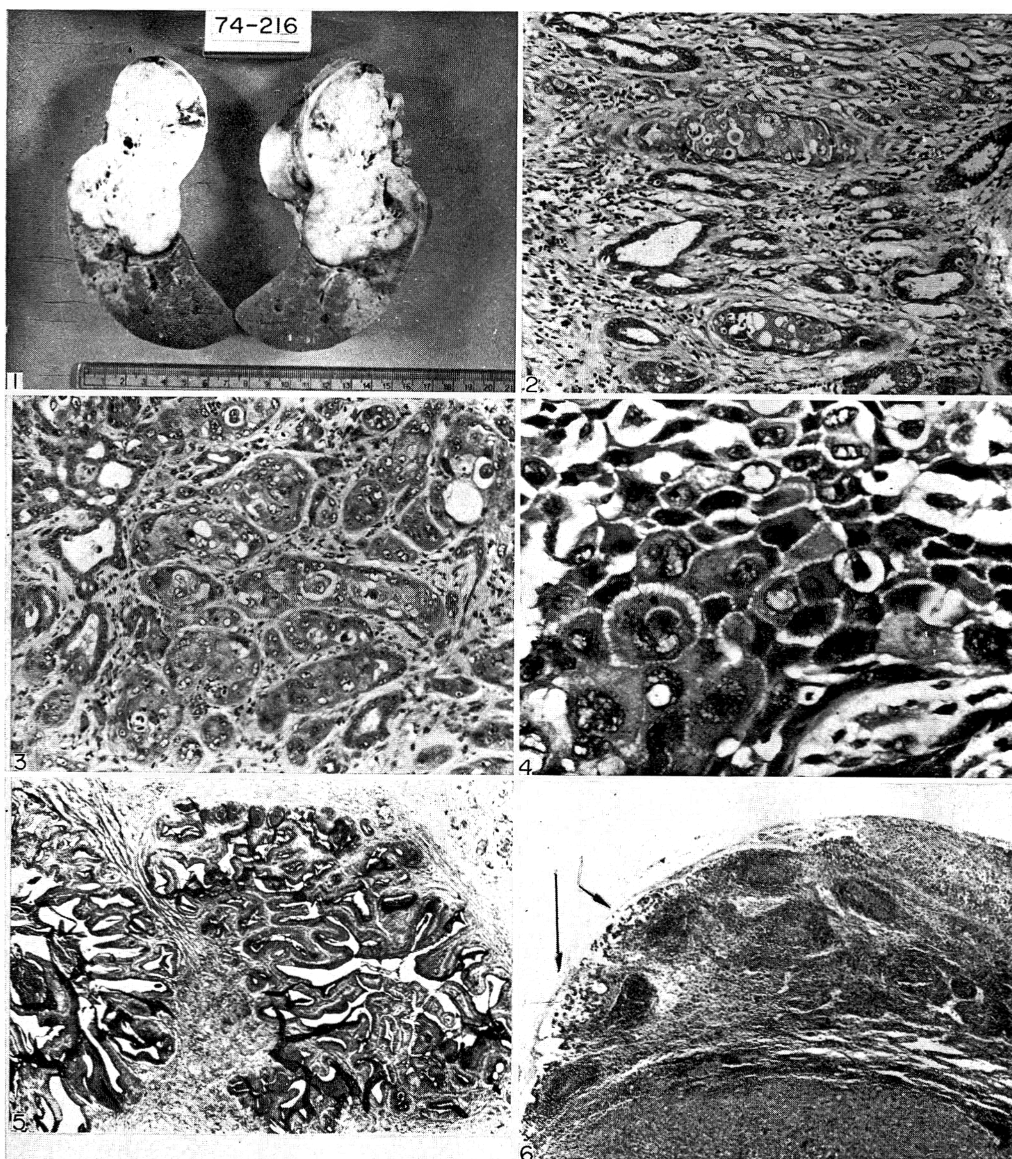

Fig. 1. Cut surface of liver and tumour from female rhesus monkey treated with aflatoxin for 5·5 yr and observed for a further 5·25 yr until moribund.
Fig. 2. Section of hepatic neoplasm in aflatoxin-treated female monkey, showing features of adenoacanthoma with a prominent adenomatous element and fibrous stroma. Haematoxylin and eosin × 100.
Fig. 3. Representative section of large areas of the same tumour exhibiting a picture of an adenoacanthoma. Haematoxylin and eosin × 100.
Fig. 4. Section of area of squamous-cell carcinoma with intercellular bridges from the same hepatic tumour. Haematoxylin and eosin × 400.
Fig. 5. Area of papillary adenocarcinoma with mucous secretion from the same hepatic tumour. Haematoxylin and eosin × 25.
Fig. 6. Metastasis of the hepatic tumour in a regional lymph node. Arrows mark the subcapsular sinuses distended with neoplastic cells. Haematoxylin and eosin × 25.

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Review Section

ENVIRONMENTAL FACTORS IN THE ORIGIN OF CANCER AND ESTIMATION OF THE POSSIBLE HAZARD TO MAN*

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Summary—The stages in the development of cancer are described as: (1) alteration of cellular function by the carcinogen; (2) reinforcing interaction by proximal altered cells—a microscopic control force; (3) failure of the restraining factors against tumorous proliferation within an organ—a macroscopic control; (4) failure of restraining forces against tumorous growth in the body as a whole. All four stages are important in human cancers. Urethane-induced lung cancer is a single-stage process atypical of cancer induction except as an example of the first stage. The relationship of dose to time of appearance of cancers is shown to have general validity as a law relating latent time to the inverse cube-root of the dose. The law is explained as the consequence of the second carcinogenesis stage, in which there is interaction of cells altered by carcinogens. Several examples of the estimation of cancer risk are presented as they apply to current problems of exposure of man to low levels of diethylstilboestrol, nitrosamines and radiation. If the cube-root of the dose applies to the estimation of the time of appearance of cancers, low-dosage exposure at some levels is virtually without risk because the expected lifespan of those exposed is exceeded by the time necessary for low concentrations of altered cells to develop into cancers.

Introduction

Cancer-causing substances have a wide range of properties. A number of inorganic chemicals are carcinogenic. Some hydrocarbons are powerfully carcinogenic, as was noted more than a century ago in the scrotal cancers of chimney sweeps and subsequently in the carcinogenicity of coal-tar products. Excesses of biologically occurring substances also cause cancer—disturbances of the growth hormone or female sex hormones are associated with cancer, while injection of cholesterol can be carcinogenic. Yet female sex hormones and cholesterol are not cancer-generating in the normal animal, including man. Mammals are abundantly endowed with these substances, but most males and females show no cancer in the absence of external causes.

Scientific investigation of the origins of cancer needs guidelines. We sometimes suffer from the tendency to generalize that any tumorous lump is a cancer. Better classification of cancers may come about when we understand the nature of this disease. Uncontrolled growth is still the most generally accepted criterion for defining a cancer. Whether the cancer occurs for unknown reasons or as a consequence of exposure to oestrogen, radia-

*This work was done under the auspices of the US Atomic Energy Commission.

†This paper was presented by invitation at the AAAS meeting in San Francisco, 25 February-2 March 1974, under the title: "Evaluation of Carcinogenic Risks of Chemical Residues". It has subsequently been expanded in collaboration with A. Grendon.

tion or a hydrocarbon, the general characteristics of uncontrolled growth (and physiological imbalances) occur, regardless of the organ system affected. There is reason to suspect, however, that the mechanisms of carcinogenesis differ for the various causative agents, although some generalizations can be made.

Dose-effect relationships

The hydrocarbons and ionizing radiation show similar dose-effect relationships with respect to carcinogenesis.

The probability of inducing a tumour per unit of dose appears to be a constant for any given carcinogenic agent. Applied to a population of N animals, the relationship may be expressed either in terms of the number (or the fraction) of animals affected with the disease or in terms of the number of tumours (or number of tumours per animal) that occurs. Designating the probability of tumour induction per unit dose as k and the dose as D, the number of tumours expected in the population is NkD, with an average of kD tumours per animal. The number of cases expected is $N(1 - e^{-kD})$, so that the fractional incidence is $1 - e^{-kD}$. A corollary is that the fraction free of tumours is e^{-kD}; thus, a graph of the logarithm of the unaffected fraction *versus* the dose should be a straight line of negative slope k, passing through the ordinate 1 at zero dose. Extrapolation of data from various experiments with several different hydrocarbons (Fig. 1a) and with radiation (Fig. 1b) tends to confirm this relationship. The ordinates in the figure are marked to show the percentage incidence of tumours but are plotted as logarithms of the percentage free of tumours. The linear relationship is quite evident in Fig. 1b and in the lower graphs of Fig. 1a, but is less so in the case of benzopyrene, which shows a diminishing effectiveness as the dose

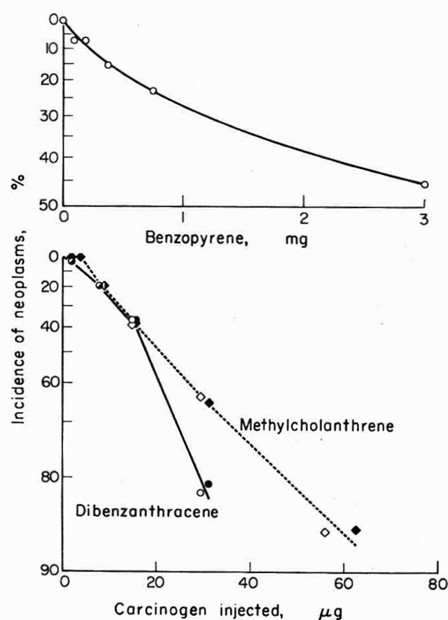

Fig. 1a. Tumour incidence in mice *v.* dose of various hydrocarbons. Upper graph: data from Poel (1959). Lower graphs: data (○, ◇) from Boyland (1958) and (●, ◆) from Bryan & Shimkin (1942). The latter authors corrected the number of mice at risk for the non-cancerous deaths in the sample before determining the percentage incidence.

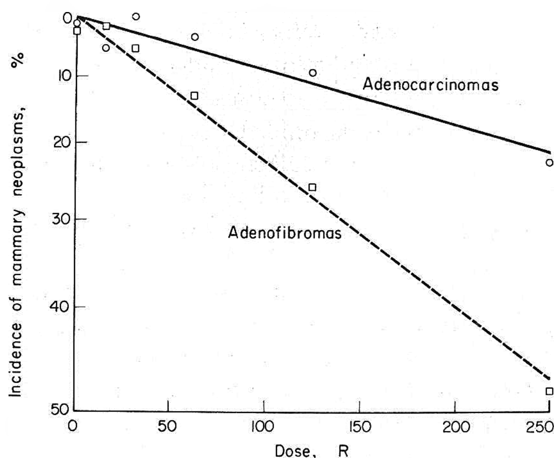

Fig. 1b. Tumour incidence in rats *v.* dose of total-body ^{60}Co radiation. Data from Shellabarger, Bond, Cronkite & Aponte (1969).

increases. This decrease in effectiveness may relate to the method of application, since the agent was applied as drops on the skin and successive drops may have had less than proportionate access to unaffected cells.

The proportionality of response to dose, in numbers of tumours induced, shows that the change to cancer is a definite break from normal control of growth with a constant probability of such damage per unit of dose over wide dose ranges. Such a relationship is consistent with all-or-nothing molecular change.

On the other hand, the relationship of time of appearance of cancers to dose suggests that the development of detectable cancers is also ruled by more complex events that are relatively influenced by dose. Extrapolation of the time for the appearance of cancers to the low-dose (and hence infrequent cancer) range leads to the conclusion that, at the low environmental levels of many of these agents, induction time begins to exceed life expectancy. In that event, the true risk effectively approaches zero.

The time interval between administration of various carcinogenic agents and the appearance of cancer shortens as the dose increases, but not proportionally. Evans (1966) noted this effect in his study of persons carrying body burdens of radium and related radionuclides, and Druckrey (1967) quantified a similar relationship in the effects of certain chemical carcinogens.

The time-effect relationship appears to be such that alteration of the dose by a thousandfold usually causes an approximately tenfold change in the time of tumour induction. When the dose of methylcholanthrene, for example, is increased from 1 μg/g body weight to 1 mg/g, the latent period of appearance of cancers in mice is reduced from 15 months to 1·5 months. The relationship may be expressed as:

$$t = t_o \left(\frac{Do}{D}\right)^{1/n}$$

where *Do* is the dose known to produce cancer with a latent period, t_o, and the usual value of *n* is 3; then the latent period, *t*, associated with any other dose, *D*, is given by the equation. A similar shortening of the time of appearance of tumours with increase of dose occurs whether single or repeated doses are used. In experimental induction of cancer, we

take the latent period to be the interval from initial exposure to the first detection of cancers. Time intervals can be measured to other end-points of cancer incidence, and from other starting points when other than single doses are involved.

A relationship that occurs widely is unlikely to be fortuitous, and the relationship between dose and time occurs similarly in all species tested and in most circumstances of carcinogenesis (Figs 2a,b). We have sought a biological model to explain the observed inverse cube-root relationship between latent period and dose and offer the following hypothesis.

Assume that the active carcinogenic agent interacts with single cells in such a way as to render some of them potentially cancerous and that the number of such potential tumour foci is proportional to the concentration of the agent in the tissues. Whether a chemical agent or radiation is involved, the number of foci per unit volume may then be expressed as $n = kD$, where D is the dose in customary units such as mg/g or rad.

Assume further that the birth-and-death process among these cells causes every incipient tumour to die without becoming overt disease, except for such foci as happen to coalesce with others before reaching the limit of growth at which the death of an individual focus is almost certain to occur. When the growth of neighbouring foci causes coalescence of the required (unknown) number to give the neoplastic colony the ability to resist the defence mechanisms of the host, a cancer has been initiated. Let us consider the time it may take from formation of the foci to initiation of this cancer.

If there are kD foci per unit volume of tissue, the mean number of foci along a random line in that volume is $(kD)^{\frac{1}{3}}$ per unit length. Thus, the mean distance between foci is inversely proportional to $D^{\frac{1}{3}}$. If we assume that the growth of cells from a focus occurs at a constant rate, the time required for a given focus to expand radially by a specified amount is proportional to that specified distance. The average time required for coalescence of a pair of adjacent foci, then, is proportional to $1/D^{\frac{1}{3}}$.

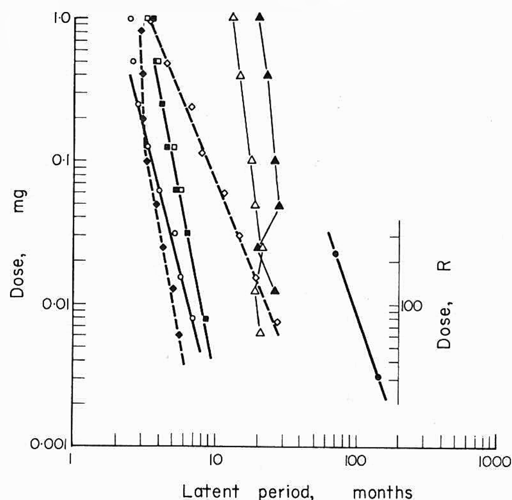

Fig. 2a. Latent period *v.* dose (— single exposure; --- continuous exposure) for various chemical carcinogens in mice and rats and *v.* dose of radiation in man. Data for hydrocarbons (treatment of mice with benzpyrene, ◆, methylcholanthrene, ○, and dibenzanthracene, ■,□) and for diethylnitrosamine in rats (◇) from Druckrey (1967), for diethylstilboestrol in C3H (△) and in A-strain castrate mice (▲) from Gass, Coats & Graham (1964) and for human leukaemia following radiation exposure (●) from Bizzozero, Johnson, Ciocco, Hoshino, Itoga, Toyoda & Kawasaki (1965).

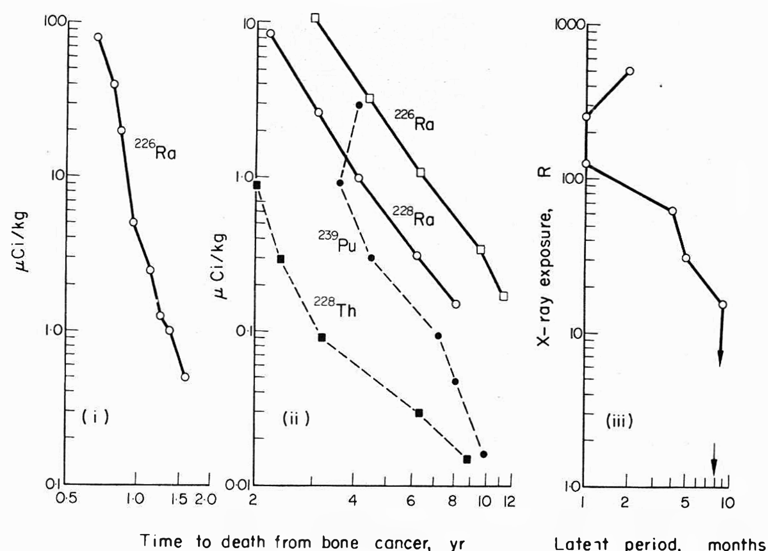

Fig. 2b. Latent period *v.* dose for bone cancer from internal radionuclides in mice (i) and dogs (ii) and for mammary cancer from X-rays in rats (iii). Data for mice from Finkel, Biskis & Jinkins (1969), for dogs from Mays, Dougherty, Taylor, Lloyd, Stover, Jee, Christensen, Dougherty & Atherton (1969) and for rats from Shellabarger *et al.* (1969).

Whether tumour development depends on the junction of two or many foci, the time-relation still holds. If, for example, it takes at least four foci to constitute a self-defending tumour colony, death would overtake any focal clone that did not happen to have at least three neighbouring foci making contact with it before one of the group died. The average time required for pairs to meet is the average time required for such groups to form.

This model, then, affords a biological basis for the observed phenomenon that latent period is inversely proportional to a fractional power of the dose of various carcinogens, with a value close to 0·33 for the exponent—but not necessarily precisely that value.

Speculation that the latent period in man may vary similarly with the inverse cube-root of the dose has been based on observations over a narrow dose range, a variation in exposure of approximately one order of magnitude. Our review of the data on the latent period for the appearance of leukaemia in the Japanese survivors of atomic bombing and on the time of appearance of radium-induced bone cancers supports and extends this hypothesis.

Leukaemia latency in survivors of atomic bombing

Leukaemia incidence above the normal rate appeared in the survivors of atomic bombing, and the risk of it was shown to be in proportion to the degree of exposure (Folley, Borges & Yamawaki, 1952; Jones, 1956a; Lewis, 1957). The quantitative relationship of radiation exposure to the incidence of leukaemia has been extensively studied; but the significance of the length of the latent period, which is much less conspicuously altered over the narrow range of variation in the degree of irradiation, has not previously been singled out for comment.

The reported increase in the incidence of leukaemia in the atom-bombed population under follow-up study in Japan showed a peak in the period 1950–1953 (Fig. 3). This marked the latent period for the group of survivors most heavily exposed to radiation, who experi-

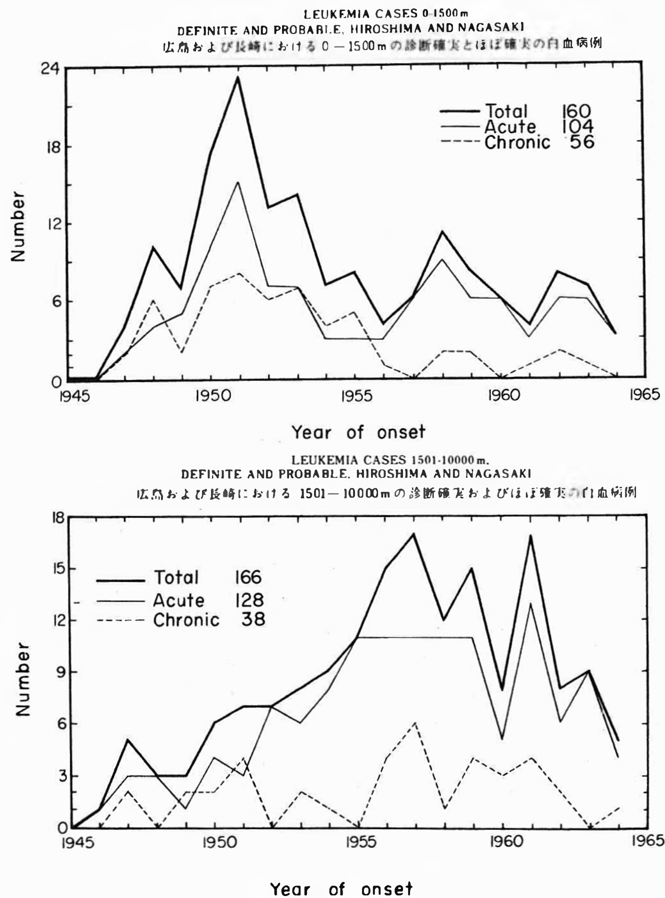

Fig. 3. Latent period for leukaemia in Japanese bomb victims *v.* distance from epicentre. Reproduced from Bizzozero *et al.* (1965).

enced a mean dose of about 230 rem (Bizzozero *et al.* 1965). A second peak occurred in the period 1958–1964; this peak was associated with the less-exposed population, who experienced a mean dose of about 32 rem (Bizzozero *et al.* 1965). Although the data are so sparse (Table 1), these two points have been plotted in the lower right quadrant of Fig. 2a and the slope of the line is seen to correspond closely to the inverse cube root of the dose relationship.

Tumour latency in humans exposed to radium

In order to study the relationship, if any, between the dose of a carcinogen, such as radiation, and the latency of its effect, it seems preferable when possible to determine the paired values of dose and latent period for individual cases rather than to deal with mean doses and mean latent periods of groups. The body of data pertaining to the carcinogenic effects of radium and mesothorium in the luminous-dial painters of the 1920s and 1930s has been extensively treated in the scientific literature. Evans, Keane, Kolenkow, Neal & Shanahan (1969) have published graphic presentations from which we have been able to

Table 1. *Leukaemia latency in survivors of atomic bombing at Hiroshima and Nagasaki*

Type of leukaemia	Mean interval (years) from time of bombing to onset of leukaemia for exposure levels (rads)* of			
	>350	100–350	<100	0
Acute lymphatic	5·5 (5)	11·6 (7)	11·8 (4)	12·2 (8)
Acute granulocytic	11·1 (7)	14·9 (10)	10·0 (7)	12·0 (28)
Chronic granulocytic	9·2 (2)	8·6 (10)	12·1 (19)	10·7 (11)
All other types	13·4 (3)	10·2 (3)	16·0 (4)	13·2 (9)
Weighted average...	9·6 (17)	11·6 (30)	12·1 (34)	12·0 (56)

*Numbers in parentheses are numbers of cases.
Based on data to September 1966, compiled by Ishimaru, Hoshino, Ichimaru, Okada, Tomiyasu, Tsuchimoto & Yamamoto (1969).

extract individual values of the quantities they have defined: CR, CRY and PRE. The quantity CR is the cumulative dose in rads averaged over the entire skeleton, from initial exposure to the time of the survey. CRY (cumulative rad years) is the sum of the products of the increments of dose and the respective elapsed time intervals since each increment was received. PRE (pure radium equivalent) is the computed amount (in μCi) of radium-226 (the isotope commonly meant by 'radium') that would have given the same dose as the mixture of radium and mesothorium (radium-228) actually ingested.

Previous investigators have regarded the time interval since first exposure as the latent period in studying the time of incidence of cancer. We believe that a more appropriate index of latency would take into account the pattern of accumulation of the dose, since it is impossible to determine whether the first increment of dose or the first N increments constituted the initiating cause. For that reason, we have computed for each individual who had developed a malignant neoplasm by the time of the survey (1967) a weighted latent period, L, by dividing CRY by CR. That process yields a weighted average time interval by assigning a weight to each interval in proportion to the dose accrued to the beginning of that interval.

The values of L determined in this way have been related to the corresponding values of PRE, with the result shown in Fig. 4. The straight line fitted to the points by the method of least squares seems a reasonable basis for extrapolation to lower values of PRE. The correlation coefficient for log PRE versus log L is $-0{\cdot}76$, indicating a close association between these variables. The equation for the fitted line yields the relation:

$$L = \frac{29{\cdot}44}{PRE^{0{\cdot}453}}$$

If we regard PRE as a simple measure of the dose, then the latent period seems to vary inversely as the 0·453 power of the dose, which is not markedly different from the inverse cube-root relation noted in connexion with other carcinogens.

Using this relation, the computed value of L when PRE equals 0·1 μCi is 83 years. Even if the entire amount of CR were received in the first year of exposure (it never is, since radium persists in the bone) and that exposure occurred at the age of 17, the individual could expect to be about 100 years old before a malignant tumour might appear. This mode of analysis lends support to the hypothesis advanced by Evans (1966) that there is

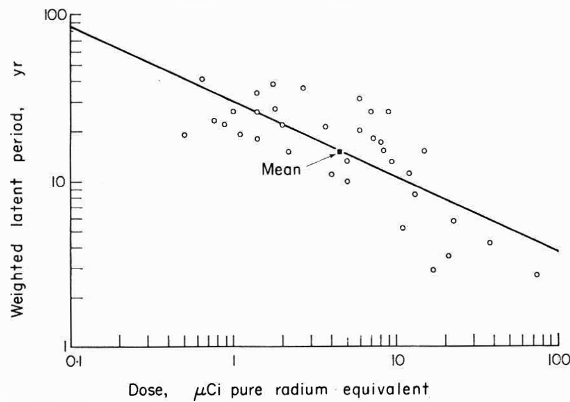

Fig. 4. Weighted average latent period *v.* radionuclide body burden for bone cancer from internal radionuclides. Data from Evans *et al.* (1969).

probably a practical threshold to the carcinogenic effect of ingested bone-seeking α-emitters—in other words, that it takes so long to induce this effect with small ingested doses of such substances that life ends for other reasons before radiogenic cancer can appear.

Nitrosamine carcinogenesis and latency

The family of dialkylnitrosamines ranges from weak to powerful in respect of carcinogenic potency. When normal laboratory rodents are treated with doses of these agents close to the acutely lethal level, they fairly uniformly develop destructive cancers. The nitrosamines and the related dialkylaminoazobenzenes and dialkylaminostilbenes induce cancers in high yield in experimental animals and, like many carcinogens, they have an anomalous usefulness as drugs for destroying cancer tissue. They have been especially well studied because of the need to know their carcinogenicity in view of their therapeutic role. The incidence of cancer produced by them at high dosage in experimental animals tends to be so high that only the time-delay in tumour appearance is useful as a measure of relative activity. It was with these compounds that Druckrey (1967) established the striking relationship that the latent period prior to the appearance of the cancers varies inversely as a fractional power of the dosage of carcinogen (Fig. 2a). Prior to the work with substances such as these, with a high cancer potency and low acute toxicity, it was not possible to gather quantitative data over a sufficiently wide range of dosage to establish precise relationships between dose and latency. Attention tended to focus, therefore, on the presence or absence of a few tumours in the low-dose range, while quantification of the time of appearance of the tumours was neglected because of the much smaller extent of its variation over this narrow range of doses.

The logarithm of dose versus the time of tumour appearance for nitrosamines is now known to be linear over a thousandfold range in dosage. The dose-response relationships of these agents are similar to those of ionizing radiation and of the well-studied carcinogenic hydrocarbons. As in the example discussed above, if the dose producing a few cancers is reduced to one-thousandth as much, then the latent period before the induction of tumours increases by a factor of 10. If the latent period for a moderate dose is, say, 10% of the lifespan, such a change would increase it to 100% of the lifespan. In that event, no observable increase in cancer is caused at the lowered dose level.

The nitrosamines are currently being studied because of their presence in certain preserved meats. They are believed to originate from chemical alteration of the food additive, sodium nitrite, especially as a result of the heat of cooking. In cured meats, sodium nitrite is both a preservative and a flavouring agent, and in canned meats, it is an important aid in preventing spoilage. Nitrosamines have been detected in commercial smoked and canned foods to the extent of a few parts per billion (ppb; b $= 10^9$) in bacon, ham, frankfurters and canned meats, and in some canned fish. In a few instances, nitrosamine levels in single samples of nitrite-treated meat have been as high as 100 ppb, but more often there is either no detectable residue or they are within the 1–3 ppb range. Since a few different nitrosamines can be present, since they appear to have equivalent actions in the induction of cancer and hence are probably additive, and since each is in the range of a few ppb, it is reasonable to assume that the average total nitrosamine content of nitrite-treated meat is about 10 ppb and certainly does not exceed 100 ppb (Wolff & Wasserman, 1972).

In the testing of individual nitrosamines, diethylnitrosamine fed continuously to rats at a level of 75 ppm caused cancers in a significant fraction of the animals after one-half of the lifespan, with an average induction time of 840 days (Druckrey, 1967). Dimethylnitrosamine was observed to induce a few cancers when fed continuously at 5 ppm, but none at all at 1 ppm (Wolff & Wasserman, 1972). It appears, then, that the limit of dosage at which a detectable effect occurs is in the range of 1 ppm for these substances. Taking all nitrosamines together, the limit of observable carcinogenic effect may be estimated to occur at about 1 ppm of the entire average diet.

If the entire diet consisted of meat and all the meats eaten were nitrite-treated, the human dietary intake of nitrosamines would be 10–100 times less than that of these laboratory animals (10–100 ppb compared with 1 ppm). There are the additional factors that man eats less food per unit of body weight than do rats or mice, and that only the cured- and canned-meat fraction of the diet is involved. These two factors contribute an additional reduction of at least 100 times in the comparison of man with the laboratory animals. Thus, the estimated magnitude of the risk to man from nitrites and nitrosamines in food is at least 1000 times less (and probably 10,000 times less) than the minimum detectable level of cancer risk in laboratory mice and rats. In addition, the effect of the lower dose on the time of tumour development should cause the lag to stretch out far beyond the human lifespan (to 10–20 times the human lifespan).

Since there may be small regions of higher concentration of nitrosamines in the feed of the experimental animals, cancer-inducing contact in the intestinal tract may be important in the animal studies. In rats fed these substances, the liver is the primary organ affected and there is some increased cancer of the oral cavity and oesophagus, but not of the stomach. As shown above, however, man would be exposed to concentrations that are less by several orders of magnitude and hence there would be a proportional reduction of any estimated cancer risks. The above estimates have not taken into consideration the possibility of combined (additive or multiplicative) interactions of nitrosamines with other cancer-potentiating substances.

There is a basis for drawing conclusions from experimental carcinogenesis in laboratory rodents for application to man:

(1) It has been shown that the risk of cancer induction varies with the degree of exposure to carcinogens in man as well as in laboratory rodents and other animals.

(2) There is evidence that the latent time for the appearance of cancer in these species after exposure to various carcinogens varies approximately as the inverse of the cube-root of the dose of carcinogen. For the nitrosamines, this relation has been established in rats, but nitrosamine-induced cancer has not been observed in man. An approximately similar relationship, however, between latent period and dose has been shown above for man in bone tumour-induction from radium exposure and in leukaemia among persons exposed to atomic bombing. Moreover, the dose-latency relation for radium induction of bone cancer shows excellent agreement between the observations on man and the data on experimental laboratory rodents. Urethane is the only carcinogen so far discovered that does not conform to the inverse cube-root (or approximately cube-root) principle, with the possible further exception of radiation-induced acute granulocytic leukaemia.

(3) No experimental situations have been uncovered in which the relationship between latent period and carcinogenic dose was different for man from that for other animal species.

The induction of lung tumours in mice by urethane

The discovery that urethane anaesthesia of mice induces lung tumours led to a study of the dose-response relation. A direct proportion between urethane dosage and the incidence of the lung-tumour nodules was reported by Henshaw & Meyer (1945) and was confirmed by others. Reported anomalies when a given dosage was fractionated led to studies in which urethane was administered in single doses over a wide range and in equivalent fractionated doses with varied intervals between the fractions (White 1972; White, Grendon & Jones, 1967). These studies extended the range of direct proportionality of effect to dosage and refined the determination of dosage by taking into account the small but progressive increase in the time of retention of the urethane with an increase in the administered dose. When corrected for the true or 'internal exposure' to urethane the dose versus lung-tumour incidence becomes precisely linear within the expectancy of biological observation. The following points are now established from a review of the urethane lung-tumour-induction system:

(1) As with other carcinogens, the number of potential tumour foci induced by urethane appears to be proportional to the exposure.

(2) The family of curves relating mean numbers of tumours per mouse at the various dosage levels to the time interval between administration of urethane and sacrifice of the animals can be used to estimate the time of appearance of the first tumour in each experimental group. We have drawn tangents to these curves at the earliest time of sacrifice and extrapolated these tangents to determine their intercepts on the time axis (Fig. 5). The interval from first administration of the agent to this point of intercept (the estimated first incidence of a tumour) is here called the latent period. It turns out that all of this family of curves have approximately the same intercept, within surprisingly close limits, indicating that, for urethane-induced lung tumours, latency is independent of dose.

(3) Shimkin, Wieder, Marzi, Gubareff & Suntzeff (1967) have reported that the yield of tumours for a given dose of urethane may be increased or decreased by adjusting the time interval between dose fractions. We believe that this anomalous effect, which has never been reproduced in other experiments, including

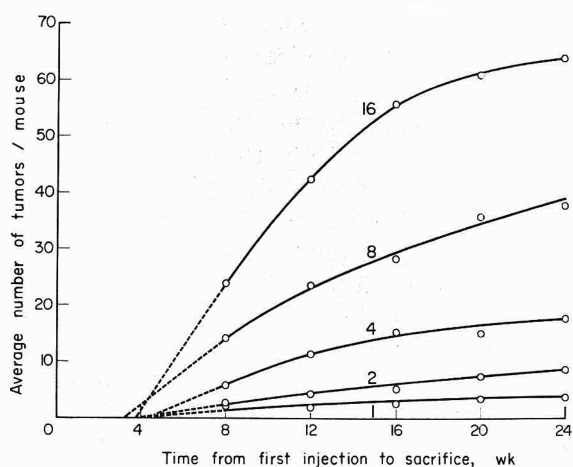

Fig. 5. Estimated time of initial incidence of lung tumours in mice exposed to urethane. The dose, in terms of the number of injections of 0·250 mg/g, is indicated on each curve. Data from White *et al.* (1967).

our own, does not truly represent the behaviour of the system. The variability of tumour yields in successive trials reported by Shimkin *et al.* (1967) suggests that some experimental factors were not well controlled. We attribute the internal consistency between dosage and tumour yield within our experiments (White, 1972; White *et al.* 1967) to the fact that Margaret White personally carried out the critical manoeuvres in all the experimental work.

We conclude that the urethane lung-tumour-induction system is dose-dependent in the number of tumour foci induced but that, consistent with the concept of risk dependence on a single chemical change in a cell, the time of appearance of the tumours is not related to dosage. Such a relationship, in our opinion, depends on the relative absence of any suppression of urethane-induced lung tumours by tissue-regulative functions.

Estimated cancer risk from diethylstilboestrol (DES)

Recent government action against the use of DES to stimulate the growth of beef cattle has resulted from the possible risk of cancer of the female genital tract of infants born to mothers who ingest DES from meat eaten during pregnancy. An estimate of the risk can be made on the assumption that it is proportional to the intake of DES from meat produced under the former regulations, which limited the amount and mode of administration and required 7 days withdrawal from DES prior to slaughtering. We shall evaluate the risk first on the basis of a set of pessimistic assumptions and then adjust the computed risk to correct to a more realistic situation.

Data from government reports (Goldhammer, 1971) indicate that the concentration of DES in the liver is ten times that in beef muscle. Since 2 ppb is the limit of sensitivity of the latest field test method (Van Houweling, 1972), 0·2 ppb in beef muscle is the limit of controllability, so let us assume that all beef is contaminated to that extent. Various weight-reducing diets recommend high levels of protein, and although some, such as 'Weight Watchers', prohibit extensive use of beef, let us assume that there is a large group of pregnant women who eat 10 oz of beef daily. At the 2 ppb level, their dose is 0·2 ×

$10^{-9} \times 284$ g or approximately 6×10^{-8} g DES/day. If they all follow the 'Weight Watcher' recommendation and substitute 6 oz of beef liver for beef muscle once a week, the average intake becomes about 10^{-7} g DES/day.

The lowest doses reported by Herbst, Kurman, Scully & Poskanzer (1972) in cases of observed vaginal cancer were 1·5 mg/day throughout pregnancy. Let us make the pessimistic assumption that the same number of cases would have occurred even if all women had received only 1·5 mg/day. The known cases that resulted after DES therapy appear to be the 49 found by Herbst's national enquiry (Herbst *et al.* 1972) and eight or nine reported by Greenwald (1972), giving a total of 57 or 58. This total could properly be reduced slightly to account for the probability that some of the cases that followed DES therapy would have occurred without such therapy; but let us assume instead that enough additional cases will be found to make the total 100. Assume also that 1% of all pregnant women received DES (Heinonen, 1973) in the period 1951–1955, cited by Greenwald (1972) as "the time of peak clinical use of DES to prevent mis-carriages", and that all cases came from this group of births.

About 20 million children were born in the period 1951–1955, about half (or 10^7) being females. If 10^5 of their mothers (1% of 10^7) received DES and 100 cases occurred among them, the pessimistic assumption is that 1·5 mg/day caused a risk of $100/10^5 = 10^{-3}$. If we make the further pessimistic assumption that there is no threshold and that this effect is linearly proportional to the dose, the risk to mothers of girls from DES in beef is—on the basis of the above pessimistic assumptions—

$$\frac{10^{-7}\text{ g DES/day}}{1{\cdot}5 \times 10^{-3}} \times 10^{-3} \cong 7 \times 10^{-8}.$$

Thus, the maximum overall risk to pregnant women is $1/2 \times 7 \times 10^{-8}$ (since about half of the births are girls) $\cong 3 \times 10^{-8}$. With approximately 4×10^6 births per year, about one birth would be affected every 8 years. This is the pessimistic estimate of incidence of such cancers, not of mortality caused by them, since, to the Congressional hearings of 20 July 1972, Herbst (1972) cited six out of seven patients "treated and . . . living and well".

Now, let us adjust these pessimistic assumptions in the direction of the real situation. The per capita consumption of beef in the United States has gradually increased from 79 g/day in 1950 to 141 g/day in 1971 (Statistical Abstract of the United States, 1973; Table 134; US Government Printing Office, Washington, D.C.). If we make a subjective allowance for the low consumption by very young children and the high consumption by men, we may estimate roughly that women consume on average about 140 g beef/day rather than the 284 g on which the previous calculation was based. The corresponding risk-adjustment factor is $140/284 = 0{\cdot}493$.

We made the assumption that the same number of cancers would have occurred even if all therapeutic doses had been at the lowest level reported; but this runs counter to our hypothesis that effects are proportional to dose, so we must correct for that overestimate. Let us call the risk to female children per mg/day R and evaluate R in order to compare it with the calculated risk of 10^{-3} per 1·5 mg/day. In the composite data of Herbst *et al.* (1972) and Greenwald (1972), the dose of DES is known for only 28 cases as against the 100 cases assumed to occur in a population of 10^5 mothers (of girls) treated with DES; so we may regard $28/100 \times 10^5$ as the population from which these cases of known dosage arose, since there is no reason to believe that these cases differ from the other 72. Call this

subpopulation P, and further divide it into P_1, P_2 and P_3, corresponding to the three dosage ranges reported by Herbst *et al.* (1972), namely 1·5–10, 10–50 and more than 50 mg/day and assume the latter to be 50–125 mg/day, making use of the comment of Greenwald (1972) on the range of highest doses. Since the numbers of cases in these three dosage classes are 5, 13, and 10, respectively, we can determine the sizes of P_1, P_2 and P_3 by assuming that cases arose in proportion to dose and that the average of each range represents the dose for that category:

$$\frac{1{\cdot}5 + 10}{2} RP_1 = 5$$

$$\frac{10 + 50}{2} RP_2 = 13$$

$$\frac{50 + 125}{2} RP_3 = 10$$

$$P_1 + P_2 + P_3 = \frac{28}{100} \times 10^5.$$

Solving for R, we get the value $5{\cdot}1 \times 10^{-5}$. The corresponding risk-adjustment factor is the ratio of this value to the previously employed $1/1{\cdot}5 \times 10^{-3}$ or 0·076.

Our initial calculation assumed that only 1% of pregnant women received DES therapy. From private discussions with physicians long engaged in the practice of obstetrics and gynaecology, it appears that about one-third of all women show some bleeding during pregnancy and that, when the use of DES was in vogue, probably one out of three or four of these may have been so treated. Let us assume, then, that 10% rather than 1% of mothers took DES, so that the population from which the affected cases arose was ten times as large as previously assumed. The risk-adjustment factor is 0·1.

The actual population at risk covered a longer time interval than the limited period, 1951–1955 when clinical use of DES was at a peak. In fact, DES therapy certainly covered the period 1947–1964, corresponding to the ages of the cancer-affected offspring reported by Herbst *et al.* (1972), and probably even longer. Although this 18-year interval is nearly four times the 5-year interval previously assumed, let us estimate that the population at risk was three times the previous estimate, since usage did not rise instantly to a maximum or decline instantly to zero. The risk-adjustment factor for this consideration is 1/3 or 0·333.

If we assume that compliance with regulations requiring 7 days withdrawal from DES reduces liver DES by at least a factor of 10 (Van Houweling, 1971a), then we must apply an adjustment factor of 0·1 or less, since the presence of DES at the 2 ppb level was reported when only 48 hours withdrawal was required, and even then in only 0·5% of several thousand inspections of liver (Yeutter, 1971).

If we assume that the 0·5% cited above represents the actual level of failure to reduce DES to 'zero' (i.e. to levels so low as to have a negligible effect on our calculations) and that the calculated DES levels ingested by man apply only to intake from that fraction of market beef, an adjustment factor of 0·005 is required.

The effects of all of these factors could operate concurrently. If they do, the risk to pregnant women would be:

$$(3 \times 10^{-8}) \times 0{\cdot}493 \times 0{\cdot}076 \times 0{\cdot}1 \times 0{\cdot}333 \times 0{\cdot}1 \times 0{\cdot}005 \cong 2 \times 10^{-14}$$

and the annual expectation of affected children would be $(4 \times 10^{6}) \times (2 \times 10^{-14}) \cong 10^{-7}$, or one case in 10 million years.

In terms of public health costs, using the estimate made by Van Houweling (1971b) that the increased cost of living if DES use in cattle feed is banned would be $700–800 million/year, plus additional costs for dealing annually with $1{\cdot}8 \times 10^{10}$ pounds of animal wastes, we would spend about $1 billion/year to save a theoretical maximum of 0·12 cancer cases/year or about $8 billion/case. Any public health official could guarantee to save many actual lives (not merely one hypothetical case of disease) for $8 billion.

The above consideration of the levels of DES in beef products under recent regulations does not take into account the fact that schedules of practical treatment of cattle with DES, followed by longer withdrawal prior to marketing, could reduce these estimated levels by another order of magnitude. Thus, the range of possible carcinogenic effects of permitting the use of DES in cattle feed under the regulations that prevailed until recently may be at most a few cases in the tens of millions of cancers that occur in our country in a lifetime, it may be as small as one case in 10 million years, and it might, in fact, be zero. As for induction of cancer in the individual who consumes the DES, the effect reported by Gass *et al.* (1964) is that DES induces mammary cancer in mice only at levels causing physiological disturbances, not at lower levels (Fig. 6). This fact is evident from a comparison of the text-figures 1 and 3 in Fig. 6; cancers appeared only at doses double those that caused loss of weight of the ovaries. Gass *et al.* (1964) found that the latent period for the appearance of these cancers was shortened only by dosages in excess of about 30 ppb and that approximately the same level was necessary to induce an increase in incidence of these cancers. The implication is that there is a genuine threshold of effect at that level.

If physiological disturbances necessarily underlie the carcinogenic hazard of DES, as these studies seem to show, we can conclude that there is no hazard from DES residues in treated beef, since the levels that occur are far below the normal variations in human oestrogen levels (Van der Molen, 1969). Thus, it appears that these amounts of DES present no hazard either to those who consume the DES or to their offspring.

Biological hypotheses concerning the induction of cancer

Induced risk of cancer, as discussed above, develops in proportion to the exposure to carcinogen. This is usually taken to indicate that cancer is a cellular change induced in at least one of the many cells exposed to a given concentration of carcinogen. Were this the only condition affecting the induction of cancer, then we should expect that the more cells that are exposed to a given concentration of a carcinogenic agent, the greater is the risk of cancer. Experimental evidence shows that such relationships do not hold between species because, in spite of great differences in size and hence in numbers of cells, each species has roughly a similar risk of cancer over the same fraction of the lifespan after exposure to the same concentration of carcinogen. An illustration of this relationship is seen in the effects of ionizing radiation on mice (Furth, Upton, Christenberry, Benedict & Moshman, 1954) and man (Harada, Ide, Ishida & Troup, 1963) exposed to separate atomic explosions. In both the mice and the human subjects, the frequency of cancers (and other degenerative diseases) that occurred in comparable fractions of lifespan increased

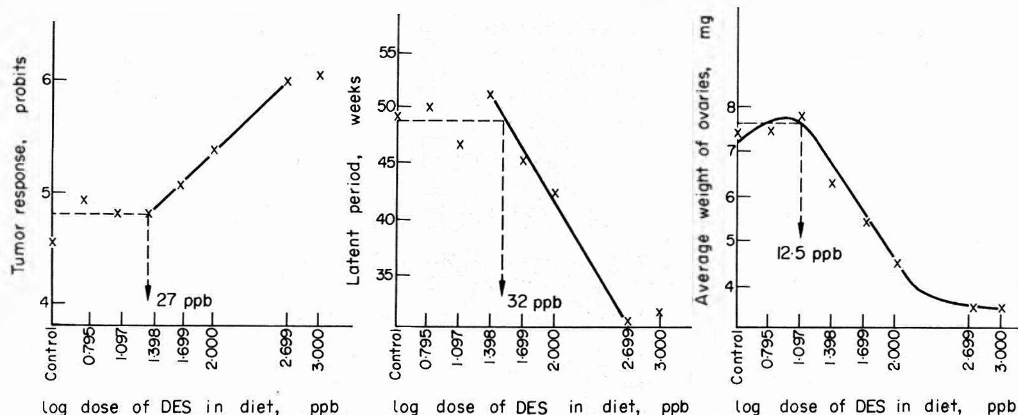

Fig. 6. Effects of dosage of diethylstilboestrol (DES) on incidence of mammary tumours. latent period and weight of ovaries in mice. Reproduced from Gass *et al.* (1964). The authors labelled these as text-figures 1, 2 and 3, reading from left to right, with the following legends:
(1) "Log dose-response curve for incidence of mammary carcinoma in C3H female mice receiving varying concentrations of diethylstilbestrol in the diet. The curve is linear ($P < 0·05$) between 25 and 500 parts per billion (ppb)."
(2) "Log dose-response curve for latent period of mammary carcinomas in C3H female mice fed diethylstilbestrol (DES) in the diet. The curve is linear between 25 and 1000 parts per billion (ppb) and has a significant slope ($P < 0·01$)."
(3) "Log dose-response curve for average weight of ovaries from C3H female mice (with tumours) that had received diethylstilbestrol (DES) in the diet. The curve is linear between 12·5 and 500 parts per billion (ppb) and has a highly significant slope ($P < 0·001$)."

to approximately the same extent at comparable levels of radiation exposure. Humans are approximately 3000 times larger than mice in body size and in numbers of cells, yet the risk of cancer for the same concentration of radiation is almost the same. Cancer must, therefore, involve alteration of some critical fraction of cells rather than of a specific number of cells. Our inference is that the critical process in carcinogenesis is a failure of organ controls over the proliferative tendency of the clone or clones of cells that form a cancer. This hypothesis is still consistent with the hypothesis that molecular changes in cells, occurring in proportion to the concentration of carcinogen, are the initiating events. The implication, however, is that the initial event runs a limited course unless the fraction of the organ affected is sufficient to cause a failure of the tissue organizational functions, and that this fraction is approximately the same for the various mammalian species. With the failure of the organic defences, the altered cells may develop into a clinically recognizable cancer.

Urethane induction of lung cancer in mice is the sole well-documented case of cancer induction in proportion to the exposure to carcinogen in which tissue-control factors appear not to be operative (though perhaps acute granulocytic leukaemia may be another example). Perhaps the cells in the lung are too dispersed to permit as great a degree of tissue control as in the solid tissues of other organs. As the force of control mechanisms diminishes, the carcinogenic response would tend to be more closely dependent on the numbers of cells affected by the agent. In that event, urethane may be more carcinogenic in man than in mice.

The observed relationship between degree of carcinogenic exposure and time of development of cancer extends from prompt cancer induction at the highest dosages to infrequent

tumours appearing near the end of the lifespan at the lowest tested dosages. The question is whether the effects of still lower dosages of carcinogens may be extrapolated to hypothetical latent periods greater than life expectancy and hence be unobservable. We believe that this is probably the case with most carcinogens, because:

(1) The orderliness of the $D^{-\frac{1}{3}}$ relationship shows no evidence of coming to an end at low dosages.

(2) As explained in the discussion of the $D^{-\frac{1}{3}}$ principle, the thousandfold range of exposure investigated in the study of some carcinogens should produce a tenfold variation in the proximity of those cells whose interactions presumably lead to cancer. Since the distances involved must be microscopic, it seems plausible that, say, another increase by a factor of ten in the range of distances may still permit cell multiplication to control the rate of interaction among focal clones. Given that condition, the $D^{-\frac{1}{3}}$ rule may be expected to hold in estimating latent periods ten times as long as those observed and perhaps longer.

A theory of the stages in the development of cancer is as follows:

(1) Changes affecting cellular function are induced by carcinogens and are presumably alterations of DNA (perhaps RNA) transmissible to daughter cells.

(2) Interactions occur between clones of altered cells; these are responsible for the relationship described between dose and time of cancer appearance.

(3) Organ changes produced by these cellular alterations may reach a level at which they cause failure of those tissue-organizing systems which normally prevent the development of a cancer.

(4) The death rate of the population of those in whom cancer has been clinically detected remains constant regardless of treatment or the duration of the disease (Jones, 1956b). The implication is that failure of the cancer-suppressing mechanism has extended from the organ of origin to the entire body.

(5) The final stage of cancer is uncontrolled growth, typical of acute malignancies. Death from other causes may, however, intervene. In cancer of the rectum, the death risk from intercurrent disease was found to be equal to the death risk from recurrence of the malignancy (Jones, 1956b).

Conclusions

On the assumption that carcinogens cause cancer risk in proportion to exposure, the risks associated with DES and nitrosamines in food have been insignificant and should not have been a cause of concern to public health agencies. Because of the observed inverse relation between latency and dose, the prevailing levels of exposure to many carcinogens, such as radiation at levels of the order of the natural background, imply a delay in potential cancer induction that exceeds the life expectancy of any individual. The relationship between the dose of many carcinogens and the time of appearance of cancer suggests that the common mechanism involved in the induction of cancer by these agents is an interaction of cells affected by the carcinogen and that the rate of this interaction depends on the proximity of the affected cells.

A similar relationship between the dose of carcinogen and the time of appearance of cancers has been demonstrated in radiation-induced human leukaemias, osteogenic sarcomas induced by radium in man and laboratory animals and osteogenic sarcomas induced by strontium-90 in laboratory animals, as well as in cancers induced by many chemical agents (including methylcholanthrene, dibenzanthracene and diethylnitrosamine)

in mice and rats. Urethane-induction of lung cancers in mice and possibly radiation-induction of acute granulocytic leukaemia in man appear to have latency that is independent of dose and is very brief. This characteristic suggests that these neoplasms are not dependent on tissue interaction but develop directly from single altered cells.

The observation that the risk of cancer induction per unit of exposure is approximately the same for man as for small laboratory rodents in spite of their great differences in size and hence in numbers of cells exposed to the agent implies that the carcinogenic process extends beyond the cellular level. Since there is a correspondence of organ systems in these species, the mechanism probably involves disturbances in the factors governing the characteristic cellular proliferation in each organ.

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*'Government Operations—Part 1' refers to the report: Regulation of Diethylstilbestrol (DES) (Its Use as a Drug for Humans and in Animal Feeds) (Part 1): Hearings before a Subcommittee of the Committee on Government Operations, House of Representatives, 92nd Congress, 1st Session, 11 November 1971. 'Government Operations—Part 2' denotes the continuation of the same hearings, designated Part 2 and dated 13 December 1971.

†'Health' refers to the report: Regulation of Diethylstilbestrol (DES), 1972: Hearings before the Subcommittee on Health of the Committee on Labor and Public Welfare. United States Senate. 92nd Congress. 2nd Session. on S.2818, 20 July 1972.

Fd Cosmet. Toxicol. Vol. 13, pp. 269–274. Pergamon Press 1975. Printed in Great Britain

BOOK REVIEWS

Nutritional Problems in a Changing World. Edited by Dorothy Hollingsworth and Margaret Russell. Applied Science Publishers Ltd., London, 1973. pp. xvi + 309. £10.00.

Philosophy aside, human life is essentially a struggle between health and disease, the outcome of which is dependent to a large extent on the quality and quantity of our food. In the developing countries of the 'Third World', quantity of food is frequently the limiting factor in the maintenance of health, but affluent societies have their own problems—typified by the chronic degenerative diseases, which are due more to dietary imbalance than to an inadequate calorific intake. Nutritional problems among the more fortunate societies were recently considered at a British Nutrition Foundation Conference held at Churchill College, Cambridge, and the proceedings of this meeting have been recorded in a lucid text, the didactic approach of which is suitable for those with a limited knowledge of the biological sciences. The thirty contributors to this volume include distinguished workers in the fields of paediatrics, nutrition and dietetics, both from the UK and overseas, and respected representatives of the food industry and appropriate government departments.

The contributors concerned with reviewing the means available for practising nutritional surveillance in Britain, Czechoslovakia, the USA and Canada all stress the superiority of information derived from epidemiological surveys among the general population over that from laboratory work with experimental animals. Nevertheless, surveillance systems that are too broad may well fail to identify specific problems, as was clearly brought out in a recent haemoglobin analysis of some 32,000 blood samples (0·016% of the US population). The data obtained were virtually meaningless until they were sorted out into groups according to age and ethnic, geographical and social status, after which division the numbers tended to be too few for statistical analysis. Since the time required for conducting and evaluating these surveys is so long, it is also important that some method be developed to feed back information to those who can use it on a day-to-day basis. Such arrangements have reached an advanced stage in Canada, where the nutritional status and dietary intake of all segments of the population are continually monitored.

The second part of the proceedings deals with aspects of infant feeding, and with the complex factors that control the dietary intake of the newborn child. Possible adverse effects of dietary imbalance include obesity, hypertension and hypertonic dehydration. No proven advancement on breast feeding exists at present, despite the numerous protagonists of innovations such as early weaning, high sodium intakes and high solute/water ratios.

On the subject of adult foods, one contributor expresses the view that the carbohydrate needs of the future could be comfortably met by large-scale *in vitro* photosynthesizing plants, and that there need be no concern over future availability of lipids. Another paper makes the point that the introduction of alternative protein sources, such as leaf or grass protein, gluten and oilseed isolates, will aid the reduction of carbohydrate intake and thus counteract the most overt form of malnutrition in western societies, namely obesity. Lipids, too, present their problems, the most widely publicized of which is the increased

risk of coronary heart disease among individuals with a high consumption of saturated fats. The recommendation that our intake of these fats be largely replaced by polyunsaturated fats has been put forward by a number of national and international groups concerned with the increasing problem of heart disease.

Several chapters are devoted to the problems of food fortification and its significance for various adult groups, including the elderly. One interesting contribution deals with the related requirements for the nutrients, calcium and vitamin D. It suggests that the UK recommendation for calcium intake may be too low for children and post-menopausal women and that the vitamin D allowance may have to be increased for older people, particularly if osteomalacia proves to be an important factor in the occurrence of fractures of the neck of the femur.

Overall, the contributors to this volume not only demonstrate a keen awareness of the nutritional problems of our society, but also provide expert opinions as to the best ways of overcoming these hazards to health.

The Second International Symposium on Microsomes and Drug Oxidations. Edited by R. W. Estabrook, J. R. Gillette and K. C. Leibman. The Williams & Wilkins Company, Baltimore, 1973. pp. viii + 486. $30.00.

Man is surrounded in his environment by a multitude of chemical substances—both those of natural origin and those that are the creation of his own ingenuity. Such compounds include natural products, food additives and preservatives, drugs, pesticides, cosmetics and industrial chemicals, and may be generally termed foreign compounds or 'xenobiotics'. The major site of metabolism of such compounds in mammals occurs in the endoplasmic reticulum microsomes of the liver.

This publication deals with the properties of the hepatic microsomal mixed-function oxidase system and particularly with the terminal haemoprotein oxygenase, cytochrome *P*-450. The book consists of a series of research papers presented at the Second International Symposium on Microsomes and Drug Oxidations held at Stanford University, California, in July 1972. The publication containing the papers presented at the First International Symposium (held in 1968) was reviewed some time ago (*Cited in F.C.T.* 1970, **8,** 546).

As cytochrome *P*-450 is an extremely atypical haemoprotein intimately associated with the endoplasmic reticulum, two series of papers are devoted to the chemistry of this material and to the structure of the microsomal membrane. Other subjects dealt with in detail include the induction and inhibition of xenobiotic metabolism, the effect of environmental factors on microsomal enzymes, and the synthesis and degradation of microsomal haemoproteins. Another group of papers is concerned with xenobiotic metabolism in extrahepatic tissues such as the kidney, lung and placenta. Of particular interest is the inclusion of a section on clinical studies of drug metabolism in man. This includes several contributions dealing with the various mechanisms involved in the metabolism of that ubiquitous drug, ethanol.

Each paper is supplemented with a list of references and, in addition, a fairly useful index is provided. The discussions that followed the presentation of some of the papers at the symposium are also reported. Altogether this monograph, which carries an impressive list

of contributors, provides an excellent overall picture of the state of knowledge, in 1972, on the mechanisms of xenobiotic metabolism.

Enzyme Inhibitors as Substrates. Interaction of Esterases with Esters of Organophosphorus and Carbamic Acids. By W. N. Aldridge and Elsa Reiner. North-Holland Publishing Company, Amsterdam, 1972. pp. xvi + 328. Dfl. 65.00.

The avowed intention of the series of monographs collectively entitled *Frontiers of Biology* is to present coherent and up-to-date views on the fundamental concepts that dominate modern biology. Several earlier volumes in the series have contained material of direct interest to the practising toxicologist, such as Volume 9 on the pyrrolizidine alkaloids. The present volume, which is number 26 in the series, also falls into this category and amply fulfils the aims established by the general editors. Drs Aldridge and Reiner are to be complimented on producing a treatise which, in addition to being an admirable and well-indexed source of reference, is a model of both general organization and detailed content.

Considered from the toxicological standpoint, it is unfortunate in some respects that the main title conceals from the casual observer the scope of the material actually considered. Thus, although apparently designed for the enzymologist, the text presents, in fact, an in-depth survey of the interaction of materials of known utility (including insecticides) from a wide range of organophosphorus compounds, carbamates and organosulphur derivatives with esterases as a group, ultimately analysing the mechanism of those reactions responsible for toxicity at a molecular level.

For the less well-informed, the authors take the precaution of embarking with a chapter establishing the ground rules of definition and nomenclature (although they wisely reserve detailed kinetic derivations for eighteen concluding appendices). Subsequent sections consider those esterase inhibitors that are in a sense quasi-substrates, having the capacity to interact specifically with the active site of B-esterases but yielding a chemically-modified enzyme molecule more or less incapable of further catalytic events, and culminate in a comparison of B-esterases with A-esterases. Particularly pleasing is the authors' use of deductive reasoning to establish mechanistic differences likely to exist between these two broad groups of esterases. Also worthy of special note is a timely account of the application of acylating inhibitors as research tools in the study of biological processes.

In their concluding remarks, the authors indicate with some degree of caution that, despite the extent of existing fundamental information, details of the catalytic mechanisms underlying the action of B-esterases remain to be elucidated, and they point to the need for continuing investigations, especially since reaction of enzymes within the group at sites other than the active centre can lead to both increased and decreased catalytic activity. Such information is clearly of prime importance to those concerned with refinements in the design of agents intended to achieve specific inhibitory functions, such as insecticides, especially where a "high safety factor for man" is sought.

In an era of multi-author review volumes, which frequently descend to little more than reference lists, it has been a refreshing experience to be taken through the lucid development of a research philosophy. This is not a book for those who are content with horizons bounded irrevocably by the execution of guidelined animal experiments, but it may be highly recommended to workers who seek deeper insights into the chemical mechanisms underlying toxicological manifestations.

Aerosol Technology in Hazard Evaluation. By T. T. Mercer. Academic Press, London, 1973. pp. xi + 394. £9.15.

This volume is a companion to *Pulmonary Deposition and Retention of Inhaled Aerosols* by T. F. Hatch and P. Gross, an earlier monograph in a series prepared under the direction of the American Industrial Hygiene Association and the United States Atomic Energy Commission. Like its stablemate reviewed earlier (*Cited in F.C.T.* 1965, **3**, 889), this later volume is clearly and concisely written and arranged in a straightforward and logical format.

An introductory chapter reviews the factors affecting the deposition, absorption and clearance of particulate matter in the lungs, and describes sampling procedures appropriate for various purposes. The emphasis of the book, as its title implies, is upon the theory of aerosol generation and characterization as far as these are relevant to the assessment of inhalation hazards. It gives comprehensive coverage to the properties of aerosols, including particle dynamics and aerosol stability, the geometric and aerodynamic aspects of measuring particle size and distribution and the pitfalls inherent in these estimations, and the quantitation of aerosol concentrations by various filtration, electrostatic, optical and piezoelectric methods. The characterization and separation of the respirable fraction of an aerosol are considered in a chapter on air samplers, while a final chapter deals with the special problems of generating a given type of aerosol for experimental purposes.

The wealth of information packed into this small book includes not only published information but a considerable amount of previously unreported work. The mathematics on which the theory is based appear to be unambiguous and, in general, it is difficult to find fault with this well-referenced text, which should be a valuable aid to anyone interested in the technology of aerosols from the viewpoint of investigating inhalation hazards.

Human Genetics. Proceedings of the Fourth International Congress of Human Genetics, Paris, 6–11 September 1971. Edited by J. de Grouchy, F. J. G. Ebling and I. W. Henderson. Excerpta Medica, Amsterdam, 1972. pp. 499. $50.00.

In the last two decades, the relevance of genetics to the practical problems of medicine and society at large has become widely appreciated. Today, growing fears of impending doom for mankind arise from the possibility that the chemical environment is causing or will cause changes in the genetic apparatus of human cells. This volume, which is the edited version of the proceedings of the Fourth International Congress of Human Genetics, presents reports of genuine scientific endeavour and rational interpretations of the results. The papers are conveniently arranged under a number of generic headings such as Population Genetics, Cell Genetics, Pharmacogenetics and so on, following in this respect the division of the Congress into eleven separate symposia. The papers fall generally into two types —those reporting experimental results and those which are summaries or commentaries on the current state of knowledge in a particular field, the latter frequently summarizing data generated by the author and others. Only a few of the papers are in French, the majority being in English.

The volume vividly illustrates the wide-ranging importance of genetics, not only in the sense of an academic subject but also in the broad spectrum of its practical implications, and it would be difficult to identify any kind of biologist who will not find something of

direct interest. The title *Human Genetics* is fully justified by the text, the great majority of which is concerned with observations made directly on man or with animal studies directly relevant to man. Thus, basic studies in molecular biology do not feature prominently, and this is an advantage. However, the inevitable problem arises for the non-specialist, in that the vocabulary of the geneticist, the statistician, the clinician, the immunologist and the pharmacologist are freely used.

A high standard of editing is reflected in the sensible arrangement of each paper, and the inclusion of adequate bibliographies for all the contributions except the brief introductory remarks presented at the beginning of some of the sessions

One disappointing feature is the rarity with which any report is made of the discussions that followed the presentation of papers. Although their reproduction presents considerable administrative and mechanistic difficulties, discussions often reveal the degree of controversy on particular issues and in addition throw up valuable suggestions for future work.

Enzyme Nomenclature. Recommendations (1972) of the International Union of Pure and Applied Chemistry and the International Union of Biochemistry. Prepared by the Commission on Biochemical Nomenclature. Elsevier Scientific Publishing Co., Amsterdam, 1973. pp. 443. Dfl. 15.00.

The post-war years were marked by an enormous growth in enzymology and in the application of this knowledge to many different branches of scientific study, including toxicology. The rapid proliferation of known enzymes during this period led to much confusion in terminology, many enzymes catalysing essentially similar reactions being given widely differing names while others totally different in type were included in the same group. Other major problem areas developed in connexion with co-enzyme nomenclature, the variety of mathematical symbols used in enzyme kinetics and the diversity of arbitrarily defined units of enzyme activity, which led to chaos in enzyme standardization.

The first major step towards overcoming these problems was the establishment in 1956 of an International Commission on Enzymes, formed by the International Union of Biochemistry in consultation with the International Union of Pure and Applied Chemistry. This Commission set out to devise systematic rules for the consistent naming of new enzymes, but at the same time sought to keep to a minimum the renaming of enzymes already well known. Several reports on enzyme classification and nomenclature were prepared by the Commission or by its successors—first the Standing Committee on Enzymes and then the IUPAC/IUB Commission on Biochemical Nomenclature—in conjunction with various other interested bodies. These reports and recommendations culminated in the 1972 version named above. The addition of new enzymes and the deletion of some old ones has left an enzyme list containing 1770 entries, compared with 874 in the 1964 list. Expert groups coped with various special fields, such as the cytochromes and oxygenases.

This report outlines at the start the general principles of enzyme nomenclature and the plan of systematic and trivial names, following this with an explanation of the basic scheme of classification and numbering used and the general and specific guidelines for recommended names. Multiple enzyme forms and enzyme precursors, units of enzyme activity, kinetic symbols and the nomenclature for electron-transfer proteins all receive separate consideration, but the bulk of the publication is taken up with the enzyme list, which provides for each enzyme its recommended name and number, the reaction catalysed, other

names and reactions and a key to relevant references. This is followed by the list of references cited (2743 in all), an index to the enzyme list, relating both recommended and systematic names to the corresponding enzyme number, and a subject index.

The layout throughout is logical and straightforward and makes the tracing of individual enzymes a relatively simple task. These recommendations have gone a long way towards clearing the confusion that has existed in this field and towards ensuring that new enzymes are introduced and identified without ambiguity. They should prove of considerable value not only to enzymologists *per se* but to all concerned indirectly with enzyme reactions.

BOOKS RECEIVED FOR REVIEW

Bread. An Assessment of the Bread Industry in Britain. The TACC Report. By Technology Assessment Consumerism Centre. Intermediate Publishing Ltd., London, 1974. pp. 89. £1.25.

Introduction to Molecular Embryology. By J. Brachet. The English Universities Press Ltd., London, 1974. pp. xi + 176. £2.45.

The Chemistry and Metabolism of Drugs and Toxins. An Introduction to Xenobiochemistry. By M. Briggs and Maxine Briggs. William Heinemann Medical Books Ltd., London, 1974. pp. xii + 386. £5.00.

Progress in Medicinal Chemistry. Vol. 10. Edited by G. P. Ellis and G. B. West. North-Holland Publishing Company, Amsterdam, 1974. pp. x + 294. $30.00.

Environmental Medicine. Edited by G. M. Howe and J. A. Loraine. William Heinemann Medical Books Ltd., London, 1973. pp. xii + 271. £6.00.

Advances in Cancer Research. Vol. 18. Edited by G. Klein and S. Weinhouse. Academic Press, London, 1973. pp. ix + 409. £12.90.

Mercury, Mercurials and Mercaptans. Edited by M. W. Miller and T. W. Clarkson. Charles C. Thomas, Illinois, 1973. pp. xvii + 386. $19.75.

Fd Cosmet. Toxicol. Vol. 13, pp. 275-284. Pergamon Press 1975. Printed in Great Britain

Information Section

ARTICLES OF GENERAL INTEREST

THE VINYL CHLORIDE PROBLEM

Since the appearance last year of reports linking several fatal cases of a rare form of liver tumour with prolonged industrial exposure to vinyl chloride, considerable attention has been paid to the possible carcinogenic and other toxic effects of this important monomer. Meetings of toxicologists have been held in Europe (*Cited in F.C.T.* 1975, **13,** 121) and the USA (Kramer, *Wall Street J.* 13 May 1974, p. 10) to assess existing data and to consider what further information is required and how this can best be obtained. While efforts are maintained to establish just how great the hazard may be, manufacturers, trade associations, trade unions, government departments and other groups concerned in various ways with the maintenance of reasonable standards of occupational safety have all been concerned with the problem of reducing exposure to gaseous vinyl chloride to a minimum (*Nature, London* 1974, **247,** 420).

The need has been recognized for a marked reduction in the threshold limit value for vinyl chloride, from the 200 ppm previously deemed appropriate to at most 50 ppm, although the results of some recent animal studies suggest the advisability of an even lower level. In the UK, the Factory Inspectorate has, in fact, set a new interim upper limit of 50 ppm for vinyl chloride exposure in PVC-manufacturing plants, with a time-weighted average exposure of 25 ppm (*Lancet* 1974, **I,** 1323). In the USA, where an emergency temporary standard issued in April reduced the ceiling value for vinyl chloride exposure from 500 to 50 ppm (*Federal Register* 1974, **39,** 12342), the Occupational Safety and Health Administration has since proposed much more stringent limitations on exposure (*Food Chemical News* 1974, **16** (8), 2).

The liver tumours that gave rise to all this concern were of an extremely rare type. Haemangiosarcomas (otherwise known as angiosarcomas, haemangioendotheliomas or haemangioblastomas, to name a few alternatives) arise from the epithelium of the blood vessels and in the past have been reported mainly in patients injected with Thorotrast, a radio-opaque material which was used as a contrast medium in radiography. It has been stated (Adam *et al. Ann. Surg.* 1972, **175,** 375, cited from Block, *J. Am. med. Ass.* 1974, **229,** 53) that, excluding these Thorotrast cases, the occurrence of only 43 of these tumours in adults had been reported in the world literature up to 1972. The actual incidence of haemangiosarcoma in the USA has been put at 20–25/year (*British Medical Journal* 1974, **1,** 590) and the occurrence of several deaths from this form of tumour within a relatively small area therefore quickly led to further investigation. These six cases among workers at a plant in Louisville, Kentucky, have been reported in some detail (Block, *loc. cit.*) and a recent count gave the total number of cases of the tumour in workers involved in PVC manufacture as 19 (Lee & Harry, *Lancet* 1974, **I,** 1316), the early reports from the USA

having been augmented by others from Sweden and Germany and also by one case in the UK. Lee & Harry (*loc. cit.*) have provided full details of the latter patient, who died when 71 years old and who had been for 20 years a process worker engaged in the polymerization of vinyl chloride.

Because of the long periods of exposure to which this and the other haemangiosarcoma patients had been subjected, it seemed likely that the cancer could be attributed to prolonged and persistent exposure to low levels of vinyl chloride. However, the possibility that the tumours arose as a result of brief periods of high-level exposure many years before cannot be altogether dismissed. All the American cases reported in detail had at one time been "pot cleaners", and Block (*loc. cit.*) makes the interesting observation that in this capacity they were, in their early years in the PVC factory, required to enter large polymerization tanks, approximately 10 ft high and 6 ft in diameter and with a 2-ft oval opening in the top, in order to chip the reaction residues from the walls. The potential for exposure was particularly great in the early years, because the residue often contained pockets of trapped gas which was liable to be released literally into the cleaner's face during the chipping operations. The subsequent development of improved cleaning techniques and stricter attention to industrial hygiene measures, including the use of respirators, has undoubtedly reduced this type of exposure considerably in more recent years, but it remains to be seen how significant this factor alone may have been in the induction of haemangiosarcoma.

Meanwhile, a recent paper (Monson *et al. Lancet* 1974, **II,** 397) has extended the question of vinyl chloride carcinogenicity beyond the narrow confines of haemangiosarcoma. It has already been remarked (*British Medical Journal loc. cit.*) that the apparent carcinogenicity of vinyl chloride would probably have been much less readily recognized if the resulting tumours had been of a more common type. The possibility that vinyl chloride exposure may also have an effect on the incidence of other forms of cancer was not lost on Monson *et al.* (*loc. cit.*), who carried out a proportional-mortality analysis of the 161 deaths recorded between 1947 and 1973 among past and present workers in the Louisville vinyl chloride polymerization plant associated with the first haemangiosarcoma reports and in the same company's production plant for vinyl chloride monomer. The mortality patterns for the two plants were similar, so all the deaths were considered together. The survey revealed that the number of deaths due to cancer of any type was 50% higher than the number to be expected on the basis of the relevant figures for the comparable section of the whole US population. The highest excess over expected mortality was found in the deaths from cancer of the liver and biliary tract, and the incidence of tumours of the brain was also unexpectedly high, as to a less marked extent was that of lung tumours. The excess incidence of fatal cancer was seen mainly in men who died before the age of 60 years. Also observed was a clear trend in the time pattern of the ratio of observed to expected deaths. No excess of deaths due to cancer was observed in the workers dying before 1965, but subsequently the proportion of these deaths increased and since January 1970 cancer has accounted for over twice as many deaths as would have been expected. While the inherent drawbacks in this type of study must not be overlooked, this report does provide an indication that some forms of vinyl chloride exposure may induce tumours in organs other than the liver.

[A.M.S. BIBRA]

LEAD EXPOSURE AND ITS SIGNIFICANCE

During the past decade, it has been widely alleged that modern man is faced with a far heavier burden of lead than were his predecessors, although some studies, including that of Barry & Mossman (*Br. J. ind. Med.* 1970, **27,** 339) have indicated that this is not necessarily the case. This argument apart, however, a major problem exists in establishing the maximum level of lead exposure that will present no overt or insidious hazard to man. Children present a particular problem in this connexion, since they may be more vulnerable than adults to the toxic effects of lead. Differences in the clinical manifestations of lead poisoning in adults and children (King *et al. Am. J. publ. Hlth* 1972, **62,** 1056) may be due, in part, to the fact that in adults about 94% of the total body burden of lead is situated in the bone tissue, while in children under 6 years old only 64% is located in the bones (Barry & Mossman, *loc. cit.*). The remaining lead is found mainly in the soft tissues and seems likely to be the fraction responsible for the known toxic effects of severe lead exposure in children. Nevertheless the situation is far from clear-cut and the question of relative vulnerability to lead toxicity may only be answered when adequate methods become available for comparing the responses of children and adults to subclinical lead levels.

Normal lead intake by children

The lead intake of children living in dwellings free from hazardous sources of lead is derived partly from the diet and partly from inhaled air. It is generally accepted that the food intake of a child between 2 and 3 years old is approximately half that of an adult, so that on the basis of a figure of 300–400 μg/day for the oral ingestion of lead by normal adults the normal oral intake of lead for a child has been estimated as 150–200 μg/day (King, *Am. J. Dis. Child.* 1971, **122,** 337). A study by L. B. Tepper (unpublished work cited from King, *loc. cit.*) indicated, however, that the lead content of the average adult diet was rather less than that quoted above, giving an estimated figure of 100–140 μg/day for a child. As regards inhalation, if one assumes a ventilation of about twice the resting volume, the 24-hour air exchange by children aged between 1 and 3 years may be estimated at about 6 m^3. Taking this figure and a normal value of 2 $\mu g/m^3$ for atmospheric lead in areas free from hazardous sources of the metal (Chow, *Chemy Brit.* 1973, **9,** 258), together with the revised estimate for lead ingestion, the minimal values for total lead intake are approximately 100–150 μg/day (King, *loc. cit.*). If we accept the maximum values quoted, however, the daily lead intake in unpolluted areas would be of the order of 150–200 μg. Some support for the latter values has come from lead-excretion studies in children (Chisolm & Harrison, *Pediatrics, Springfield* 1956, **18,** 943; cited from King, *loc. cit.*). This average daily intake represents 50–66% of the maximum daily permissible intake of lead for children—set at 300 μg by a US Public Health Service *ad hoc* committee in 1971 (King, *loc. cit.*).

This level was specified without the benefit of more recent data pointing to a greater gastro-intestinal absorption and retention of lead in children than in adults (King *et al. loc. cit.*). Balance studies in eight healthy children (aged 3 months–8·5 years) have since been reported by Alexander *et al.* (Proceedings of the International Symposium on Environmental Health Aspects of Lead, Amsterdam, October 1972, p. 319, Commission of the European Communities, Luxembourg, 1973; cited from Lin-Fu, *New Engl. J. Med.* 1973, **289,** 1289). These studies demonstrated a 53% absorption rate for ingested lead and an average retention of 18%, whereas earlier studies in adults (*Cited in F.C.T.* 1967, **5,** 716) indicated that less than 10% of ingested lead was absorbed. On this basis, Alexander *et*

al. (*loc. cit.*) proposed that the limit of daily lead intake in children up to 5 years of age should be set at 10 μg/kg.

Estimation of the body burden of lead

In another article, Lin-Fu (*New Engl. J. Med.* 1972, **286,** 702) reviewed the literature on lead intoxication, concluding that "the mean blood lead level of the urban population without undue intake, expressed in micrograms per 100 ml, is between the teens and lower twenties, the upper limit of normal should be no higher than 40 μg per 100 ml".

If we take the arbitrary figure of 40 μg/100 ml as the maximum blood-lead level that can be taken to indicate no undue absorption, it becomes clear that a significant proportion of young children living in urban areas throughout the world may be accumulating an undesirable body burden of this element. In New York, for example, the results of a mass screening programme in 1970 revealed that more than 4000 out of some 80,000 children screened had blood-lead levels of at least 60 μg/100 ml and some 25% had levels of 40 μg/100 ml or more (Guinee, *Am. J. Med.* 1972, **52,** 283). Simpson *et al.* (*Hlth Servs Rep.* 1973, **88,** 419) reported the results of neighbourhood surveys in 27 US cities, aimed at assessing the proportion of children aged 1–6 years who were exposed to lead-based paints in their homes and who showed evidence of an excessive lead burden. In 25 of these cities, limited venepuncture sampling for atomic absorption spectrophotometry or dithizone analysis, together with more extensive fingerstick sampling and anodic stripping voltammetry, indicated that about 6% of the children had blood-lead levels at or about 40 μg/100 ml. In two of the cities, however, a much more extensive venepuncture screening was carried out, and this indicated that these elevated lead levels were present in around 30% of the children, some 95% of whom were coloured.

Although the majority of reports of potential lead intoxication in children have centred on major metropolitan areas, there is reason to believe that smaller communities may also be exposed to uncomfortably high levels of this element. In a survey of lead poisoning in 14 Illinois cities of intermediate size (with populations between 10,000 and 150,000), for example, blood specimens were obtained from 6151 children aged between 1 and 6 years and nearly 20% of these infants were found to have blood levels of at least 40 μg lead/100 ml (Fine *et al. J. Am. med. Ass.* 1972, **221,** 1475). The percentage of subjects showing undue absorption of lead of this order in the individual cities varied from 9·6 to 31·3%.

In view of the known ability of lead to cross the human placenta (*Cited in F.C.T.* 1966, **4,** 533), it might be expected that infants born to mothers living in an urban environment would have higher levels of lead in their umbilical-cord blood than infants born to mothers residing in a suburban area. Scanlon (*Am. J. Dis. Child.* 1971, **121,** 325) appears to have pioneered work in this field, in a study involving 28 infants born in Boston. The average lead content of the cord blood of infants from metropolitan Boston was 22·1 μg/100 ml, compared with a level of 18·3 μg/100 ml for infants from the suburban area of the city, a difference not considered to be statistically significant. Essentially the same conclusion was reached by Harris & Holley (*Pediatrics, Springfield,* 1972, **49,** 606) in their survey of 24 mothers, 11 from suburban middle class backgrounds and 13 from an urban ghetto. There were no statistically significant differences between the blood-lead levels of the two groups, and the mean value for the cord blood was nearly 13 μg/100 ml with a range of approximately 10–20 μg/100 ml. The same range was found in the maternal blood, which showed a mean of slightly less than 14 μg/100 ml. Urban foetal and maternal blood-lead values

recorded by Rajegowda *et al.* (*J. Pediat.* 1972, **80,** 116) ranged from roughly 10 to 30 μg/100 ml, and correlated well with the results of previous work.

Children living in rural areas, although generally exposed to lower atmospheric lead concentrations than their urban counterparts, cannot be considered free from the hazards of lead exposure. A recent survey of 230 rural children aged 1–5 years (Cohen *et al. J. Am. med. Ass.* 1973, **226,** 1430) revealed blood-lead levels of 40 μg/100 ml or more in 9%, although none of these children showed any signs of lead poisoning.

Sources of lead exposure

It appears that in the past the main source of lead in cases of childhood poisoning has been the peeling lead paint and lead-impregnated plaster often found in old, badly maintained homes. Thus, of 1155 children treated for lead poisoning in Chicago between 1967 and 1968, 78% had a history of pica associated with ingestion of paint and plaster (Sachs *et al. Pediatrics, Springfield* 1970, **46,** 389; cited from Lin-Fu, *New Engl. J. Med.* 1973, **289,** 1229). It is significant that in the survey made by Cohen *et al.* (*loc. cit.*) 18 out of 19 rural dwellings in which flaking surface coatings were found yielded at least one paint, plaster or putty sample containing more than 1% lead. According to recent calculations, the accidental ingestion of only 1 cm^2 (65 mg) of a 10-layered paint chip with a 1% lead content may add 650 μg, or over twice the permissible daily intake, to the body burden of lead (American Academy of Pediatrics Committee on Environmental Hazards, *Pediatrics, Springfield* 1972, **49,** 918).

Other lead sources that can present a specific problem in children include pencils coated with paint containing a high proportion of lead chromate and the lead present in some toothpaste tubes. Schoplowsky (*HSMHA Hlth Rep.* 1971, **86,** 961) has reported finding 12·5% lead in the surface coating of one brand of pencil, while some toothpastes have been found to have acquired more than 180 μg lead/g from their containers, the outer coatings of which sometimes contain more than 5% lead (Berman & McKiel, *Archs envir. Hlth* 1972, **25,** 64; Shapiro *et al. J. Am. dent. Ass.* 1973, **86,** 394; both cited from Lin-Fu, *New Engl. J. Med.* 1973, **289,** 1229). Other writers have suggested that the coloured printing inks used in magazines and comics may be a significant source of lead for paper chewers (Hankin *et al. Clin. Pediat.* 1973, **12,** 654; Joselow & Bogden, *Am. J. publ. Hlth* 1974, **64,** 238; Tinker, *New Scient.* 21 March 1974, p. 731).

Lin-Fu (*New Engl. J. Med.* 1973, **289,** 1229) draws attention to a store of lead in the environment that may sometimes be overlooked. A recent analysis of street dirt in Washington, D.C., revealed a lead concentration as high as 12·8 mg/g in samples collected at a busy road junction, and many samples from other areas contained 4–8 mg/g, while the surface soil in a Los Angeles park had a lead content of over 3 mg/g. These are, of course, extreme examples, but they must still be viewed in the context of the daily intake of 300 μg lead estimated as acceptable for children.

Much has already been written about the possible contribution of atmospheric lead to the daily lead burden of urban dwellers and the importance of this source in connexion with blood-lead levels in children. In this connexion, Lin-Fu (*ibid* 1973, **289,** 1229) makes the point that devices used for measuring lead concentrations in ambient air are seldom placed at the level at which children breathe, although the concentrations vary markedly with altitude. One study cited demonstrated that concentrations 20 m above ground were only 50% of those at 1·5 m (Petrova *et al. J. Hyg. Epidem. Microbiol. Immun.* 1966, **10,** 383).

Toxicological significance of high blood-lead levels

While discussion continues on the relative significance of various sources of environmental lead (*Nature, London* 1974, **248,** 469), there remains the need to establish the clinical significance of elevated blood levels of the element. For some years there has been growing concern over possible untoward effects in children whose blood-lead concentrations lie in the 40–60 μg/100 ml range. In some cases, a high incidence of clinical abnormalities has been reported, as in a recent survey conducted in Birmingham (Betts *et al. Br. med. J.* 1973, **1,** 402) in which 9/24 children (36%) with blood-lead levels of 37–60 μg/100 ml were anaemic, as opposed to only 3/21 children (14%) with blood-lead levels in the normal range. This was in keeping with a significant correlation ($P < 0{\cdot}002$) between blood-lead and haemoglobin concentrations at times of diagnosis. The inhibitory effect of lead on haem formation is well-documented, but it is important to recognize that iron deficiency could have potentiated this effect.

As blood-lead levels reach above the normal range, it is possible that certain biochemical functions may be impaired in the absence of overt symptoms. We have previously referred to an observed impairment of haem synthesis, resulting from inhibition of erythrocyte δ-aminolaevulinic acid dehydrogenase (ALAD), an inhibition which increases progressively as blood-lead levels increase from 4 to 33 μg/100 ml (Hernberg & Nikkanen, *Lancet* 1970, **i,** 63). This inhibition is accompanied by increased excretion of δ-aminolaevulinic acid and type III coproporphyrin, the increase becoming measurable at blood-lead levels of around 40 μg/100 ml (*Lancet* 1973, **i,** 87).

Although these early haematological abnormalities cannot be ignored, they are known to be reversible, and are therefore regarded less seriously than the possible subclinical neurological effects of the metal. As an example of subclinical neuropathy, Catton *et al.* (*Br. med. J.* 1970, **ii,** 80) have presented evidence of a minor degree of peripheral nerve damage in a group of 19 workers involved in manufacturing lead accumulators. Standards of hygiene in the factory were poor and seven of the workers had blood-lead levels of 120 μg/100 ml or more, while six others were in the 80–119 μg/100 ml range and the remaining six were in the 40–80 μg/100 ml range. Seven had haemoglobin levels below 12 g/100 ml, indicating considerable exposure to lead, but none showed any overt clinical signs of any neurological lesion. Nevertheless, nerve-conduction velocity studies revealed a defect in peripheral nerve function in some of the workers and there appeared to be some association between the abnormal electrical findings, a low haemoglobin value and the duration of exposure to lead.

In the survey by Betts *et al.* (*loc. cit.*) eight children were found to have a blood-lead concentration greater than 99 μg/100 ml, and all these suffered from encephalopathy, one case of which proved fatal. On the whole, however, the possible effects of subclinical encephalopathy have received little attention. According to David *et al.* (*Lancet* 1972, **ii,** 900), one manifestation of this may be hyperactivity in children. These workers compared groups of hyperactive children with others of similar social, racial and economic background. More than half of the hyperactive children had blood-lead levels above 24·5 μg/100 ml, compared with approximately one quarter of the controls. In addition, 62% of the hyperactive subjects had post-penicillamine urinary lead levels above 80 μg/litre (indicative of significant lead accumulation in bone as a result of earlier exposure) compared with only 21% of the control subjects. Only eight of the hyperactive children had a history of previous lead poisoning. All of these showed raised lead levels in blood and urine, but the

findings of this study indicate that many of the other hyperactive children had raised body stores of lead. It is also significant that replies to a questionnaire revealed a much higher incidence of histories of lead exposure for the hyperactive children than for the controls. This apparent correlation merits further investigation.

Pueschel *et al.* (*J. Am. med. Ass.* 1972, **222,** 462) have recently achieved what may be regarded as a breakthrough in investigations of subclinical lead poisoning by studying the psychological, as well as the neurological, effects in young children known to have a high lead burden. In a survey conducted in a working-class area of Boston, 705 children below school age were screened by analysis of the lead content of their hair, and in this way 98 were found to possess a markedly increased lead burden. This condition was considered confirmed when further studies revealed a blood-lead content above about 50 μg/100 ml and/or a urinary lead output of 500 μg/24 hours after chelation therapy. The 58 children thus identified were then given a comprehensive examination, which was repeated after a further 1·5 years, steps having been taken to eliminate excessive lead exposure during this intervening period. During the initial examination, nearly 25% of the children exhibited fine motor dysfunction and slightly impaired balance, compared with less than 10% in a control population. The majority of children were classified in the 'low-average' range of mental ability, the mean intelligence quotient (IQ) being 86, with a standard deviation of 15. Of the 42 patients located after 1·5 years, 32 were reported to be living in acceptable environmental conditions. Once again, about 25% of the children exhibited impairment of fine motor activity and poor co-ordination, and the same proportion showed some reduction in gross motor function. In keeping with this, Visual Motor Integration Test results did not alter significantly upon retesting. A significant improvement in some areas of intellectual ability was noted, however, and an average increase of eight IQ points was recorded. A combination of factors could have brought about these changes, which are in any case difficult to interpret since no control population was included in the study.

The results obtained by de la Burdé & Choate (*J. Pediat.* 1972, **81,** 1088) are more meaningful in this respect, since simultaneous evaluations were made on 70 4-year-olds unduly exposed to lead and on a further group of 72 children of the same age and with comparable socio-economic backgrounds but no known unusual exposure to the metal. Although the exposed group had shown no overt clinical symptoms, only 35% of these could be considered 'normal' with respect to all psychological testing procedures, which included estimations of IQ, motor performance and behaviour. In comparison, 65% of the control group performed 'normally' in all the areas tested. Failure in the fine motor tests, for example, occurred almost twice as often, and deviations in overall behaviour ratings occurred almost three times as frequently in lead-exposed children than in controls. Blood-lead levels in the experimental groups ranged from 30 to 100 μg/100 ml, but this parameter was not investigated in the control group, so that it is possible that the differences found were due to environmental factors other than lead.

Another study, however, carried out on a relatively homogeneous London population, produced rather different findings (Lansdown *et al. Lancet* 1974, **I,** 538). This included built-in controls, since it covered the children of families whose homes were situated at varying distances from a factory responsible for emitting lead into the atmosphere, so that the children were subject to lead exposure of varying degrees of severity. The distance of the home from the factory was reflected in the blood-lead levels of the children, but no correlation was established between these blood levels and measurements of mental function (intelligence, reading attainment and behaviour). This study, which has not been with-

out its critics (Bryce-Smith & Waldron, *ibid* 1974, **I,** 1166; David, *ibid* 1974, **I,** 866; Landrigan *et al. ibid* 1974, **I,** 1167) indicated that, in this population, social factors were of more importance than lead exposure in determining mental development.

Conclusion

Although behaviour has a very complex aetiology in which social factors play an important part, there is clearly a possibility that neurotoxic chemicals such as lead may influence intellectual capacity or behaviour when present in the environment at levels that fail to elicit any overt toxicological manifestations. While this must still be regarded as an open question, it could be a problem in urban and suburban areas where children are exposed to high atmospheric concentrations of lead and where blood-lead levels well above normal values are frequently recorded. A note of caution should be introduced here, however, since elevations in blood-lead levels have often been criticized as indices of the overall picture of exposure. This is because transitory changes in blood-lead levels reflect actual periods of exposure or ingestion and then decline as lead is taken up by other body tissues. On the other hand, calcified tissues are known to store lead and would be expected to represent a more accurate index of lead intake. Bone biopsies are generally impractical, for obvious reasons, but deciduous teeth may offer a valuable alternative, since they are physically stable and have fixed times of formation and exfoliation. In a recent American study (Needleman *et al. Nature, Lond.* 1972, **235,** 111), for example, the mean lead content of primary teeth from suburban controls was 11·1 ± 14·8 ppm, while for ghetto children it was 51·1 ± 109·0 ppm. When the same group of workers determined the distribution of lead in human permanent and primary teeth (Shapiro *et al. Envir. Res.* 1972, **5,** 467), comparable levels of the metal were found in coronal dentine, root dentine and enamel, but raised levels were found in the superficial layers of the secondary dentine (i.e. those layers nearest to the pulp). Since this tissue is in intimate contact with blood and its calcification is a continuous process, continuing throughout the life of the tooth, a method has been developed for the analysis of secondary dentine with a view to providing a useful means of identifying subclinical lead poisoning (*idem, Clinica Chim. Acta* 1973, **46,** 119). Such methodology, combined with the development of psychological test profiles of the type previously elaborated for work on carbon disulphide poisoning (*Cited in F.C.T.* 1973, **11,** 151) may help to establish whether subclinical levels of lead can evoke permanent neurological damage in children or adults.

[J.J.-P. Drake—BIBRA]

OCHRATOXIN IN EXPERIMENTAL AND FARM ANIMALS

The widespread distribution of ochratoxin A (OA), a metabolite of *Aspergillus ochraceus*, other aspergilli and *Penicillium viridicatum* (*Cited in F.C.T.* 1973, **11,** 903) has given rise to concern, since the toxin has been associated with liver toxicity in farm stock (*ibid* 1967, **5,** 730), with kidney disease in some species, including pigs (*ibid* 1975, **13,** 159) and possibly also with cases of bovine abortion (*ibid* 1972, **10,** 606). Samples of mouldy grains, beans and peanuts collected in Canada, for example, have been found to contain 0·02–27 ppm OA (Scott *et al. J. agric. Fd Chem.* 1972, **20,** 1103).

Changes similar to those seen in livestock fed mouldy feeds have been induced experimentally in animals fed OA, and in an attempt to establish more clearly the degree of responsibility of OA for mould-induced farm toxicoses, Munro *et al.* (*J. Am. vet. med. Ass.* 1973, **163**, 1269) have studied the acute and subacute effects of feeding OA to rats. Male Wistar rats fed a diet supplemented with levels of OA between 2·4 and 24 ppm for 2 weeks showed impairment of renal function and a slight increase in urinary protein content and there was a significant rise in blood urea nitrogen in animals fed the 9·6 or 24 ppm level. The kidneys of these rats showed accelerated hyaline droplet degeneration, principally in the proximal tubules but, in those given 24 ppm, also in the distal tubules, loop of Henle and upper connecting ducts. The livers of rats fed 9·6 or 24 ppm OA showed reduced cell growth, cytoplasmic hyalinization and pyknosis of scattered single cells. Contrary to earlier indications (*Cited in F.C.T.* 1969, **7**, 401) but in line with a more recent study (*ibid* 1974, **12**, 432), there was a reduction in liver glycogen, possibly as a result of a reduction in food consumption. In male and female rats fed 0·2–5 ppm OA for 90 days, kidney lesions were similar to those seen in the shorter study but were less marked, affecting mainly the proximal tubules. Hyaline changes and pyknosis were more extensive at the 1 and 5 ppm feed levels than at the 0·2 ppm level. Half of the animals treated in this way were killed only after a further 90 days on the control diet, and at the end of this period, liver-glycogen levels were still reduced.

As the authors indicate, this study does not go very far towards determining the significance of OA in the aetiology of naturally-occurring mould toxicoses. In addition to further data on the occurrence of the toxin in foods and feeds, formal studies on its toxicity and metabolism in food-producing animals are essential if a clear indication is to be obtained of the relative importance of OA and other mycotoxins and the extent to which diet and various environmental factors influence the pathogenesis of the observed lesions. One such study, in pigs, has been reported fairly recently, by a group which also examined the effects of OA in dogs.

Szczech *et al.* (*Vet. Pathol.* 1973, **10**, 135 & 219) have described the clinical and pathological effects in beagles given 0·2–3·0 mg OA/kg body weight/day orally either as the pure toxin or as a rice culture. Most of the dogs were killed *in extremis* on or before day 14 of treatment and all suffered anorexia, weight loss, emesis, blood-stained rectal mucus, a high temperature, dehydration and prostration. Indications of renal damage were prominent, the urine having a low specific gravity and containing granular casts, necrotic renal epithelial cells and raised levels of protein, glucose and enzymes, although serum-enzyme levels were not affected. Serum-enzyme studies gave no indication of hepatic dysfunction. Autopsy revealed mucohaemorrhagic enteritis of the caecum, colon and rectum, tonsillitis, and enlargement, oedema and focal necrosis of the lymph nodes. Renal necrosis and desquamation of the epithelial cells were most prominent in the proximal convoluted tubules. Lymphoid-tissue necrosis occurred principally in the germinal centres of the spleen, tonsils and thymus and the lymph nodes and lymphoid nodules of the intestinal tract, but was not found in the bone marrow. Slight to moderate centrilobular necrosis and fatty change occurred mainly in the livers of dogs given 0·2–0·3 mg/kg for 11–14 days.

A subsequent paper by Szczech *et al.* (*ibid* 1973, **10**, 347) reported a reduction in feed intake and body weight and the occurrence of diarrhoea, polyuria, polydipsia and dehydration in pigs fed a diet containing 0·6 or 1·8% OA as a rice culture or given the pure toxin in a daily oral dose of 1·0 or 2·0 mg/kg. The former groups survived for 16–22 days, the latter for only 3–6 days. Haematological findings reflected a progressive degree

of dehydration and the white cell counts were indicative of tissue necrosis. The lesions of the gastro-intestinal mucosa were moderate to severe and were less restricted in location than those in the dog, but the lymphoid-tissue necrosis was less severe and hepatic lesions were limited to some perilobular fatty change. Renal necrosis again occurred mainly in the epithelium of the proximal convoluted tubules, which were dilated.

The renal lesions induced by OA were thus similar to those seen with citrinin, but the latter toxin has not been associated with enteritis or diarrhoea. Although citrinin has been strongly favoured as the probable cause of Danish porcine nephropathy, a recent report suggested that OA may be the more important aetiological factor (*Cited in F.C.T.* 1975, **13**, 159).

Following the finding that cultures of *A. ochraceus*, isolated from samples of mouldy hay suspected of inducing bovine abortion, produced a high rate of foetal resorption in rats (*ibid* 1972, **10**, 606), Munro *et al.* (*loc. cit.*) carried out some preliminary studies on the placental transfer and foetal toxicity of OA in sheep. Only trace amounts of OA appeared in the foetal blood and none was detected in the amniotic fluid, despite a maternal dose of 1 mg OA/kg given iv over 20 minutes and resulting in maternal blood levels of 8–3 μg/ml over the subsequent 12 hours. Both ewes appeared normal 8 hours after the infusion but died between 12 and 24 hours after treatment. The foetal blood levels found in this limited study suggest that the foetotoxicity of OA is unlikely to be significant, at least in the sheep.

However, ip injection of OA to mice in a single dose of 5 mg/kg on one of days 7–12 of pregnancy increased prenatal mortality, decreased foetal weight and caused various foetal malformations (Hayes *et al. Teratology* 1974, **9**, 93). The malformations were few in the offspring of mice injected on day 7 or 12, the highest incidence being associated with injection on day 8. Among the grossly apparent anomalies, exencephaly and malformations of the eyes, face, digits and tail predominated, while additional skeletal defects involved the ribs and vertebrae. The most severe malformations were found in some foetuses exposed to OA on day 8 or 9 of development. These foetuses were exencephalic, anophthalmic or microphthalmic and had a very marked median facial cleft completely dividing the facial area. In considering these findings, it should be remembered that this study involved injection of OA rather than the oral administration more directly relevant to a food or feed contaminant and also that teratogenic effects in the mouse are not necessarily reproducible in other species. Nevertheless, it demonstrates that the mouse placenta does not act as an effective barrier to this mycotoxin.

[A.M.S.—BIBRA]

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TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS

AGRICULTURAL CHEMICALS

2817. Monitoring exposure to arsenicals

British Medical Journal (1974). Arsenic makes a comeback. *ibid* **3,** 487.

Arsenical compounds are widely used as herbicides and for the destruction of insect pests, but their highly poisonous nature has tended to restrict their use, particularly as less toxic and equally efficient chemicals for performing the same tasks have become available. There are some situations, however, where arsenical compounds still appear to be the compounds of choice, a case in point being the control of weeds and seedling trees in forest areas.

The Editorial cited above discusses this problem, pointing out that, in both Britain and the USA, such forestry work involves hand-cutting and clearing and the use of knapsack sprayers. In Britain, the use of simple mixtures has proved adequate and the wearing of gloves and goggles by the sprayers has apparently provided satisfactory protection, but in the USA, relatively toxic preparations are still used, including dimethylarsinic acid (cacodylic acid). Despite the various forms of protective clothing used, workers apparently absorb a considerable amount of organic arsenic, some of which they excrete in the urine. A recent study (Wagner & Weswig, *Archs envir. Hlth* 1974, **28,** 77) showed that arsenic could be recovered from the urine during the first week of exposure. The urinary content of arsenic rapidly declines after exposure ceases, but it is not known whether all the arsenic absorbed is eventually eliminated. In an evaluation of the reliability of blood and urine levels as an indication of the degree of exposure to arsenicals, it was found that total urinary excretion was a better measure of any exposure to arsenic than random tests of blood or urine.

Because of an apparently increasing interest in the use of arsenicals both as herbicides for specific applications and as supplements in animal feedstuffs to counteract various pathogens of particular significance under modern conditions of animal husbandry, it is essential to keep a close watch in order to minimize the hazard such use may present. Residues of arsenic in the edible tissues of treated stock seem unlikely to present problems, but the possible conversion of cacodylic acid to arsine by fungi after its application could constitute an inhalation hazard to forestry workers.

2818. Cholinesterase values in industrial exposure to dichlorvos

Menz, M., Luetkemeier, H. & Sachsse, K. (1974). Long-term exposure of factory workers to dichlorvos (DDVP) insecticide. *Archs envir. Hlth* **28,** 72.

Various inhalation studies carried out on dichlorvos (*O,O*-dimethyl-2,2-dichlorovinyl 1-phosphate; DDVP) have indicated that continuous exposure (24 hr/day) for several days to concentrations of about 0·25 μg/litre is needed to produce any significant decrease in plasma cholinesterase (ChE) activity (*Cited in F.C.T.* 1974, **12,** 768). The US threshold limit value for industrial exposure has been set at 1·0 μg/litre.

In the study described above, 11 male and two female factory workers were exposed to an average dichlorvos concentration of 0·7 μg/litre on each working day over a period of 8 months in the course of either the production or processing of a dichlorvos-releasing product. General medical examination of the workers, as well as blood tests, were carried out at regular intervals both during and after the exposure period. ChE and erythrocyte acetylcholinesterase (AChE) were measured weekly during the period of exposure and monthly for the following 4 months.

Repeated exposure to 0·7 μg dichlorvos/litre was found to inhibit ChE activity by at least 60%, whereas AChE activity was only slightly affected (up to 35%). Inhibition of ChE activity was noted within a few days of the start of exposure, while AChE inhibition developed much more slowly. One month after exposure had ceased, ChE and AChE activities were found to have returned to normal physiological levels. The other haematological investigations and the medical examinations revealed no changes attributable to exposure to dichlorvos. Because of the rapidity with which dichlorvos disappears from the blood, the authors conclude that the ChE value is not only important as an indication of dichlorvos exposure, but is a more practical index than direct measurement of dichlorvos in the blood.

2819. Morfamquat and the kidney

Balogh, K. & Merk, F. B. (1973). Ultrastructure of renal collecting tubules following ingestion of a bipyridinium herbicide (morfamquat). *Experientia* **29**, 1101.

The bipyridylium herbicide known as morfamquat has been reported to cause degeneration of the proximal tubules or mitochondrial proliferation in the collecting ducts of the medullary-papillary zone of the rat kidney (*Cited in F.C.T.* 1970, **8**, 465).

The paper cited above describes the effects of feeding morfamquat dichloride at a level of 0·015% in the diet to 2-month-old male Sprague–Dawley rats. The animals were killed after 6, 12 and 24 wk and the parotid glands, thyroid, liver and collecting ducts of the kidney were examined by electron microscopy. The only ultrastructural change appeared in the renal collecting ducts, in which a constant finding, most marked at wk 24, was an overall increase in lysosomal bodies. These lysosomes, which were 0·2–1·2 μm in diameter, were enclosed in single-unit membranes and often contained small vesicular inclusions or whorls of packed membranes. Cellular debris was present in the lumina of the collecting ducts. There was no evidence of the mitochondrial proliferation in the collecting ducts previously described after the feeding of 0·015% morfamquat (*ibid* 1970, **8**, 465).

2820. A teratogenic anthelmintic

Duncan, W. A. M. & Lemon, Phyllis G. (1974). The effects of methyl-5(6)-butyl-2-benzimidazole carbamate (parbendazole) on reproduction in sheep and other animals. VIII. Teratogenicity in the rat. *Cornell Vet.* **64**, Suppl. 4, 97.

Parbendazole is a veterinary anthelmintic used in the treatment of nematode infestations and is effective in a variety of animal species in dosages in the range of 10–50 mg/kg/day given for several days (Actor *et al. Nature, Lond.* 1967, **215**, 321). Following reports that malformed lambs had been born to ewes dosed with this drug in early pregnancy (Saunders *et al. Cornell Vet.* 1974, **64**, Suppl. 4, 7), experiments have been conducted to investigate its teratogenic potential in the rat.

Various dilutions of parbendazole in water were administered by gavage to rats on days 6–15 of pregnancy. Animals given 12·5, 25 or 50 mg parbendazole/kg/day were killed on

day 19 and those given 0·5, 1, 2, 5 or 10 mg/kg/day were killed on day 21 of pregnancy. In all the former groups, the frequency of resorptions was much higher than that in control animals, and there was some evidence of a reduced incidence of implantation sites. Numbers of implantation sites did not differ from the control value in the groups given 0·5–10 mg/kg/day and no dose-related incidence of resorptions could be detected in these low-dosage groups. The average weights of foetuses from rats that had been given 10 mg/kg/day or more were significantly lower than the mean control weight, and the former were less mature for their gestational age.

The threshold dose for teratogenic effects was 10 mg/kg/day, at which level a slight increase in the incidence of major deformities was noted and there was a tendency towards abnormal ossification and the development of a fourteenth rib. At 12·5 mg/kg/day, the drug produced a 20% malformation rate (0% among controls), and at the highest dosage level 75% of the survivors were malformed. Skeletal and facial malformations were most common, although central nervous system defects were also evident. All the dosage levels administered were well tolerated by the adult rats.

PROCESSING AND PACKAGING CONTAMINANTS

2821. No explanation for meat-wrappers asthma

Van Houten, R. W., Cudworth, A. L. & Irvine, C. H. (1974). Evaluation and reduction of air contaminants produced by thermal cutting and sealing of PVC packaging film. *Am. ind. Hyg. Ass. J.* **35,** 218.

Exposure to fumes liberated during the cutting of polyvinyl chloride (PVC) film with hot wire has resulted in a form of respiratory distress, which has been termed 'meat-wrappers asthma' because of the frequent use of this process in meat packaging (*Cited in F.C.T.* 1975, **13,** 145). Reports of this respiratory irritation have led to studies of the pyrolysis products of PVC film under various conditions, and the authors named above have investigated the fumes produced by the actual machines used for the cutting and sealing process.

They found that even when the machines were severely misused to produce the maximum amount of fume, both the concentration of hydrochloric acid and the total particulate matter in the operator's breathing zone remained well below the occupational threshold limit values. The PVC films used in this work contained up to 30% dioctyl adipate plasticizer and this was found to account for 75–80% of the total particulate matter in the fumes. The authors point out that the limited toxicity data on this ester do not suggest that the atmospheric concentrations encountered under these conditions would present any inhalation hazard. Nevertheless, in the interests of the general reduction of air contamination in the working environment and since contamination from these machines varied directly with the operating temperature, they sought the lowest wire temperature at which the cutting process could be efficiently performed. For the type of film investigated, this temperature was found to be about 104°C.

THE CHEMICAL ENVIRONMENT

2822. Chlorhexidine as a soft lens sterilant

Davies. Mollie (1973). Rabbit eye irritation from bactericides in soft lens soaking solutions. *J. Pharm. Pharmac.* **25**, 134P.

A commonly-used ophthalmic preservative, benzalkonium chloride (BAK), may provoke occasional allergic reactions when used in this way (*Cited in F.C.T.* 1973. **11**, 1157) and has been shown to be a skin irritant when applied in high concentration to experimental animals (*ibid* 1974, **12**, 164). However, the rabbit eye was found able to tolerate daily administration of up to eight drops of an acidic solution containing 0·004% BAK, alone or with ethylenediaminetetraacetic acid (EDTA), when the drops were administered at 30-min intervals (Sussman & Friedman, *Am. J. Ophthal.* 1969, **68**, 703). Chlorhexidine diacetate (CHDA), used at a concentration of 0·01% as an alternative agent for the sterilization of certain eye drops, also evokes occasional allergic reactions in man (*Cited in F.C.T.* 1974, **12**, 799).

Because hydrophilic contact lenses absorb and concentrate bactericides and prolong their contact time with the cornea, it was considered essential to assess the safety of bactericides for this purpose by the use of a lens/animal model. Hydron soft lenses were equilibrated in bactericidally-effective concentrations of BAK (0·0025% plus 0·01% EDTA), CHDA (0·005%) or chlorocresol (0·1%), all formulated in isotonic solution at neutral pH, and were then fitted into rabbit eyes for 6 hr/day for up to 21 days, with overnight storage in the bactericidal solutions. After a few days, chlorocresol and BAK had produced severe irritation, which prevented further treatment, while CHDA produced a persistent mild irritation and, in two of the three rabbits, a corneal opacity. The latter effect developed towards the end of the treatment and histological examination confirmed stromal swelling and disorientation of collagen lamellae. Further experiments with CHDA at concentrations of 0·01–0·002% revealed toxic effects at all levels. Their severity and time of appearance depended on the concentration used; with weaker solutions the effects became evident only after several months. It was concluded that CHDA was not a suitable bactericide for use in solutions for soaking soft lenses and work is continuing to find a safer and more effective alternative.

2823. Hair depigmentation by *p*-cresol

Shelley, W. B. (1974). *p*-Cresol: cause of ink-induced hair depigmentation in mice. *Br. J. Derm.* **90**, 169.

In an earlier issue (*Cited in F.C.T.* 1972, **10**, 284), we reported on a paper which suggested that the depigmenting properties of two phenolic derivatives were due to the presence of a *p*-substituent in the benzene ring. A further *p*-substituted phenol, previously identified as having skin-depigmenting properties, has now been implicated in a similar effect on animal hair.

A study was undertaken to identify the chemical component responsible for a previously reported loss of hair pigment in mice exposed to contact with an indelible laundry-ink. The ink and its pure components, each in a concentration of 0·5% in acetone, were applied three times weekly for 6 wk to the plucked or clipped skin of female agouti mice or of black male mice of the C57BL/6J strain, and hair colour was observed for the following 6 months.

p-Cresol was identified as the active component, since it induced depigmentation of both skin and hair in the agouti as well as the black mice. In the agouti animals, bands, whorls and plaques of completely white hair were noted, but the most consistent finding was a loss of melanin granules from the root and shaft of each hair, with only the extreme distal portion retaining the pigment. In the black mice, patches of complete pigment loss included the tips of the hairs. These *p*-cresol effects persisted throughout the 6-month observation period. White surface patterns were observed in control animals painted with 8-hydroxyquinoline, but neither *o*- nor *m*-cresol application resulted in any sign of depigmentation over the 6-month period.

The essentially permanent pigment losses were related to a specific sensitivity of the follicular melanocytes to the toxic effects of *p*-cresol. A simple inhibition of tyrosinase would not be expected to affect new cycles of hair formation. More likely, therefore, is the previously suggested mechanism, that the enzyme tyrosinase acts on certain phenols to form free-radical derivatives which provoke lipid peroxidation, leading to permanent melanocyte damage or destruction (Riley, *J. Path.* 1970, **101,** 163; *idem, Br. J. Derm.* 1971, **84,** 290).

2824. Dimethylformamide and the human pancreas

Chary, S. (1974). Dimethylformamide: A cause of acute pancreatitis? *Lancet* **II,** 356.

In our last issue (*Cited in F.C.T.* 1975, **13,** 152), we referred to acute toxicity studies on certain alkylformamides, noting that both *N*-*n*-butyl- and *N*,*N*-di-*n*-butylformamides could induce severe pancreatitis in rats. The letter cited above now suggests a possible association between dimethylformamide (DMF) exposure and the development of acute pancreatitis in man.

DMF is used as a solvent in the spinning of polyacrylonitrile fibres, and two factory workers exposed to the solvent were found, on admission to hospital, to be suffering from upper abdominal pain radiating to the back, nausea, vomiting, an erythematous rash on hands and forearms and tenderness of the epigastrium. Unfortunately the serum-amylase level was only measured in one patient, in whom it was markedly higher than normal. Gall-bladder and bile-duct X-rays revealed no abnormalities.

A raised serum-amylase level is a diagnostic feature of acute pancreatitis, and the clinical picture of these cases was consistent with this type of lesion. Subsequent contact with DMF was associated with occasional epigastric discomfort in one patient, and both men remained free of symptoms after changing their jobs. The three other workers at the factory were all found to suffer from intermittent gastro-intestinal disturbance, erythema and pruritus, especially after ingestion of alcohol. Serum-amylase levels in these workers were measured before and after shifts, and at the latter time were found to be at the upper end of the normal range.

2825. Methoxyflurane metabolism

Van Dyke, R. A. & Wood, Catherine L. (1973). Metabolism of methoxyflurane: Release of inorganic fluoride in human and rat hepatic microsomes. *Anesthesiology* **39,** 613.

It is now established that the anaesthetic, methoxyflurane (MF), can cause mild renal damage in some individuals during its clinical use (Mazze & Cousins, *Can. Anaesth. Soc. J.* 1973, **20,** 64). This nephrotoxicity has been attributed to the inorganic fluoride (F^-) formed during its biodegradation, but the exact mode of metabolism has not been established. To supplement existing knowledge in this area, the workers cited above investigated

in vitro the amount of F^- released by the enzymatic metabolism of MF in microsomal fractions obtained from rat and human liver.

No obvious pattern emerged in the amount of F^- produced in a given MF concentration by the enzyme activity in measured samples of human microsomes. In particular, enzyme activity was not inversely proportional to the substrate concentration in this situation. The lack of uniformity in the response of different samples suggested a wide individual variation in microsomal activity, which showed no apparent correlation with age or sex. In contrast, microsomes obtained from three strains of rat (Long–Evans, Fischer 344 and Sprague–Dawley) showed a high intra- and inter-strain uniformity in enzymatic activity for this reaction, suggesting a similarity in the F^--releasing enzymes in these strains. The results of human and rat studies did agree, however, in the absence of any inhibition of MF metabolism to F^- at high substrate concentrations.

Moreover, the metabolism of MF by microsomes of human or rat origin yielded a product whose acid-lability resulted, at low pH, in the formation of additional F^- and formaldehyde. The identity of this acid-labile intermediate has not been established, but two possibilities are methoxydifluoroacetic acid, which has previously been detected among the products of MF metabolism (Holaday *et al. Anesthesiology* 1970, **33,** 579) and methoxyfluorodichloroethylene. Both compounds would yield not only formaldehyde and F^- by non-enzymatic degradation but could also be a source of oxalic acid, which has been demonstrated in the urine after MF anaesthesia.

2826. Peripheral neuropathy from methyl *n*-butyl ketone inhalation

McDonough, J. R. (1974). Possible neuropathy from methyl *n*-butyl ketone. *New Engl. J. Med.* **290,** 695.

The brief letter cited above refers to reports that have indicated a possible connexion between industrial exposure to methyl *n*-butyl ketone (MBK) and the development of peripheral neuropathy. Because of this possibility, studies have been undertaken on the effects of administration of MBK by various routes to several species of animal.

In one of these preliminary studies, nerve changes characteristic of peripheral neuropathy were found in six rats, which had been exposed to an atmospheric level of 1300 ppm MBK for 6 hr daily on 5 days/wk for 4 months. These findings, since confirmed by a second laboratory, were induced by exposures far in excess of the threshold limit value, which is 100 ppm, and further studies are planned to establish the maximum inhalation level that will fail to induce the effect.

2827. Methylene chloride TLV too high?

Ratney, R. S., Wegman, D. H. & Elkins, H. B. (1974). In vivo conversion of methylene chloride to carbon monoxide. *Archs envir. Hlth* **28,** 223.

For many years, methylene chloride (CH_2Cl_2) was considered to be perhaps the safest of the chlorinated hydrocarbon solvents. Recently, however, evidence was presented that the body converts inhaled CH_2Cl_2 to carbon monoxide (CO), and it was demonstrated that at the existing threshold limit value (TLV) of 500 ppm, the solvent might produce carboxyhaemoglobin (COHb) levels in exposed workers greater than those permitted from CO itself (*Cited in F.C.T.* 1974, **12,** 162). These observations have received further support from the study cited above.

Workers exposed to 180–200 ppm CH_2Cl_2 had pre-exposure COHb levels of about 4·5%, as measured by alveolar CO concentrations, and this level doubled after one 8-hr

exposure period. Before work commenced on the following day, the COHb content had returned to pre-exposure levels. The 24-hr time-weighted average COHb level was 7·3%, compared with 2·7% for personnel exposed to 35 ppm CO and 3·8% for those exposed to 50 ppm CO (the current TLV for CO in the UK and USA).

In the light of these findings, the authors suggest that the controlling factor in setting a TLV for CH_2Cl_2 should be its endogenous conversion to CO, rather than its more widely-recognized narcotic effects or hepatotoxicity. They calculate on this basis that a TLV of 75–100 ppm would be appropriate for CH_2Cl_2 rather than the 250 ppm limit tentatively adopted in 1973 by the Committee of the American Conference of Governmental Industrial Hygienists (*Michigan's Occupational Health* 1973, **19** (1), 3).

NATURAL PRODUCTS

2828. From sterigmatocystin to aflatoxin

Hsieh, D. P. H., Lin, M. T. & Yao, R. C. (1973). Conversion of sterigmatocystin to aflatoxin B_1 by *Aspergillus parasiticus*. *Biochem. biophys. Res. Commun.* **52,** 992.

Sterigmatocystin, produced by *Aspergillus versicolor*, needs little more introduction than the aflatoxins. Orally administered sterigmatocystin has been estimated to have a hepatocarcinogenic potency only about ten times lower than that of aflatoxin B_1, which has a similar chemical structure, and like aflatoxin, it has been nominated as a possibly important carcinogen in terms of human health in some parts of the world (Purchase & van der Watt, *Fd Cosmet. Toxicol.* 1968, **6,** 555).

Both toxins contain a bifuranomethoxybenzene ring, and sterigmatocystin has been postulated as an intermediate in several hypothetical schemes for aflatoxin biosynthesis, but direct evidence for the conversion of sterigmatocystin to aflatoxin has not hitherto been available. Now, however, [^{14}C]sterigmatocystin prepared in a [1-^{14}C]acetate-supplemented culture of *A. versicolor* has been used to demonstrate that the toxin can be efficiently converted to aflatoxin B_1 by a culture containing cells of *A. parasiticus*. The possibility that enzyme activity might be involved in this conversion was eliminated by a demonstration that no radioactive aflatoxin was isolated from control flasks containing autoclaved cells, 90–98% of the added radioactivity being recovered in the residual sterigmatocystin. The detailed experimental findings in this study indicate a possible biosynthetic pathway leading from 5-hydroxysterigmatocystin to sterigmatocystin and then to aflatoxin B_1.

2829. Introducing flavutoxin

Kirksey, J. W. & Cole, R. J. (1973). New toxin from *Aspergillus flavus*. *Appl. Microbiol.* **26,** 827.

Aspergillus flavus achieved notoriety in connexion with its production of aflatoxin, but it has since been shown to produce several other toxic metabolites, including aspertoxin (*Cited in F.C.T.* 1968, **6,** 550), kojic acid, a tremorgen, oxalic acid and various derivatives of aspergillic acid (*ibid* 1967, **5,** 402). Further screening of two *A. flavus* isolates obtained from peanuts has added to this collection a non-fluorescent nitrogen-containing metabolite which has been shown by both physical and chemical studies to differ from all the previously reported compounds produced by this fungus.

The new toxin, which has been named flavutoxin, was found to be toxic to day-old cockerels, the mean lethal oral dose being 19 mg/kg. The corresponding dose for aflatoxin B_1 is lower (about 3·5 mg/kg), but the value for flavutoxin still indicates a high degree of toxicity.

Further studies are in progress on the toxic effects of this metabolite in various vertebrates and on its chemical structure.

2830. The mouldy corn menace

Smalley, E. B. (1973). T-2 toxin. *J. Am. vet. med. Ass.* **163,** 1278.
Ellison, R. A. & Kotsonis, F. N. (1974). In vitro metabolism of T-2 toxin. *Appl. Microbiol.* **27,** 423.

Mouldy corn toxicosis in farm animals involves varied signs and symptoms, including refusal to eat, general digestive disturbances and failure to gain weight. In severe cases, haemorrhagic lesions of the stomach, heart, intestine, lungs, bladder and/or kidneys may lead to the animal's death. In cool northern regions, *Fusarium tricinctum* is consistently the most toxic fungus isolated from mouldy corn. Strains of this fungus produce several toxic metabolites, including a butenolide (4-acetamido-4-hydroxy-2-butenoic acid γ-lactone; *Cited in F.C.T.* 1971, **9,** 604), the oestrogen zearalenone (6-(10-hydroxy-6-oxo-1-undecenyl) β-resorcylic acid lactone; *ibid* 1969, **7,** 667) and several 12,13-epoxytrichothecenes (*ibid* 1974, **12,** 586). A notable member of the latter class is T-2 toxin (3-hydroxy-4,15-diacetoxy-8-(3-methylbutyryloxy)-12,13-epoxy-Δ^9-trichothecene).

In rats and other animals, T-2 toxin has been shown to induce necrotic lesions and haemorrhages in the intestines, liver and kidneys. Absorption appeared to be most marked through the small intestinal wall and the skin. The oral LD_{50} in swine and rats was about 4 mg/kg and in rainbow trout about 6·5 mg/kg (*ibid* 1970, **8,** 334). Long-term feeding of low doses of T-2 toxin did not induce tumours in rats or trout. Effects reported in rats have included a fall in body temperature, an increased leucocyte count, a rise in blood-clotting time, an increase in blood cholesterol and a lengthening of barbiturate sleeping time. In a herd of 35 dairy cows, seven of which died over a 5-month period, illness was attributed to consumption of mouldy corn containing T-2 toxin at a level of at least 2 ppm, providing an intake for each cow of 20–30 mg T-2 toxin daily. The author suggests the possible value of diagnostic tests for T-2 toxin based on its ability to inhibit seed germination and retard the growth of young plants, cultures of plant tissues and yeasts.

The second paper cited above reports some preliminary studies on the metabolism of T-2 toxin, with a view to establishing whether the toxic effects that have been associated with it are due to the toxin itself or to a metabolite. Exposure of T-2 toxin to simulated human gastric juice and subsequent thin-layer chromatography of the products showed that the toxin was stable in this medium for over 1 hr and suggested that hydrolysis in the gut would generally be slight and that most of the toxin would be absorbed intact into the blood stream.

Incubation of T-2 toxin with the 9000 **g** supernatant of a homogenate of either human or bovine liver resulted in its conversion to a deacetylated product, designated HT-2 toxin. This metabolic reaction occurred more rapidly with the human than with the bovine liver homogenate. It was not produced when T-2 toxin was incubated with human plasma, and its production was thought to be brought about by the activity of a liver esterase. Preliminary toxicity studies in birds and mice indicated that the deacylation reaction did not

markedly reduce the toxicity of T-2 toxin, since the potency of the toxin and its metabolite was similar in both species.

2831. Tannic acid in the liver

Konstantinov, A. & Ivanov, A. (1973). Electron-microscopic changes of the liver and kidneys in chickens treated with tannic acid. *Zentbl. VetMed. A*, **20**, 426.

More than a decade ago (Korpássy, *Prog. exp. Tumor Res.* 1961, **2**, 245), attention was drawn to a suggestion that tannic acid might be hepatocarcinogenic in rats and mice, but at least one study has since failed to provide support for this view (*Cited in F.C.T.* 1968, **6**, 677). Over this period, a number of authors have reported that tannic substances in feeds have a depressant effect on the development and reproductive capacity of chicks (*ibid* 1968, **6**, 677; *ibid* 1971, **9**, 923) and such findings have stimulated the workers cited above to test the possible long-term effects of parenterally administered tannic acid in this species. Electron-microscopic studies were carried out on the liver and kidneys of chickens injected sc with a 2% aqueous solution of tannic acid either in a single dose of 200 mg/kg body weight or in multiple doses of 200 mg/kg given at 7-day intervals. The birds given a single injection were killed the following day, while those given repeated injections were killed after varying intervals between 30 and 258 days.

As early as 24 hr after a single injection, granular intranuclear inclusions were present in some parenchymal cells of the liver and of the kidney tubules. After repeated weekly injections, similar inclusions occupied more than one third of the intranuclear space. Many inclusions were clearly surrounded by a monolayer capsule. Cells with nuclear inclusions frequently showed degeneration or disintegration of the mitochondria, and string-like formations in the cytoplasm of these cells were thought to be the debris of mitochondrial capsules. Inclusions similar to those in the nuclei of the liver cells were also found in the cytoplasm of some of the Kupffer cells.

Histochemical studies have supported earlier suggestions that the observed inclusions are probably iron-containing protein products. The authors consider, however, that they are a manifestation of cellular repair rather than of any carcinogenic potential of tannic acid. In support of this view, attention is drawn to the presence of surrounding capsules and to the fact that no hepatomas or cholangiomas were produced even in animals given repeated injections of tannic acid over a period of 258 days.

COSMETICS, TOILETRIES AND HOUSEHOLD PRODUCTS

2832. Biodegradation of alkyl and aryl sulphonates

Thysse, G. J. E. & Wanders, Thea H. (1974). Initial steps in the degradation of *n*-alkane-1-sulphonates by *Pseudomonas*. *Antonie van Leeuwenhoek*, **40**, 25.

Willetts, A. J. (1973). Microbial metabolism of alkylbenzene sulphonates. Fungal metabolism of 1-phenylundecane-*p*-sulphonate and 1-phenyldodecane-*p*-sulphonate. *Antonie van Leeuwenhoek* **39**, 585.

The first step in the bacterial breakdown of *n*-alkane 1-sulphonate detergents is the fission of the carbon-sulphur bond and the formation of a fatty acid (Thysse & Wanders, *Antonie van Leeuwenhoek*, 1972, **38**, 53). The first paper cited above reports a study of the

mechanism of this initial reaction catalysed by two strains of Pseudomonas. Four possible mechanisms were explored: (a) hydrolysis of the carbon–sulphur bond, (b) α,β-dehydrogenation, (c) insertion of oxygen into the carbon–sulphur bond and (d) α-hydroxylation. The results were consistent only with mechanism (d), catalysed by an *n*-alkane-1-sulphonate α-hydroxylase. The 1-hydroxy-*n*-alkane sulphonate so formed hydrolyses spontaneously to the corresponding aldehyde and bisulphite.

The properties of the inducible enzyme were studied in a crude extract of a strain of Pseudomonas grown in minimal medium containing *n*-octane 1-sulphonate as the sole carbon and energy source. The enzyme has complex kinetics and was found to depend for its action on the presence of molecular oxygen and $NADH_2$. Anaerobic degradation of alkane sulphonates by Pseudomonas is therefore impossible. The specificity of the hydroxylase was low and secondary alkyl sulphonates were also attacked.

It must be noted that the sulphonates principally used in this study, namely *n*-pentane and *n*-octane 1-sulphonates are not used commercially as detergents, but it appears that detergents based on sulphonated paraffins or olefins are likely to be readily biodegradable, though not under anaerobic conditions.

A related group of compounds, the alkylbenzene sulphonates (ABS), are used as detergents both domestically and industrially. Studies on the biodegradation of ABS have been confined to bacterial metabolism, but the second paper cited above reports the breakdown of 1-phenylundecane *p*-sulphonate and 1-phenyldodecane *p*-sulphonate by the fungus *Cladosporium resinae*, which can utilize ABS homologues as a sole source of carbon and sulphur. On the basis of experiments using either whole cells or cell-free extracts, a pathway for this fungal degradation was deduced. The results indicated that the initial reaction was a desulphonation of the aromatic moiety by an inducible enzyme dependent on the presence of molecular oxygen. Subsequently the alkyl side-chain was progressively removed by a β-oxidation pathway to leave the aromatic nucleus, which was metabolized by a sequence of reactions involving first hydroxylation and then oxygen-dependent cleavage by an "ortho cleavage" pathway.

All three stages of ABS degradation by the fungus are essentially similar to those found in the bacterial breakdown of these compounds (Willetts & Cain, *Biochem. J.* 1972, **129,** 389), except that the desulphonation step catalysed by *Cl. resinae* results in the direct release of sulphate anion, whereas bacterial desulphonation leads to the release of sulphite anions into the medium and the concomitant hydroxylation of the desulphonated aromatic moiety.

METHODS FOR ASSESSING TOXICITY

2833. Serum enzymes as indicators of hepatotoxicity

Korsrud, G. O., Grice, H. G., Goodman, T. K., Knipfel, J. E. & McLaughlan, J. M. (1973). Sensitivity of several serum enzymes for the detection of thioacetamide-, dimethylnitrosamine- and diethanolamine-induced liver damage in rats. *Toxic. appl. Pharmac.* **26,** 299.

This group's study of the value of various serum enzymes as early indicators of carbon tetrachloride-induced liver damage (*Cited in F.C.T.* 1973, **11,** 930) has now been followed by a similar assessment in connexion with the hepatotoxic effects of thioacetamide (TA),

dimethylnitrosamine (DMNA) and diethanolamine (DEA). The tests were again carried out in male rats, which were fed a semi-purified diet for 12 days, fasted for 6 hr, given a single oral dose of the test compound and then killed 18 hr later. Serum analyses of ten enzymes, 19 amino acids and urea were performed terminally and livers were studied by light and electron microscopy.

Sorbitol dehydrogenase was found to be the most sensitive of the enzyme indicators studied, showing an increase in activity with a dose of 9·4 mg TA/kg or 5·1 mg DMNA/kg. Increased activities of seven other enzymes (isocitric dehydrogenase, fructose-1-phosphate aldolase, glutamic–oxalacetic and glutamic–pyruvic transaminases, ornithine–carbamoyl transferase and malic and lactic dehydrogenases) were recorded with higher doses of these two compounds. A dose of 800 mg DEA/kg increased the activity of six of these enzymes, but ornithine–carbamoyl transferase and lactic dehydrogenase were only affected by 1600 mg DEA/kg, a dose which also induced glutamic dehydrogenase and fructose-1,6-phosphate aldolase. Activities of several enzymes were dose-dependent.

Serum levels of arginine were decreased by 25·4, 13·7 and 800 mg/kg doses of TA, DMNA and DEA, respectively, and in the first two cases ornithine levels were increased. DEA at 800 mg/kg had a variable effect on ornithine, but increased the serum levels of urea.

Morphological changes were detectable at dosage levels below those required to affect serum enzyme levels, evidence of histological damage being present in at least some of the animals treated with 3·5 mg TA/kg, 1·9 mg DMNA/kg and 200 mg DEA/kg. Nevertheless, serum-enzyme studies have obvious practical advantages over biopsy or autopsy, and the data presented here support the conclusion of the earlier study, suggesting that sorbitol-dehydrogenase activities, which were dose-dependent over a wide range of doses of each test chemical, offer the best chance of detecting minimal hepatic damage.

CANCER RESEARCH

2834. Calculi, alkaline urine and bladder tumours

Flaks, Antonia, Hamilton, J. M. & Clayson, D. B. (1973). Effect of ammonium chloride on incidence of bladder tumours induced by 4-ethylsulfonylnaphthalene-1-sulfonamide. *J. natn. Cancer Inst.* **51,** 2007.

The importance of contact with urine and of the presence of calculi as factors in the development of tumours in bladders implanted with paraffin-wax pellets has been demonstrated in the rat (*Cited in F.C.T.* 1974, **12,** 592). The study cited above was designed to show whether the incidence of bladder tumours induced in mice by 4-ethylsulphonylnaphthalene-1-sulphonamide (ENS) could be altered by changing the pH of the urine and thus affecting the formation of calculi.

Prolonged dietary administration of ENS to mice results in the production of an alkaline urine, the formation of bladder calculi, the development of a marked degree of hyperplasia of the bladder epithelium and frequently the induction of bladder tumours. When mice were given 1% ammonium chloride (NH_4Cl) in their drinking-water as well as 0·01% ENS in the diet, the pH of the urine was kept almost within the normal range (5·6–7·2 compared with 5·5–6·7 in the controls). After treatment for 52 wk these mice showed some

mild hyperplasia of the bladder epithelium but were free of both bladder tumours and calculi. After a comparable period, the group of 26 mice treated only with ENS included 18 with moderate or severe epithelial hyperplasia, 13 with bladder calculi, seven with bladder tumours and 14 with hydronephrosis. The range of urinary pH in this group was 6·9–9·0. In two of these mice there was no correlation between the presence of calculi and tumour formation.

These results are in accord with previous evidence that the presence of calculi may play an important role in bladder-tumour induction in rodents. They also support an earlier suggestion that the bladder changes induced by ENS are largely due to rises in urinary pH and irritation from calculi rather than to the direct action of the chemical on the bladder epithelium.

Fd Cosmet. Toxicol. Vol. 13, pp. 297–302. Pergamon Press 1975. Printed in Great Britain

LETTERS TO THE EDITOR

LONG-TERM ADMINISTRATION OF ARTIFICIAL SWEETENERS TO THE RHESUS MONKEY (*M. MULATTA*)

Sir,—In testing chemical compounds for carcinogenic potential, emphasis has traditionally been placed on the use of rodents, notably mice (Grasso & Crampton, 1972). Clayson (1974) has recently reviewed the artefacts and problems likely to be encountered in experimental induction of cancer in the urinary bladder of rats and mice. Early efforts to explore systematically the capacity of hydrocarbon carcinogens and oestrogens to induce tumours in non-human primates proved discouraging (Engle *et al.* 1943; Hisaw *et al.* 1937; Iglesias & Lipschutz, 1947; Pfeiffer & Allen, 1948; Scott & Wharton, 1955). Recent studies have demonstrated that certain monkeys and other non-human primates are sensitive to a variety of carcinogens and hormonal preparations (Hamner, 1973; Hull *et al.* 1969; McClure & Graham, 1973; O'Gara *et al.* 1971; Schepers, 1964; Vorwald *et al.* 1966). Contrary to the belief that the latent period for tumour induction extends over a major part of the lifespan of these animals, neoplasia has developed within a few months in some instances (McClure & Graham, 1973) and in approximately 1–2 yr in others (Adamson *et al.* 1974; Kelly *et al.* 1966). The only systematic effort to explore the potentialities of a wide variety of carcinogens in rhesus and cynomolgus monkeys has been made by Adamson *et al.* (1974), who have concluded that non-human primates are, in fact, good models for the evaluation of potential carcinogens. Adamson and his colleagues are currently engaged in studies of the effects of administering cyclamate (100 or 500 mg/kg/day, 5 times/wk) and saccharin (25 mg/kg/day, 5 times/wk) to groups of ten rhesus monkeys; these experiments are now in their fifth year (R. H. Adamson, personal communication 1974).

In similar studies carried out at this Institute, sodium cyclamate in aqueous solution has been given orally to two female rhesus monkeys for 6·7 yr and to one male monkey for 6·4 yr, in each case at a dose level of 200 mg/kg/day, on 6 days/wk. Similarly, sodium saccharin* has been administered at dose levels of 20, 100 and 500 mg/kg/day on 6 days/wk to groups comprising, respectively, two, two and three rhesus monkeys of each sex, with three animals of each sex being retained as controls. After 5·4 yr on this dosage regimen, five male and six female monkeys remain in the treated groups. Of the other three animals originally on saccharin, one died of intercurrent infection and two had to be killed because of inadvertent injuries. At autopsy, the bladders of these three monkeys were examined with special care; no evidence of hyperplastic or neoplastic change was found in this organ or in any others.

As to the appearance and behaviour of the animals, no changes attributable to the treatment have been noted at any time in any group. Analysis of the components of serum (glucose, urea nitrogen, sodium, potassium, chloride, glutamate–pyruvate trans-

*The original batch of sodium saccharin (provided by Squibb & Sons, New York) was found by the Health Protection Branch, Health and Welfare, Canada, to contain 2·4 ppm *o*-toluenesulphonamide. In 1971, a new lot of sodium saccharin was obtained from Pfaltz and Bauer, Inc., Flushing, N.Y. This contained 3·2 ppm o-toluenesulphonamide (Stavric *et al.* 1975).

aminase and alkaline phosphatase) and measurement of routine haematological parameters (red and white cell counts, differential leucocyte count, haemoglobin and haematocrit) have been carried out at about 6-monthly intervals, and no consistent deviation from normal values has been observed in any group. Body weights have been recorded monthly in all groups. In the cyclamate group, after 6 yr on test, the one male exceeded in body weight both surviving male controls, and the two females exceeded in body weight those of two of the three female controls. In the saccharin series, the group receiving 20 and 100 mg/kg/day from the outset grew more rapidly than the controls, but the 500 mg/kg/day group began to fall slightly behind the controls in mean body weight from yr 2 of the test. However, even the greatest difference observed between these groups ($6{\cdot}4 \pm 0{\cdot}6$ *v.* $7{\cdot}2 \pm 0{\cdot}8$ kg at 4 yr) was not statistically significant.

A question of considerable interest concerns the possibility that these animals have developed some type of metabolic adaptation in the course of long-term administration of the synthetic sweeteners. When this question was studied earlier in the same animals, by Parekh *et al.* (1970) for cyclamate and by Byard & Golberg (1973) for saccharin, there was no evidence of significant metabolic adaptation. To study the question further, a 24-hr urine collection was obtained from each of the cyclamate monkeys during yr 7 of the test, and from the saccharin monkeys during yr 6. Examination of the urine specimens revealed no abnormal components. Cyclohexylamine was determined in the former samples by extraction into chloroform at pH 12; the bases were returned to dilute hydrochloric acid, re-extracted into a much smaller volume of chloroform and analysed by the method of Derse & Daun (1966). Sulphamoylbenzoate was determined in the samples from the saccharin monkeys by the method of McChesney & Golberg (1973). No cyclohexylamine was found in two of the cyclamate samples, but in the third (from a menstruating female) an amount equivalent to 0·3% of the daily dose of cyclamate was found. None of the animals on saccharin was converting this compound to sulphamoylbenzoate at the time of this study.

Until an animal dies or is killed, one cannot exclude the possibility that it has developed lymphosarcoma (Fitzhugh *et al.* 1951), urinary calculi (Lessel, 1971), bladder tumours of the type reported by Allen *et al.* (1957) following the implantation of saccharin–cholesterol pellets in the bladder, or papillary bladder tumours of the type reported by Price *et al.* (1970) following the long-term feeding to rats of a 10:1 cyclamate–saccharin mixture. However, urinary sediments obtained from the three female monkeys on 500 mg saccharin/kg/day were examined after the animals had been on test for 3·6 and 4·5 yr. The earlier examination was made by Dr. C. Alastair Moodie of the Canadian Health and Welfare Department (Health Protection Branch, Ottawa) and the later one by Dr. M. J. Iatropoulos of this Institute. The sediments were prepared for examination by staining by the Papanicolaou method. In neither case was evidence of the presence of precancerous cells seen.

The capacity of a weak carcinogen to elicit bladder tumours in the rhesus monkey has never been established. To that extent our negative findings suffer from a lack of a positive control group. Equally, while certain strong carcinogens have a relatively short latent period in monkeys, weak ones may require a lifetime to become manifest. Nevertheless, our experience to date tends to reinforce the mounting evidence of non-carcinogenicity of cyclamate and saccharin (Brantom *et al.* 1973; Schmähl, 1973).

Our thanks are due to Dr. R. H. Adamson for information concerning his experiments and to Dr. C. A. Moodie for cytological examination of urinary sediments. This research

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L-GLUTAMIC AND L-ASPARTIC ACIDS—A QUESTION OF HAZARD?

Sir,—Observation of irreversible necrosis in the hypothalamic neurons in the brains of 10-day-old mice given a single oral dose of glutamic or aspartic acid led Olney & Ho (*Nature, Lond.* 1970, **227**, 609) to claim that the health of young children could be adversely affected by the consumption of these compounds. This group considered that susceptibility was not limited to newborn animals and put the thresholds for brain damage from orally administered monosodium glutamate at 0·5–1, 1–1·5 and 1·5–2 g/kg for 10-day-old, weanling and older mice, respectively (Olney, *Science, N.Y.* 1969, **164,** 719; Olney & Sharpe, *ibid* 1969, **166,** 386; *idem, ibid* 1970, **167,** 1017; Olney *et al. J. Neuropath. exp. Neurol.* 1972, **31,** 464). Assuming a similar shift with age in the toxic threshold for man puts infants or children at risk from doses of 0·5–1·5 g/kg and adolescents and adults from 1·5–2 g/kg.

L-Aspartic and L-glutamic acids account together, on average, for some 30% of animal proteins and some 39% of vegetable proteins, so that in highly industrialized countries they constitute about 35% of total dietary protein. The amino acids are readily liberated in the gut and absorbed, and there is no reason to suspect that the action of this abundant dietary supply of L-glutamate and L-aspartate differs from that of the L-isomers administered directly in the form of their alkali salts.

Total daily intakes of these two amino acids from the diet have been calculated as 1·4 g by infants consuming only human milk (about 500 ml) and 6–12 g by weaned infants weighing about 5 kg and consuming 0·5–1·0 litre of cows' milk daily. The toxic threshold of 0·5–1 g/kg assumed by Olney & Ho (*loc. cit.*) for infants would therefore be exceeded by the intake of these amino acids in milk alone. For the average 10-yr-old child (weighing 32 kg), the daily intake of protein is about 1·25 g/kg or a little more, corresponding to a daily intake of aspartic and glutamic acids of at least 0·44 g/kg. Young children could therefore easily exceed the lower limit of the estimated risk threshold of 0·5–1·5 g/kg for this age group merely from consumption of ordinary food. This would suggest that children, especially those with an above-average protein intake, would be continuously at risk from brain damage. For adolescents and adults, the average daily intake of these amino acids can be calculated as at least 0·6 g/kg, a figure on the safe side of the alleged risk threshold of 1·5–2 g/kg for this group but providing a relatively small safety margin. Such a situation must be regarded as highly improbable.

We are convinced, therefore, that the views propounded by Dr. Olney and his colleagues require critical reassessment in the light of animal studies conducted in a second laboratory. The need for observations liable to have important repercussions in the public-health field to be confirmed in more than one research centre is particularly marked in the present case, in which the claims being made suggest that infants and

children are regularly consuming quantities of aspartic and glutamic acids considered to be toxic, while the levels consumed throughout adolescence and adult life are close to the toxic range.

Of relevance to the question of aspartic acid consumption is the recent development of the sweetening agent, aspartame, a dipeptide (mol wt 294) consisting of aspartic acid (mol wt 133) and phenylalanine (mol wt 165) and with a sweetening potency nearly 200 times that of sugar. If the total sugar intake (amounting to some 100 g/day in most of western Europe and to nearer 150 g/day in Sweden and the USA) were replaced by aspartame, consumption of the latter would amount to 0·5 or 0·75 g/head/day. However, since aspartame cannot be used in foods requiring prolonged cooking, a more realistic calculation would be based on the equivalent in sweetening potency of half of the total sugar consumption, namely 0·25 or 0·375 g aspartame, corresponding to 0·11 and 0·17 g aspartic acid respectively. Taking an overall daily intake of about 0·14 g, therefore, and a body weight (weighted average) per head of population of about 50 kg, the daily intake of aspartic acid derived from aspartame would not exceed 0·003 g/kg. Using this estimate and the figures given above for the total amount of aspartic and glutamic acids derived from the protein of the daily diet, it can be calculated that the intake of aspartic acid from a relatively high consumption of aspartame would be 1·2–2·3, 0·7 and 0·5% of the dietary aspartic and glutamic acids ingested, respectively, by the weaned infant weighing 5 kg, the 10-yr-old child weighing 32 kg and the 60-kg adult (consuming 100 g protein).

If an increase of 1–2% in the intake of these amino acids is assumed to offer a hazard of potential brain damage, a comparable increase in protein intake must be assumed to pose a similar hazard. In fact, protein intakes above those cited are not unusual, and it would therefore seem rash to extrapolate to man Dr. Olney's interesting research observation which, at this stage, must be assumed to be species specific.

Hypothalamic lesions have apparently also been demonstrated (J. W. Olney, unpublished observations 1974) in mice given 2–2·5 g aspartame/kg by intubation when 10–20 days old. This high dose level would correspond to an intake of 120–150 g by a 60-kg man, a quantity 300–600 times greater than that equivalent to the sweetening potency of half of the total sucrose in the human diet. Tests involving such overdosage are as unrealistic as wondering what would happen to a man consuming 300–600 times more sugar than usual.

The validity of using an intubation technique to administer a relatively large volume of fluid in a single dose to animals weighing only a few grams is also questionable. In view of the osmotic disturbance and consequent histological changes likely to result from such a procedure, the interpretation of these toxicological studies must remain a dubious exercise until more experiments have been carried out.

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AN ALTERNATIVE METHOD FOR THE DECOMPOSITION OF NITROSAMINES IN SOLUTION

Sir,—Recently Gangolli *et al.* (*Fd Cosmet. Toxicol.* 1974, **12,** 168) reported a method for the destruction of aqueous solutions of nitrosamines. The method was based on the reduction of nitrosamines in alkaline solutions with aluminium. We now suggest an alternative procedure, based on the halide ion-catalysed denitrosation of nitrosamines to the corresponding secondary amines. This alternative method may have certain advantages over the reduction method, such as in the time required to bring about the change.

We have been concerned, from a mechanistic viewpoint, with the rearrangement and denitrosation of aromatic nitrosamines in acid solution (Williams & Wilson, *J. chem. Soc., Perkin Trans. 2*, 1974, p. 13, for example) and have recently established the mechanism of the denitrosation process (Biggs & Williams, *ibid*. in press). Denitrosation is conveniently brought about in acid solution by Cl^-, Br^-, SCN^- or I^- (X^-) if a nitrite 'scavenger', such as sulphamic acid, urea, hydroxylamine, hydrazine or hydrazoic acid, is present in sufficient excess to remove the nitrosyl halide, NOX, as soon as it is formed. This prevents the occurrence of the reverse reaction between the formed secondary amine and NOX, which would regenerate the nitrosamine. As expected, the reactivity increases along the series $Cl^- < Br^- < SCN^- < I^-$, there being a factor of 15,000 covering the whole reactivity range. Denitrosation also occurs in the absence of added X^-, but at a much reduced rate, being presumably brought about by attack of a water molecule. In all cases denitrosation is acid-catalysed, the rate-determining step (k) being the reaction between X^- and the protonated form of the nitrosamine. For the reaction of *N*-methyl-*N*-nitrosoaniline with iodide at 30°C, 10^4kK is 6300 sec^{-1} (where K is the equilibrium constant for the protonation). This means that if the iodide ion concentration is 0·1 M and the solution is 1 M in sulphuric acid, the observed rate constant for the disappearance of the nitrosamine is 0·16 sec^{-1}, which corresponds to a half-life of about 4 sec. Clearly this could be reduced further by increasing the concentration of either the acid or the iodide ion. Thiocyanate ion is almost as effective.

The concentration of the nitrite scavenger (sulphamic acid etc.) is immaterial, as long as it exceeds some critical value which ensures the irreversibility of the denitrosation. We have worked mostly at concentrations of nitrosamine of the order of 1×10^{-4} M and have found that a tenfold excess of sulphamic acid, hydroxylamine, hydrazine or hydrazoic acid more than meets the kinetic requirement, whereas urea (which has been used widely as a nitrite trap) is much less efficient (Williams, *Chem. Commun.* 1974, p. 324) and is therefore needed in much higher concentration. To date all our measurements have concerned *N*- and ring-substituted nitrosoanilines, but it seems very probable that rapid and irreversible denitrosation of other (aliphatic and heterocyclic) nitrosamines can be effected by this procedure.

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MEETING ANNOUNCEMENTS

GENETIC HAZARDS FROM ENVIRONMENTAL AGENTS

A Symposium on Genetic Hazards to Man from Environmental Agents, sponsored by the Genetics Society of Canada, The Canadian Department of National Health and Welfare and the International Association of Environmental Mutagen Societies, will be held in the Government Conference Centre, Ottawa, on 26–28 May 1975. The topics to be considered are:

Relevance of Mutagenic Assays to Man; Certainties and Uncertainties
Interrelations of Mutagens, Carcinogens and Teratogens
Protecting the Public from Mutagens: National Strategies
Epidemiological Detection of Mutagens, Carcinogens and Teratogens
Human Exposure: How Much is Too Much? (Panel Discussion)

Participants include B. Ames, C. Auerbach, I. Barrai, B. Bridges, G. Butler, F. J. de Serres, J. W. Drake, F. C. Fraser, D. Janerich, H. Kalter, B. J. Kilbey, M. S. Legator, J. A. Miller, J. R. Miller, A. B. Morrison, C. Ramel, W. G. Röhrborn, C. R. Scriver, H. E. Sutton and S. Wolff.

Further information may be obtained from Mrs. J. Renaud, Room 1–5, Health Protection Building, Health and Welfare Canada, Tunney's Pasture, Ottawa, K1A OL2, Ontario, Canada.

INTERNATIONAL CONGRESS OF NUTRITION

The Xth International Congress of Nutrition, sponsored by the International Union of Nutritional Sciences (IUNS), will be held on 3–9 August 1975 at the Kyoto International Conference Hall, Kyoto, Japan. Host to the Congress, with the support of the Japanese Government, will be the Science Council of Japan. "Through Science and Nutrition to Human Well-being" will be the theme of the Congress, which is open to all interested persons.

Further information may be obtained from Prof. Masao Kanamori, Secretary General, Xth International Congress of Nutrition, c/o Kyoto International Conference Hall, Takaraike, Sakyo-ku, Kyoto 606, Japan (telephone: 075-791-3111).

FORTHCOMING PAPERS

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

The reliability of a procedure for the determination of nitrosamines in food. By K. Goodhead and T. A. Gough.

Short-term toxicity of ethylene chlorohydrin in rats, dogs and monkeys. By B. L. Oser, K. Morgareidge, G. E. Cox and S. Carson.

The potential mutagenicity of dieldrin (HEOD) in mammals. By B. J. Dean, S. M. A. Doak and H. Somerville.

Mycotoxine in Nahrungsmitteln. IV. Der Einfluss verschiedener Verpackungsfolien auf das Wachstum von *Aspergillus flavus* und die Bildung der Aflatoxine B_1 und G_1 auf einigen Schnittbrotarten. By J. Reiss.

The embryotoxic and teratogenic effects of CTAB, a cationic surfactant, in the mouse. By B. Isomaa and K. Ekman.

Experimental modification of photocarcinogenesis. I. Fluorescent whitening agents and short wave UVR. By P. D. Forbes and F. Urbach.

Experimental modification of photocarcinogenesis. II. Fluorescent whitening agents and simulated solar UVR. By P. D. Forbes and F. Urbach.

Experimental modification of photocarcinogenesis. III. Simulation of exposure to sunlight and fluorescent whitening agents. By P. D. Forbes and F. Urbach.

Absence of toxic and carcinogenic effects after administration of high doses of chromic oxide pigment in subacute and long-term feeding experiments in rats. By S. Ivankovic and R. Preussmann.

Long-term toxicity studies on oxidation hair dyes. By C. Burnett, B. Lanman, R. Giovacchini, G. Wolcott, R. Scala and M. Keplinger.

Absorption gastro-intestinale, chez le rat, de l'anisole, du *trans*-anéthole, du butylhydroxyanisole et du safrole. By P. Fritsch, G. de Saint Blanquat et M. T. Canal. (Short paper)

The reaction of nitrite with pesticides. II. Formation, chemical properties and carcinogenic activity of the *N*-nitroso derivative of *N*-methyl-1-naphthyl carbamate (carbaryl). By G. Eisenbrand, O. Ungerer and R. Preussmann. (Short paper)

Effect of subacute oral administration of zinc ethylenebis(dithiocarbamate) on the thyroid gland and adenohypophysis of the rat. By L. Ivanova-Chemishanska, D. V. Markov, S. Milanov, D. D. Strashimirov, G. I. Dashev and G. A. Chemishanski. (Short paper)

A case for using inbred strains of laboratory animals in evaluating the safety of drugs. By M. F. W. Festing. (Review paper)

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Aims and Scope

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

Some other Pergamon Journals which may interest readers of *Food and Cosmetics Toxicology:*

Annals of Occupational Hygiene

Archives of Oral Biology

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