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Research Section

SHORT-TERM TOXICITY STUDY OF SORBITAN MONOLAURATE (SPAN 20) IN RATS

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(*Received 13 March 1978*)

Abstract—Rats were fed sorbitan monolaurate (Span 20) at dietary levels of 0 (control), 2·5, 5 or 10%, or lauric acid at 5%, for 13 wk. Dose-related reductions in the rate of body-weight gain were associated with reduced intakes of diet containing Span 20. There were similar reductions with lauric acid. Animals fed 5 or 10% Span 20 had reduced haemoglobin concentrations and packed cell volumes, but the other haematological parameters and serum-enzyme levels were normal. Variations in organ weight were mainly associated with the lower body weights. Those thought to be related to treatment were increases in relative liver and small-intestine weights in the rats given 10% and 5 or 10% diets, respectively, and increases in kidney weight at all dose levels. Periportal fat-containing vacuoles were demonstrated in the livers of the rats fed 10% Span 20 but the increased relative kidney weights were not accompanied by histological changes or impairment of renal function and no abnormal constituents were present in the urine. In view of the kidney enlargement it was not possible to estimate a no-untoward-effect level in this study.

INTRODUCTION

Span 20® (sorbitan monolaurate) is an oil-soluble non-ionic surface-active compound. It is used primarily as a water-in-oil emulsifier, but finds application also as a spreading, penetrating and antifoaming agent and has been used as an auxiliary emulsifier in cosmetic and pharmaceutical preparations.

In 1972, the Food Additives and Contaminants Committee re-examined its requirements for the further testing of sorbitan esters of fatty acids and rescinded the requirement for long-term tests on individual esters, with the proviso that a single study be conducted on sorbitan monostearate in a second rodent species as a representative of the group. No restrictions were imposed on the use of sorbitan monolaurate in the Emulsifiers and Stabilisers in Food Regulations 1975 (Statutory Instrument 1975, no. 1486). In 1974 the EEC authorized Span 20 for use in foodstuffs for up to 5 yr, after which it would be subject to review (European Economic Community, 1974). Span 20 is permitted in the USA under Sec. 178.3400 (Emulsifiers and/or surface active agents; formerly Sec. 121.2541) of the Code of Federal Regulations.

Since the toxicology of the Span and Tween emulsifiers has been reviewed in detail by Elworthy & Treon (1967), only a brief review of the effects of Span 20 is required here. Ten male rats tolerated single oral doses of 20 g Span 20/kg without any apparent harmful effects during a 2-day observation period (*Food and Cosmetics Toxicology*, 1970). Rats exposed to Span 20 (25% in the diet) for up to 59 days, lost weight throughout the test period (Harris, Sherman & Jetter, 1951a). Many developed nasal haemorrhages after 1–5 days and did not groom themselves normally. Toward the end of the study gangrenous tails developed in some rats and eventually sloughed off. Only one rat survived the test period. This animal was stunted and, at autopsy, was found to have fatty changes in the liver. In a second group the dose of Span 20 was increased gradually from 5 to 25% over the initial 10-day period. The rats ate little food and remained inactive. Again nasal haemorrhages developed. Gangrenous tails and legs developed in rats that survived for 5 wk. Increased haemoglobin levels, fatty liver and increased relative weights of the brain, kidneys, heart, spleen, lungs and liver were found.

The same authors (Harris, Sherman & Jetter, 1951b) fed Span 20 to hamsters at dietary levels of 5–15% for 68 days. At the lower level the only gross sign of abnormality was a lower rate of body-weight gain. At the 15% level, diarrhoea was evident initially but later disappeared. The death rate in these animals was greater than in the controls. No difference in blood chemistry was detected and no change in relative organ weights was evident. Histopathological examination indicated irritation of the gastro-intestinal tract and mild degeneration of the kidney tubules together with inhibition of gonadal maturation.

Fitzhugh, Schouboe & Nelson (1960) fed Span 20 to rats at dietary levels of 15, 20 or 25% for 23 wk. Dose-related reductions in body-weight gain were evident at all dietary levels and there was a significant increase in deaths at the 25% level. There was a progressive enlargement of the common bile duct with

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each increase in dosage and gangrene of the tail occurred at all treatment levels. Histologically liver damage was pronounced and lung changes were present. In a preliminary study (J. C. Krantz, Jr., unpublished data 1945; cited from Joint FAO/WHO Expert Committee on Food Additives, 1974) rats fed 0, 1 or 4% Span 20 for 6 wk displayed a slightly slower rate of body-weight gain. Limited haematological and histological studies revealed no abnormalities. Similar studies with Rhesus monkeys showed no change in growth rate and no abnormalities on histological and haematological examination. The same report described some long-term feeding studies in which groups of rats were given dietary levels of 0 or 5% Span 20 for 2 yr. There were no adverse effects on body weight, haematology, serum chemistry, organ weights or the histological examination. Rmgrose & Waller (1959) fed Span 20 to cockerel chicks at dietary levels up to 1% and detected no adverse effects.

Treon (1963) showed that Span 20 was non-irritant when applied to the rabbit eye or human skin. Setälä (1961) tested the effects of Span compounds on tumour production in the skin of mice. Most Span compounds were ineffective, but when Span 20 was applied to the skin with "sufficient frequency", some skin tumours developed.

The present paper describes a short-term study in rats carried out as part of a programme for evaluating the safety of Spans. These investigations were requested by the Food Additives and Contaminants Committee (1972).

EXPERIMENTAL

Materials. The samples of Span 20 and lauric acid were supplied by Honeywill Atlas Ltd., Carshalton, Surrey. The Span 20 was prepared by the esterification of sorbitol with edible lauric acid and consisted of approximately 95% of a mixture of the partial esters of sorbitol and its mono- and dianhydrides with lauric acid. The specification of the material, which complied with the *British Pharmaceutical Codex, 1973*, and the Emulsifiers and Stabilisers in Food Regulations 1975 (Statutory Instrument 1975, no. 14[illegible]), was as follows: Amber-coloured, oily liquid at 2[illegible] specific gravity at 25°C, approx. 1; acid nu[illegible]er, 40–70; saponification value, 158–170; hydroxyl number, 330–358; water content, max 1·0%; sulphated ash, max 0·25%; arsenic, max 3 ppm; lead, max 10 ppm; copper, max 50 ppm; zinc, max 25 ppm.

The lauric acid complied with the following specification: white solid at 25°C; specific gravity at 25°C, approx. 1; acid number, 266–274; saponification value, 268–276; water content, max 0·5%; titre, max 27°C; unsaponifiable matter, max 0·5%; iodine value, max 12; arsenic, max 3 ppm; lead, max 10 ppm.

Animals and maintenance. Rats of a Wistar strain obtained from a specified-pathogen-free breeding colony (A. Tuck & Son, Rayleigh, Essex) were fed ground Spratts Laboratory Diet No. 1 and allowed access to water *ad lib.* The animal rooms were maintained at 20 ± 1°C with a relative humidity of 50–70%.

Experimental design and conduct. Groups of 15 male and 15 female rats, housed five to a cage, were fed a diet containing 0 (control), 2·5, 5·0 or 10·0% Span 20 for 90 days. In addition a similar group of male and female rats was fed a diet containing 5% lauric acid, which provided approximately the same intake of lauric acid as the 10% level of Span 20. Additional groups of five rats of each sex were fed 0, 5 or 10% Span 20 or 5% lauric acid for 2 or 6 wk. The animals were weighed and the food and water intakes of each cage of five rats were measured weekly. At the end of the appropriate feeding period, the rats were killed by exsanguination under barbiturate anaesthesia and blood samples were collected from the aorta for haematological examination. Serum analyses were carried out on the blood collected at wk 6 and 13. At autopsy, any macroscopic abnormality was noted and the brain, pituitary, thyroid, heart, liver, spleen, kidneys, adrenal glands, gonads, stomach, small intestine and caecum were weighed. Samples of these organs and of the lung, salivary glands, aortic arch, thymus, various lymph glands, 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. Oil Red O staining to demonstrate fat was carried out on frozen sections of fixed liver selected randomly from animals of each group.

The blood was examined for haemoglobin concentration and packed cell volume together with counts of erythrocytes, reticulocytes and total and differential white blood cells. Serum samples from the female rats at wk 6 and 13 and from the males at wk 13 were analysed for total protein and albumin, and the activities of glutamic–oxalacetic transaminase, glutamic–pyruvic transaminase and lactic dehydrogenase were determined in serum from all the animals at wk 6 and 13.

Urine collected over a 6-hr period during the final week of each treatment period was examined for appearance, microscopic constituents and content of albumin, glucose, ketones, bile salts and blood. At the same time renal concentration and dilution tests were conducted in which measurements were made of the specific gravity and volume of urine produced during a 6-hr period of water deprivation, during a 2-hr period following a water load of 25 ml/kg and during the 4 hr following a 16-hr period of water deprivation. The latter concentration test was not carried out at wk 2. The number of cells in the urine was determined in the sample collected for 2 hr after the water load.

Paired-feeding study. Twenty male rats, housed individually, were divided into two groups of ten and given diet containing either 0 (control) or 10% Span 20, each treated animal being paired with a litter-mate control. The diet containing Span 20 was offered *ad lib.* and the food intake of each rat was measured daily. The weight of food given to each control rat was the same as that eaten by its litter-mate during the previous 24 hr. All the animals were allowed free access to water, the intakes being recorded. The rats were weighed weekly and killed at the end of the 13-wk treatment period for examination and measurement of organ weights, as described above.

Table 1. *Mean data on body weights and consumption of food and test material for rats fed diets containing 2·5–10% Span 20 or 5% lauric acid for 13 wk*

Diet	Dietary level (%)	Body weight (g) at day 0†	1	27	55	90	Food consumption (g/rat/day) at day 0†	1	27	55	90	Mean food consumption (g/rat/day)	Mean intake of test compound‡ (g/kg/day)
Males													
Control	0	86	93	263	371	432	13·3	14·5	21·3	22·0	18·9	20·2	0
Span 20	2·5	85	87	238**	332**	389**	10·7	13·3	19·2	19·4	17·5	18·5*	2·1
	5	85	86	214***	294***	350***	9·6	10·1	16·4	18·7	17·8	16·9***	4·2
	10	84	81**	179***	263***	316***	7·5	7·8	16·0	17·1	15·9	15·4***	8·0
LA	5	87	88	247*	349	409	7·2	9·8	18·5	19·9	17·1	17·4***	3·7
Females													
Control	0	69	75	176	221	250	10·5	11·7	15·7	16·3	13·7	15·3	0
Span 20	2·5	71	73	162**	205*	233	9·1	9·3	13·1	15·5	14·7	14·7	2·3
	5	71	70*	152***	198**	225**	7·3	6·8	12·9	15·5	14·3	13·6*	4·5
	10	71	68**	141***	172***	191***	4·6	5·9	11·7	12·8	12·3	11·8***	8·4
LA	5	71	71	166*	213	240	5·8	8·5	13·9	14·3	11·7	12·9***	4·0

LA = Lauric acid

† Pretreatment value on day 1 of study.

‡ Calculated from data on body weight and food consumption.

Body-weight values are means for groups of 15 animals, and food consumption values are means for three cages of five animals. Values marked with asterisks differ significantly (Student's *t* test for body weight and White (1952) test for food consumptions) from the controls: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

RESULTS

Short-term feeding study

No deaths occurred and no abnormal behaviour was seen during this study. The body weights of the rats given diet containing 5 or 10% Span 20 were lower than those of the controls within 48 hr of the start of treatment and remained less, to a statistically significant extent, throughout the study (Table 1). A similar but less marked difference in body weight was found in rats given diet containing 2·5% Span 20. The latter differences did not reach statistical significance until after 6–9 days of treatment. The body weights of the rats given diet containing 5% lauric acid were lower than those of the controls, the difference being statistically significant during the first half of the treatment period. Comparison of the Span 20-treated rats with those given lauric acid revealed statistically significant differences only for the 5 or 10% Span 20 diets.

The average food intake (Table 1) over the whole treatment period was lower in the animals given Span 20 or lauric acid than in the controls. The magnitude of these differences increased with increasing dietary concentration. Although they persisted throughout the study they were most marked in the first few days of feeding.

The water intake of the female rats given lauric

Table 2. *Mean water-consumption values for rats fed diets containing 2·5–10% Span 20 or 5% lauric acid for 13 wk*

Diet	Dietary level (%)	Water intake (ml/rat/day) on day 0†	13	27	55	69	90	Mean intake (ml/rat/day)
Males								
Control	0	18·9	25·3	24·5	25·3	24·5	23·1	24·1
Span 20	2·5	16·9	27·1	26·4	24·7	24·5	23·0	25·7
	5	17·8	26·6	26·7	26·9	27·5	26·3	25·8**
	10	14·6	23·7	25·4	27·1	29·3	26·1	24·1
LA	5	12·5	21·6	23·1	26·6	26·7	22·8	22·7
Females								
Control	0	16·5	23·7	24·1	23·3	24·9	22·0	22·5
Span 20	2·5	17·0	24·9	26·9	26·1	27·3	22·3	23·7
	5	14·1	24·2	24·5	26·4	25·2	22·4	23·1
	10	13·1	23·3	19·7	24·0	21·4	21·6	20·4*
LA	5	11·2	19·7	19·5	20·5	22·8	17·9	18·5***

LA = Lauric acid

† Pretreatment level on day 1 of study.

Values for water consumption are means for three cages of five rats. Those marked with asterisks differ significantly from the control values (ranking method of White (1952) for overall means): $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

acid was slightly less than that of the controls throughout the study, resulting in a statistically significant reduction in the overall mean value (Table 2). In the males fed lauric acid, the water intake was lower only during the first half of the study, so that the reduction in the overall mean value was not statistically significant. Slightly lower values for most of the weekly water intakes and a significantly lower overall mean value were evident also for the females given 10% Span 20, but there were no comparable differences in the males given the same diet. With the lower dietary levels of Span 20 there was no consistent or overall lowering of water intake. The overall intake by males fed 2·5 or 5% Span 20 was slightly raised (107% of the control intake).

Further comparisons between the test animals and the controls demonstrated lower values for haemoglobin concentration and packed cell volume at all three examinations, the effect being related to dose (Table 3). In all the red blood cell counts except that for females at wk 2, lower values were apparent at the lower treatment levels but higher values were found at the highest treatment level. However, not all of these differences were statistically significant. There was a tendency for higher reticulocyte counts especially in the males at wk 6 and 13 and towards treatment-related reductions in the total leucocyte counts. The latter values were statistically significant only in the males at wk 13. No consistent changes in the differential leucocyte counts were apparent. There were no statistically significant differences between the controls and the lauric acid-treated rats except in the males at wk 13 when there were lower leucocyte counts and haemoglobin concentrations and higher reticulocyte counts. The results of the serum analyses were similar in the test and control rats.

The analysis of the urine collected over a 6-hr

Table 3. *Mean haematological findings in rats fed diets containing 2·5–10% Span 20 or 5% lauric acid for 2, 6 or 13 wk*

Sex and diet	Dietary level (%)	Hb (g 100 ml)	PCV (%)	RBC (10^6 mm^3)	Retics (% of RBC)	Leucocytes: Total (10^3 mm^3)	Leucocytes: Differential (%) N	E	L	M
				Wk 2						
Male										
Control	0	14·4	44	6·7	3·4	6·7	11	1	87	1
Span 20	5	13·6*	41*	6·6	1·5**	5·6	12	1	86	1
	10	13·0**	41*	7·5	4·4	5·5	14	1	84	1
LA	5	13·6	43	7·1	3·6	5·0	14	0	85	1
Female										
Control	0	14·7	44	7·0	1·9	5·0	16	1	82	1
Span 20	5	14·2	41*	6·7	1·1	3·9	11	0	88	1
	10	13·0***	40**	6·3*	2·7	3·4	11	0	88	1
LA	5	15·1	44	7·1	1·2	3·0	10	1	89	0
				Wk 6						
Male										
Control	0	15·6	47	7·2	1·2	5·3	11	1	87	1
Span 20	5	14·0**	42**	6·9	0·8*	5·0	8	0	91	1
	10	13·2***	41**	8·5*	2·4**	4·4	14	1	84	1
LA	5	15·7	46	7·5	0.6*	4·1	13	0	87	0
Female										
Control	0	15·0	44	7·1	0·7	5·3	13	1	85	1
Span 20	5	13·9***	41*	6·5*	0·6	4·0	12	2	85	1
	10	14·2*	41	8·0*	1·0	4·4	12	0	88	0
LA	5	15·5	45	7·0	0·6	3·4	13	1	85	1
				Wk 13						
Male										
Control	0	15·3	45	8.0	0.3	6·0	14	1	84	1
Span 20	2·5	15·2	44	7·2*	0·6	4·4**	18	1	80	1
	5	14·2**	43**	7·1*	0·7*	4·6*	16	1	81	2
	10	13·7***	41***	8·2	0·9*	4·8*	16	1	83	1
LA	5	14·7***	45	7·9	1·0**	4·3**	17	1	82	0
Female										
Control	0	14·6	45	7·0	0·9	3·7	17	1	81	1
Span 20	2·5	14·8	45	7·2	0·7	3·7	14	1	84	1
	5	14·0**	42**	7·0	0·7	3·6	13	1	85	1
	10	12·3***	39***	8·1**	0·8	4·2	15	1	82	2
LA	5	15·1	44	7·5	1·3	3·4	15	1	83	1

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

Values are the means for groups of five rats at wk 2 and 6 and groups of 15 at wk 13. Those marked with asterisks differ significantly (Student's *t* test) from the control values: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Basophils did not account for more than 0·5% of the leucocytes in any group and inclusions were not seen in the erythrocytes.

Table 4. *Mean values for renal concentration/dilution tests and urinary cell excretion of rats fed diet containing 2·5–10% Span 20 or 5% lauric acid for 13 wk*

Diet	Dietary level (%)	Cell excretion (10^3/hr)	Concentration: Specific gravity 0–6 hr	Concentration: Specific gravity 16–20 hr	Concentration: Volume (ml) 0–6 hr	Concentration: Volume (ml) 16–20 hr	Dilution test (2 hr): Specific gravity	Dilution test (2 hr): Volume (ml)
Males								
Control	0	3·0	1·063	1·069	2·8	0·9	1·006	7·7
Span 20	2·5	3·0	1·052	1·079*	2·0*	0·3	1·005	7·8
	5	6·8	1·053	1·080	1·3***	0·1	1·005	7·4
	10	3·7	1·056	1·084***	1·8*	0·4	1·006	7·9
LA	5	2·6	1·054	1·074**	1·3**	0·4	1·006	7·0
Females								
Control	0	1·9	1·061	1·083	1·5	0·4	1·007	2·6
Span 20	2·5	3·5	1·099	1·072	0·9	0·1	1·007	3·6
	5	2·6	1·092	1·072	0·9	0·1	1·008	3·3
	10	4·6	1·092	1·080	0·8	0·1	1·011	2·5
LA	5	3·8	1·098	1·084	1·1	0·0	1·005	3·8

LA = Lauric acid

The figures are means for groups of 15 rats and those marked with asterisks differ significantly (ranking method of White, 1952) from the appropriate control values: $*P < 0·05$; $**P < 0·01$; $***P < 0·001$.

period showed no differences between the groups in the incidence of rats with blood, glucose, ketones or the various concentrations of albumin in the urine. There was a tendency for the treated rats to produce less urine with a higher specific gravity, although these values were not always statistically significant (Table 4).

There were many statistically significant differences in organ weight and relative organ weight (Table 5) between the treated and control rats. Although the brain weights were, in general, similar in all the groups, the values for the Span 20-treated rats were higher than those of controls when expressed relative to body weight. Similar patterns of normal or even slightly low organ weights giving high values when expressed relative to the depressed body weights were encountered with the heart, various parts of the gastro-intestinal tract and testes. The spleen weights were lower than the control values but the relative values were comparable in all the groups. The liver weights of rats given 10% Span 20 were higher than those of the controls and the difference was more marked in the relative values. Relative liver weights were also higher at wk 2 and 6 in females given 5% Span 20. The kidney weights of rats given 5 or 10% Span 20 were, in general, higher than the control values and the relative kidney weights were higher also at the lowest dietary level. Few of the organ weights of the rats given lauric acid differed significantly from the control values and the only difference seen also in the relative values was a slightly higher stomach weight in females after 6 wk.

On histological examination, periportal vacuolation was found at all three intervals in the livers of rats of both sexes fed the top dietary level. Fat staining showed an increase in periportal fat in females on the 5 and 10% dietary levels and in males fed 10% Span 20. Early nephrosis was observed in both test and control animals of both sexes, more commonly in the males than the females. No treatment-related increase was found in the fat in the cortical tubules of the kidneys. One marked finding was of calcium concretions in the kidneys of female rats given lauric acid.

Signs of early respiratory disease were a common finding in this study in both test animals and controls even at the interim examinations. The incidences of all other histological findings were low and were again comparable in control and treated animals.

Paired-feeding study. The body weights of the rats given 10% Span 20 in their diet were lower than those of the pair-fed control animals (Table 6) but the difference was much less than with the *ad lib.* controls. Early in this study the treated rats drank more water than the controls, but the mean consumption over the 13-wk period was similar in both groups. The liver and kidney weights of the treated animals were significantly higher than the control values (13·09 *v.* 8·72 g, $P < 0.01$, and 2·50 *v.* 2·08, $P < 0.001$, respectively), and, as in the main study, the relative weights of these two organs as well as those of the heart and small intestine were higher (Table 7).

DISCUSSION

The results reported in this paper conflict in some respects with those of other workers. Harris *et al.* (1951a) observed nasal bleeding, gangrene of the tail and increased haemoglobin levels, none of which were seen in the present study, but they also used a much higher treatment level (25%).

The lower body weights are probably largely attributable to the lower food intake. This suggestion is supported by the smaller difference in body weight in the paired-feeding study. The most marked effect on food intake was seen in the first few days of the study, suggesting unpalatability. The smaller gain in body weight in the treated rats in the paired-feeding study may indicate that the effect was in part due to a toxic action. On the other hand this type of treatment may lead to differences in feeding patterns between treated animals that are restricting their own food intake and pair-fed controls. Such differences in feeding pattern could result in different weight gains.

Table 5. *Mean relative organ weights of rats fed diets containing 2·5 10% Span 10 or 2% lauric acid for 2, 6 or 13 wk*

Sex and diet	Dietary level (%)	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 2														
Male														
Control	0	0·86	0·37	3·78	0·38	0·81	0·62	3·97	0·45	27·5	1·24	3·9	8·8	208
Span 20	5	1·05**	0·40	3·66	0·34*	1·02***	0·69	4·51	0·42	32·1	1·40	4·3	10·9	164***
	10	1·04***	0·41***	5·14***	0·36	0·92*	0·77	4·72*	0·43	29·5	1·48*	3·5	8·6	164***
LA	5	0·90	0·42*	3·61	0·35	0·79	0·66	3·90	0·39	30·9	1·26	4·1	9·3	194
Female														
Control	0	1·08	0·38	3·39	0·36	0·82	0·64	4·03	0·50	47·2	76·9	5·1	8·2	147
Span 20	5	1·23	0·42	3·68	0·38	1·13***	0·75*	4·53	0·58	54·4	78·0	4·7	8·4	129
	10	1·29*	0·43	5·08**	0·34	1·10**	0·77*	5·02	0·55	50·3	71·3	4·6	8·5	125*
LA	5	1·14	0·41	3·46	0·34	0·81	0·70	3·88	0·42	47·7	78·6	5·2	7·1	144
Wk 6														
Male														
Control	0	0·61	0·29	2·80	0·22	0·63	0·46	2·27	0·29	25·5	1·14	2·7	4·3	307
Span 20	5	0·67	0·30	2·95	0·22	0·93***	0·56*	2·78*	0·33	22·3	1·19	2·5	4·8	275
	10	0·75**	0·34**	4·25***	0·25	0·77	0·61**	2·89***	0·32	22·0	1·38**	2·8	5·3	236***
LA	5	0·63	0·31	2·90	0·21	0·65	0·51	2·50**	0·33	20·7	1·11	2·5	5·5	294
Female														
Control	0	0·91	0·32	2·62	0·25	0·64	0·51	2·94	0·37	36·9	57·1	4·9	7·3	188
Span 20	5	0·98	0·31	3·00*	0·27	1·02***	0·64***	3·36	0·39	37·9	56·8	5·1	7·4	174
	10	1·07***	0·33	4·10**	0·24	0·98**	0·63***	3·61	0·36	29·7	50·7	4·4	7·1	160**
LA	5	0·84*	0·32	2·83	0·22	0·67	0·55*	2·71	0·33	31·6	57·3	5·5	6·8	206*
Wk 13														
Male														
Control	0	0·46	0·25	2·51	0·17	0·56	0·39	1·86	0·25	12·8	0·94	2·2	3·5	418
Span 20	2·5	0·51*	0·27	2·38	0·17	0·63***	0·40	1·93	0·25	15·9**	0·98	2·3	3·9	377**
	5	0·55***	0·28	2·54	0·18	0·75***	0·44***	2·14***	0·30*	15·7*	1·05	2·3	3·7	338***
	10	0·60***	0·31***	3·67***	0·17	0·71***	0·52***	2·51***	0·31**	14·5	1·15***	2·6*	4·0	305***
LA	5	0·48	0·25	2·46	0·16	0·53*	0·40	1·90	0·24	13·3	0·87	2·1	3·7	399
Female														
Control	0	0·72	0·30	2·50	0·20	0·60	0·49	2·37	0·32	25·8	47·1	4·9	4·9	245
Span 20	2·5	0·78*	0·31	2·44	0·20	0·69***	0·51	2·60	0·34	26·5	48·5	4·8	4·5	227*
	5	0·79*	0·32	2·67	0·21	0·88***	0·54*	2·63*	0·33	29·0	47·9	4·5	5·3	221**
	10	0·90***	0·35***	4·39***	0·20	0·97***	0·65***	3·29***	0·33	25·7	50·4	4·3	5·6	190***
LA	5	0·74	0·32	2·51	0·20	0·60	0·51	2·41	0·32	28·2	48·5	4·7	5·2	233

LA = Lauric acid

† Weights of this organ are expressed in mg/100 g body weight.

‡ Weights of female gonads are expressed in mg/100 g body weight.

Figures are means for groups of five (wk 2 and 6) or 15 (wk 13) rats. Those marked with asterisks differ significantly (Student's *t* test) from control values: *$P < 0·05$; **$P < 0·01$; ***$P < 0·001$.

Table 6. *Mean body weights and water and Span 20 intakes for male rats fed diets containing 10% Span 20 for 13 wk and for their pair-fed controls*

Parameter	Duration of feeding (wk)	Mean values for rats fed dietary level (%) of 0	10
Body weight (g)	0†	74·6	76·2
	4	207·0	193·7*
	8	299·4	274·1*
	13	357·6	331·8
Water intake (ml/rat/day)	0†	15·3	21·6*
	4	25·1	25·6*
	8	26·0	23·7
	13	25·9	27·9
	0–13	24·9	23·7
Span 20 intake‡ (g/kg/day)	1	—	12·5
	4	—	8·0
	8	—	6·3
	13	—	4·7
	1–13	—	7·9

† Pretreatment value on day of test.
‡ Calculated from data on body weight and food consumption.
Values are means for groups of ten rats and those marked with an asterisk differ significantly (ranking method of White, 1952) from the control value: $*P < 0.05$.

Loss of weight was reported by Harris *et al.* (1951a) in animals fed Span 20 at dietary levels as high as 25%.

Again in contrast to Harris *et al.* (1951a), who reported an increased haemoglobin level, significant decreases in haemoglobin were evident in the present study at the 5 and 10% dietary levels of Span 20. The cause of this decrease is uncertain. There is no evidence from the data that it was due to bone-marrow depression or haemolysis. An increased splenic weight would have been an indication of a haemolytic process, but the reverse was apparent. The explanation is unlikely to be nutritional since there was a high red cell count at the top treatment level and, in other studies where there was a comparable lowering of body weight and food intake, no such effect was observed.

As in the study of sorbitan mono-oleate (Span 80) reported by Ingram, Butterworth, Gaunt, Grasso & Gangolli (1978), many of the organ weights that were normal or lower than the controls were high when expressed as relative values. These included the weights of the brain, heart, spleen, gastro-intestinal tract and testes. A similar effect was found in rats given a standard diet in an amount restricted to 90% of the normal intake (M. Sharratt, unpublished data 1970), and no lower organ weights were found in the treated animals in the pair-fed experiment. This finding is therefore not considered to be an effect of the treatment. Similarly no such effects were found in the

Table 7. *Relative organ weights of male rats fed diets containing 10% Span 20 for 13 wk and of their pair-fed controls*

Organ	Terminal body weight (g)...	Relative organ weights (g/100 g body weight) for rats fed a dietary level (%) of 0	10
		364	336
Brain		0·49	0·54
Heart		0·28	0·33***
Liver		2·40	3·85***
Spleen		0·16	0·17
Kidneys		0·57	0·76***
Stomach		0·47	0·51
Small intestine		1·93	2·28*
Caecum		0·27	0·29
Adrenals†		13·6	18·8
Testes		0·95	1·01
Pituitary†		2·53	2·26
Thyroid†		4·01	5·07

† Relative weights of these organs are expressed in mg/100 g body weight.
Values are the means for groups of ten rats and those marked with asterisks differ significantly (Student's *t* test) from the control values: $*P < 0.05$; $***P < 0.001$.

lauric acid controls, in which there was no comparable loss in body weight. Gaunt, Hardy, Grasso, Gangolli & Butterworth (1976) observed a similar effect in rats fed cyclohexylamine hydrochloride and associated the effects with lower body weights due to reduced food intakes. Thus it is considered that the effect on these organs was not due directly to treatment but rather to a reduced food intake resulting from unpalatability.

Significantly higher relative weights of the kidneys were recorded at wk 13 at all treatment levels and in the interim examinations generally at the 5 and 10% levels. At the 10% level a higher relative liver weight was apparent at all three examination times. Neither of these relative enlargements resulted from the reduced food intakes, as they occurred in the test animals also in the supplementary experiment. Since similar enlargements were not seen in the animals fed lauric acid, they appear to be attributable to the feeding of Span 20.

The increase in relative kidney weight apparent at all dietary levels was not associated with any unusual histological findings or with any increase in the severity or incidence of the normal background renal histology. The differences in the renal concentration tests involved decreased volumes of urine and increased specific gravities, which cannot be regarded as indicative of kidney damage. These observations were made against a background of slightly reduced intake and may be the result of normal water economy. Similarly there were no abnormal constituents in the urine that might be indicative of renal damage. It is difficult to assess the significance of the enlarged kidney in the absence of functional or histological evidence of damage. Further work is needed to elucidate this point, but, in the absence of evidence to the contrary, it is assumed that the increased relative kidney weights are indicative of an adverse effect.

Calcium concretions in the kidney were an isolated finding in the female rats given lauric acid. Such concretions were not seen in any other groups in the present experiment, but they have been seen previously in untreated female rats (I. F. Gaunt, J. Hardy, S. D. Gangolli, K. Butterworth and A. G. Lloyd, unpublished data 1975).

The periportal fat and vacuolation found in the livers of animals fed the upper dietary levels is in agreement with the findings of Fitzhugh *et al.* (1960) who fed 15–25% Span 20 to their rats. They were not due solely to the high fatty acid level fed to the animals since no such effect was observed in the rats fed an equivalent amount of lauric acid. It is possible that the fat infiltration was associated with inanition, but similar effects have not been found in comparable studies in these laboratories; for example rats fed di-(2-ethylhexyl) phthalate lost weight and had enlarged but histologically normal livers (Gray, Butterworth, Gaunt, Grasso & Gangolli, 1977).

The higher weights of the gastro-intestinal tract both in the main study and in the pair-fed animals could indicate irritation of the alimentary tract by Span 20. However, no such effect was seen on histological examination, although such irritation without higher weights of the stomach and small intestine was observed by Harris *et al.* (1951b) in hamsters fed dietary levels of 5–15% Span 20 for 68 days.

Although the demonstrated enlargement of the kidney could have been a response to the need to excrete the compound or its metabolites, the existence of this effect at all dietary levels prevented the establishment of a no-untoward-effect level in the present experiment. A further study is in progress in which Span 20 is being given to rats at lower dietary levels in an attempt to obtain a no-untoward-effect level. Particular attention is being paid to possible effects on the kidneys.

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LONG-TERM TOXICITY STUDY OF SORBITAN MONOSTEARATE (SPAN 60) IN MICE

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Abstract—Sorbitan monostearate (Span 60) was given to groups of 48 male and 48 female mice at dose levels of 0 (control), 0·5, 2·0 or 4·0% of the diet for 80 wk. There was no evidence of carcinogenic activity at any of these dose levels. The treatment had no adverse effects on the death rate or rate of body-weight gain. Both male and female mice receiving 4·0% Span 60 showed enlargement of the kidneys and a higher incidence of nephrosis compared with controls. Other organ-weight changes appeared unlikely to be directly related to treatment, as did a significant depression in the total leucocyte count in the blood of female but not of male mice given 4·0% Span 60. The no-untoward-effect level in this study was considered to be 2·0% of the diet, or approximately 2600 mg/kg body weight/day.

INTRODUCTION

Span 60® is a proprietary name for the food-grade emulsifier sorbitan monostearate, a mixture of partial stearic and palmitic esters of sorbitol and its mono- and dianhydrides (*Food Chemicals Codex*, 1972). It is used, either alone or in combination with other emulsifiers, in bread, confectionery coatings, cakes, cake fillings and mixes, ice-cream, whipped vegetable-oil toppings and fat–water emulsions. It functions as a flavour-dispersing agent in both foods and beverages and is used in some pharmaceutical creams for topical use.

In the UK the use of Span 60 is not restricted under the Emulsifiers and Stabilisers in Food Regulations 1975 (Statutory Instrument 1975, no. 1486). It is included in Annex II of the relevant directive of the European Economic Community (1974) and its use is allowed in Belgium, Denmark, Eire, Luxembourg and the Netherlands. It is permitted as a direct food additive in the USA under Sec. 172.842 (formerly Sec. 121.1029) of the Code of Federal Regulations.

The insolubility and waxy nature of Span 60 makes it unsuitable for parenteral administration. The apparent oral LD_{50} value, using a 25% aqueous slurry, was 31 g/kg in rats (Eagle, 1952; Eagle & Poling, 1956), but this was said to involve excessive dosage volumes. J. D. Brandner (unpublished data 1973*) found that the maximum practical dose was 15·9 g/kg and this did not cause any deaths. When ten rats received a diet containing 50% sorbitan monostearate (J. C. Krantz, Jr., unpublished data 1947*) each had ingested about 3 g of the ester within 10 hr and all appeared unharmed after 3 days.

Young rats fed on diets containing 1 or 4% sorbitan monostearate for 6 wk showed no effect on weight gain, nor were there any significant histological changes in the liver, kidneys, intestine and bladder (J. C. Krantz, Jr., unpublished data 1946*). Chow, Burnett, Ling & Barrows (1953) added Span 60 at 5 or 15% to the basal soyabean-meal diet of rats for 14 wk and observed no signs of toxicity, except that the rats receiving the emulsifier consumed less food and grew more slowly than controls. However, since rats force-fed with diet containing 15% Span 60 gained weight at the same rate as the controls, it was concluded that the reduced weight gain was not a toxic effect of the emulsifier.

Mice, hamsters and dogs tolerated Span 60 at dietary levels of 5 or 10% for up to 1 yr without apparent ill effect, although a dietary level of 15% caused gastrointestinal disturbances and a reduced food intake (Allison, Rosenthal & Mills, 1952). Oser & Oser (1956a,b & 1957a,b) studied the effects of 5, 10 and 20% Span 60 in a nutritionally adequate diet on male and female rats for their lifetime (2 yr) and on three succeeding generations. There was a small but significant depression of growth rate in males given the 20% level. No effect was seen on gestation and fertility at any dose level, but survival of the newborn animals and maternal lactation were slightly diminished at the 20% level. The only other abnormalities were some enlarged but histologically normal livers and kidneys at the 20% level and to a lesser extent at 10%. Similar results were obtained by Fitzhugh, Bourke, Nelson & Frawley (1959), who fed Span 60 at dietary levels of 2–25% to rats for 2 yr, and at 5% of the diet to dogs for 19–20 months. In rats given diet containing 25% Span 60 there was an increase in deaths, a reduced growth rate, liver and kidney enlargement and slight fatty change in some livers. With a lower (10%) dietary level, only an increase in the number of deaths was apparent. A dietary level of 5% had no effect on dogs.

Several investigations have dealt with the effects of emulsifiers on fat absorption by the intestine and on the gut flora. Tidwell & Nagler (1952) found no effect in rats on the rate of fat absorption, degree of fat splitting or intestinal motility 2–3 hr after oral intubation with 6 or 20% Span 60 in olive oil.

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*Cited from Joint FAO/WHO Expert Committee on Food Additives, 1974.

Using sorbitan monostearate labelled with ^{14}C in either the stearic or the polyol moiety, Wick & Joseph (1953a) showed that the polyhydric components were almost completely eliminated from the body within 48 hr of administration of a single oral dose of 500 mg/kg. At least 90% of the emulsifier, when fed to rats in oily solution, was hydrolysed to stearic acid and anhydrides of sorbitol. The same authors (Wick & Joseph, 1953b) were unable to detect any deposition of fatty esters of sorbitol in the tissues of rats that had consumed radioactively labelled sorbitan monostearate at a level of 0·1% of the diet for 28 days.

There was a decrease in total intestinal organisms in rats that had consumed diet containing 25% sorbitan stearate for 4 or 8 wk (Bourke & Fitzhugh, 1953), but dietary levels up to 10% showed no similar effect (György, Forbes & Goldblatt, 1958). At the 25% level, the reduction in micro-organisms correlated with a reduction in weight gain, but the bactericidal activity of the emulsifier was not considered to account fully for the effects on body weight.

A single dose of 20 g sorbitan monostearate did not alter gastric motility or gastric acidity significantly in man (Steigmann, Goldberg & Schoolman, 1953), while repeated daily doses of 6 g given for 28 days to nine subjects had no effect on the intestinal gas pattern, the gastric emptying time or the passage of barium through the gut. This was confirmed by Waldstein, Schoolman & Popper (1954). Also 32 or 37 daily doses of 4 g sorbitan monostearate given to two children had no adverse effects on the gastrointestinal tract, or on blood and urine analyses (Preston, Hunt, McNair Scott & Spur, 1953).

A report of the Food Additives and Contaminants Committee (1970) considered the available data and called for long-term toxicological information on Span 60 in a rodent species other than the rat. The present paper describes the results of an 80-wk feeding study in mice designed to meet this requirement.

EXPERIMENTAL

Materials. The sample of Span 60 was supplied by Honeywill-Atlas Ltd., Carshalton, Surrey. It was prepared by the esterification of sorbitol with edible stearic acid and consisted of approximately 95% of a mixture of the partial esters of sorbitol and its mono- and dianhydrides with stearic acid. It conformed to the requirements of the *Food Chemicals Codex* (1972) and the Emulsifiers and Stabilisers in Food Regulations 1975 (Statutory Instrument 1975, no. 1486) and complied with the following specification: Cream-coloured granular waxy solid at 25°C; acid number, 5·0–10·0; saponification value, 147–157; hydroxyl number, 235–260; water content, max 1%; sulphated ash, max 0·25%; arsenic, max 3 ppm; lead, max 10 ppm; copper, max 50 ppm; zinc, max 25 ppm.

Animals and diet. Mice of the TO strain obtained from a specified-pathogen-free breeding colony (A. Tuck & Son, Rayleigh, Essex) were given free access to Oxoid pasteurized breeding diet, supplemented with vitamin K, and water. They were housed in an animal room maintained at 20 ± 1°C with a relative humidity of 50–70%. Male mice were caged singly to avoid fighting, and females were housed four to a cage.

Experimental design and conduct. Groups of 48 male and 48 female mice were fed diet containing 0 (control), 0·5, 2 or 4% (w/w) Span 60 for 80 wk. The animals were observed frequently for general condition and behaviour. Any mouse showing signs of ill health was kept under close observation and, in the case of the females, was isolated, to be returned to its cage if its condition improved, or killed and autopsied if its condition deteriorated. Any animal found dead was autopsied unless this was prevented by severe autolysis or cannibalism. Sixteen male mice from each group were weighed at the commencement of the study and subsequently at approximately monthly intervals. At wk 12 and 52 blood samples were collected from a caudal vein of ten male and ten female mice from the control group and from the groups fed 2 or 4% Span 60. At wk 80 blood samples were taken from the aorta of all surviving mice when they were killed for autopsy. The samples were examined for haemoglobin concentration and packed cell volume, as well as for counts of total erythrocytes and leucocytes. Differential white cell counts were performed on samples from the control and 4%-Span 60 groups. Slides for the demonstration of reticulocytes were prepared, but were not examined in the absence of effects on the erythrocytes.

At the end of the study all surviving animals were killed by exsanguination from the aorta under barbiturate anaesthesia after an overnight fast. At autopsy any macroscopic abnormalities were noted and the brain, heart, liver, kidneys, spleen, stomach and small intestine were weighed. Samples of these organs together with the caecum, salivary gland, thyroid, thymus, adrenal glands, lymph nodes, pancreas, pituitary, testes, seminal vesicles, prostate, ovaries, uterus, urinary bladder, lungs, trachea, oesophagus, colon, rectum, spinal cord, skeletal muscle, eye and Harderian gland and any other tissue that appeared abnormal were preserved in 10% neutral buffered formalin. The tissues were embedded in paraffin wax and sections were stained with haematoxylin and eosin for histopathological examination. Smears of femoral bone marrow were prepared and stained but were not examined.

RESULTS

No effects on condition or behaviour were seen during the study in the mice fed Span 60. Deaths occurred in all groups during the course of the experiment, but there was no relationship between the number of deaths at any time and the dietary level of Span 60 (Table 1). During the course of the study there were no significant differences in mean body weight between male mice fed Span 60 and control male mice, except at wk 37 when the mice given diet containing 4% Span 60 had a significantly lower weight than the controls (Table 2). Haemoglobin concentration and packed cell volume were similar in treated and control mice throughout the study, but at wk 80 there was a slight but significantly higher total erythrocyte count in male mice fed 4% Span 60 (Table 3). In female mice fed 4% Span 60, there were significantly lower numbers of total leucocytes

Table 1. *Deaths occurring in mice fed Span 60 at 0–4·0% of the diet for 80 wk*

Dietary level (%)	Total no. of mice dead at wk				
	20	40	60	70	78
Males					
0	0	1	7	14	18
0·5	0	1	11	16	20
2·0	0	2	12	19	24
4·0	0	1	3	14	20
Females					
0	0	1	4	21	26
0·5	0	2	7	13	21
2·0	0	3	16	21	28
4·0	3	4	12	21	31

Figures represent the number of animals dead or killed *in extremis* from groups of 48 treated or control mice. There was no significant positive trend in the cumulative number of deaths due to treatment ($P > 0·05$) using the logrank conditionally expected numbers method (Peto & Pike, 1973).

Table 2. *Mean body weight of male mice fed Span 60 at 0–4·0% in the diet for 80 wk*

Dietary level (%)	Body weight (g) at wk											
	0†	4	8	13	18	22	28	33	37	50	63	76
0	30	31	38	41	41	42	42	44	47	46	47	52
0·5	30	34	38	42	43	44	45	46	44	47	49	48
2·0	31	34	38	41	43	44	44	46	45	48	49	49
4·0	29	32	37	39	39	41	42	43	42**	44	44	48

†First day of feeding.
The figures are the means for 16 animals from each group and that marked with asterisks differs significantly (Student's *t* test) from the control weight: $^{**}P < 0·01$.

at all three examinations, whereas in the corresponding males the number of leucocytes was higher at wk 2 and did not differ significantly from that of the controls at wk 52 or 80. In the female animals fed 4% Span 60 there was a significantly higher percentage of neutrophils at wk 80.

When mice were weighed before autopsy at wk 80 after an overnight fast, the body weights of the males fed 0·5 or 4% Span 60 were significantly lower than those of controls (Table 4). Only in these two groups of treated male mice were there statistically significant deviations in the mean organ weights in comparison with the controls. The organs affected were the brain, kidney and stomach (in the group on 0·5% Span 60), liver (in the 4% group) and spleen (in both groups), all of which were lighter than control values. When the organ weights were expressed relative to body weight, however, the relative brain weights in the 0·5 and 4% groups and the kidney weight in the 4% group were significantly higher than the control values. In female mice, by contrast, the mean spleen weight, however, the relative brain weights in the 0·5 groups were higher than those of the controls although the values were statistically significant only in mice fed 2% Span 60. Significantly higher kidney weights compared with the control values were seen in the females fed 4% Span 60. The mean stomach weight in female mice fed 2% Span 60 was lower than the control value, as was the mean brain weight of those given 0·5% Span 60. When expressed relative to body weight, the stomach weights of the 0·5 and 2% Span 60 groups were lower than the control values but the relative spleen weight of the mice fed 2% Span 60 and the relative kidney weight of those fed 4% were significantly higher than those of the controls.

The histopathological findings were similar in all groups of mice, apart from an increased incidence of nephrosis in the kidneys of both male and female mice fed 4% Span 60 (Table 5). A single mouse (male, 2% group) showed calcification of the stomach. Chronic inflammation of the skin was apparent in one male control, two males given 0·5%, one male given 2% and four males given 4% Span 60.

Most of the tumours found in the study occurred either with comparable frequency in the test and control groups or more frequently in the controls (Table 6). One carcinoma of the liver occurred in the group of female mice fed 4% Span 60 without any similar tumours in control or lower-dose groups, but similar liver tumours were identified in male mice, one in the control group and one each in the 0·5 and 4% treatment groups. Generalized lymphosarcomas occurred in all four groups of female mice, the numbers affected being one, five, three and two in the 0, 0·5, 2 and 4% groups respectively. Two other

Table 3. *Results of haematological examinations of mice fed Span 60 at 0–4·0% in the diet for 12 or 80 wk*

Sex and dietary level (%)	No. of mice examined	Hb (g/100 ml)	PCV (%)	RBC (10^6/mm^3)	Leucocytes: Total (10^3/mm^3)	Leucocytes: Differential (%) N	E	L	M
Wk 12									
Male									
0	10	16·9	53	8·89	17·8	11	0	87	2
2·0	10	18·4	55	8·89	21·8	—	—	—	—
4·0	10	18·3	51	8·54	24·2*	8	1	90	1
Female									
0	10	18·8	58	8·41	23·4	10	1	88	1
2·0	10	19·2	56	8·86	28·6	—	—	—	—
4·0	10	18·4	54*	8·48	14·5**	10	0	88	2
Wk 80									
Male									
0	30	12·7	44	5·93	10·8	20	1	78	1
0·5	28	12·9	44	6·36	12·1	—	—	—	—
2·0	25	13·0	47	6·49	11·5	—	—	—	—
4·0	26	13·3	46	6·74*	12·2	17	1	81	1
Female									
0	23	14·1	48	7·07	11·0	17	0	82	1
0·5	26	13·9	46	6·98	10·7	—	—	—	—
2·0	21	13·9	44	6·69	10·2	—	—	—	—
4·0	14	13·1	43	6·38	7·3**	27*	1	71	1

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

Values are means for the numbers of mice stated and those marked with asterisks differ significantly (Student's *t* test) from the control values: $*P < 0.05$; $**P < 0.01$. The arcsin transformation was used for percentage values. Basophils did not constitute more than 0·5% of the leucocytes in any group.

Table 4. *Organ weights and relative organ weights of mice fed Span 60 at 0–4% in the diet for 80 wk*

Sex and dietary level (%)	No. of mice examined	Brain	Heart	Liver	Spleen	Kidney	Stomach	Small intestine	Terminal body weight (g)
Absolute weight (g)									
Male									
0	30	0·49	0·24	2·03	0·17	0·69	0·39	2·06	43
0·5	27	0·48*	0·23	1·84	0·13*	0·63*	0·34*	2·05	38***
2·0	25	0·48	0·23	1·88	0·15	0·65	0·41	2·10	43
4·0	27	0·48	0·22	1·83*	0·12**	0·68	0·37	1·93	39***
Female									
0	23	0·49	0·17	1·83	0·15	0·44	0·50	2·05	38
0·5	27	0·47*	0·17	1·77	0·20	0·44	0·43	1·86	41
2·0	21	0·49	0·17	1·78	0·22*	0·45	0·41*	2·00	38
4·0	18	0·49	0·19	1·84	0·20	0·52**	0·43	1·86	36
Relative organ weight (g 100 g body weight)									
Male									
0		1·16	0·55	4·74	0·40	1·62	0·91	4·80	
0·5		1·26*	0·61	4·82	0·35	1·67	0·89	5·40	
2·0		1·15	0·53	4·44	0·36	1·54	0·97	4·97	
4·0		1·26*	0·58	4·80	0·31	1·79*	0·96	5·07	
Female									
0		1·31	0·45	4·88	0·42	1·19	1·34	5·57	
0·5		1·21	0·43	4·43	0·49	1·12	1·07*	4·72	
2·0		1·35	0·45	4·89	0·59*	1·23	1·10*	5·50	
4·0		1·40	0·52	5·23	0·55	1·48**	1·20	5·29	

Values are means for the numbers of animals shown and those marked with asterisks differ significantly (Student's *t* test) from the control values: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Table 5. *Incidence of histological findings (excluding tumours) in mice fed Span 60 at 0·4·0% in the diet for 80 wk*

Tissue and finding	Number of mice affected							
	Males fed dietary level (%) of				Females fed dietary level (%) of			
	0	0·5	2·0	4·0	0	0·5	2·0	4·0
No. of mice examined...	39	37	38	40	34	34	30	30
Liver								
Fatty degeneration	6	5	4	3	6	10	3	4
Hyperplastic nodule	2	0	2	0	2	1	1	0
Lung								
Chronic inflammation	2	1	4	0	1	1	1	1
Kidney								
Nephrosis	15	19	14	27**	25	15	18	30**
Heart								
Fibrosis	9	9	4	8	0	0	0	0
Stomach								
Calcification (chronic degeneration)	0	0	1	0	0	0	0	0
Chronic inflammation	0	0	0	0	1	0	0	0
Salivary gland								
Chronic inflammation	0	0	0	0	1	0	0	0
Harderian gland								
Chronic inflammation	0	0	0	0	1	0	0	0
Brain								
Calcification (chronic degeneration)	0	0	1	0	0	0	0	0
Bladder								
Chronic inflammation	1	1	1	1	6	1	0	0
Testes								
Chronic degenerative changes	5	8	3	4	—	—	—	—
Ovaries								
Follicular cysts	—	—	—	—	17	16	16	14
Uterus								
Chronic inflammation	—	—	—	—	0	0	2	0
Amyloid degeneration	6	14*	9	9	17	9	10	15

Values represent the incidence of the finding among the numbers of mice shown and those marked with asterisks are significantly greater (chi-square test) than the contol values: $*P < 0.05$; $**P < 0.01$.

isolated malignant tumours were found; one was a pancreatic islet-cell adenocarcinoma (male, 2%) and the other a subcutaneous fibrosarcoma (female, 2%). The non-malignant neoplasms found in mice fed Span 60 without comparable findings in controls or in the highest dose group, were a luteoma and a papillary cystadenoma of the ovary (0·5 and 2% groups respectively), a uterine leiomyofibroma (2%), two well-differentiated renal adenomas (males, 2%), a pancreatic islet-cell adenoma (male, 0·5%) and a well-differentiated squamous papilloma of the skin (male, 2%). The only benign tumour found at the highest dietary level without a comparable control finding was the solid pulmonary adenoma found in a female.

DISCUSSION

It is unlikely that the marked reduction in mean terminal body weight in male mice given diet containing 0·5 or 4% Span 60 reflected any true toxic effect of the compound, since the body weights of the male group fed 2% and of all the female groups on Span 60 were similar to those of the controls and the group showing the only other statistically significant body-weight deviation (males on the highest dietary level at wk 37) had a lower mean body weight than the other groups from the beginning of the study. The low terminal body weights in the males on 0·5 or 4% Span 60 do not account entirely for the lower brain, stomach, spleen, liver and kidney weights seen in these groups since, when expressed relative to body weight, the brain and kidney weights were significantly higher than the control values. However, an increase in relative brain weight is a common finding in rats that have failed to gain weight normally (Carpanini, Gaunt, Hardy, Gangolli, Butterworth & Lloyd, 1978; Gaunt, Hardy, Grasso, Gangolli & Butterworth, 1976; Gray, Butterworth, Gaunt, Grasso & Gangolli, 1977) and decreases in absolute and increases in relative organ weights have been reported

Table 6. *Incidence of tumours in mice fed Span 60 at 0–4·0% of the diet for 80 wk*

Tissue and tumour	Number of mice affected							
	Males fed dietary level (%) of				Females fed dietary level (%) of			
	0	0·5	2·0	4·0	0	0·5	2·0	4·0
No. of mice examined...	39	37	38	40	34	34	30	30
Liver								
Well-differentiated haemangioma	1	0	0	0	0	0	0	0
Liver-cell carcinoma	1	1	0	1	0	0	0	1
Lung								
Adenoma—papillary	8	5	5	5	1	0	2	3
—solid	1	0	1	0	0	0	0	1
Kidney								
Well-differentiated adenoma of cortical tubules	0	0	2	0	0	0	0	0
Ovary								
Luteoma	—	—	—	—	0	1	0	0
Papillary cystadenoma	—	—	—	—	0	0	1	0
Skin								
Squamous papilloma	0	0	1	0	0	0	0	0
Subcutaneous tissue								
Fibrosarcoma	0	0	0	0	0	0	1	0
Generalized								
lymphosarcoma	1	1	0	0	1	5	3	2
Harderian gland								
Papillary adenoma	1	1	2	1	0	0	3	2
Pancreas								
Islet-cell adenoma	0	1	0	0	0	0	0	0
Islet-cell adenocarcinoma	0	0	1	0	0	0	0	0
Uterus								
Leiomyofibroma	—	—	—	—	0	0	1	0
Endometrial polyp	—	—	—	—	1	0	1	0

The tumour incidences in the numbers of mice shown showed no significant differences ($P < 0·05$ by the chi-square test) between the treated groups and the controls.

in rats fed restricted quantities of control diet (Feron, de Groot, Spanjers & Til, 1973; Peters & Boyd, 1966; Schwartz, Tornaben & Boxhill, 1973). It is likely that similar effects would be seen in mice, and the probability that the deviations in brain, spleen, liver and stomach weights are associated with body-weight changes rather than with some direct toxic effect receives further support from the lack of any dose-related effect on these organs in the females.

This argument cannot be applied, however, to the high relative kidney weights, which were significantly increased in both males and females on the 4% Span 60 diet and were associated with a significantly high absolute value in the females and a significantly high incidence of nephrosis in both sexes compared with that in the other groups and the controls. In long-term studies in rats (Fitzhugh *et al.* 1959; Oser & Oser, 1956a,b & 1957a,b), there were signs of increased kidney weight, although Oser & Oser (1957b) considered that the histopathological changes were insufficient to account for the hypertrophy, and invoked the presence of some kind of physiological stress to account for their results. In the present study the association of the larger kidneys with an increased incidence of nephrosis suggests an effect of treatment, and the dietary level having this effect was lower than those that were effective in the rat studies. However, Oser & Oser (1957b) found renal enlargement in rats given 10% Span 60 and, in relation to body weight, the intake in the present experiment was probably equal to or greater than that in the rats.

The significantly reduced counts of total leucocytes in female mice receiving 4% Span 60 in the diet at wk 12, 52 and 80 are unlikely to have been due to the ingestion of a high level of Span 60 since no comparable effect was seen in the males and the differential counts did not indicate any consistent changes in the percentages of the different types of white cells.

The finding of an increased incidence of amyloidosis in male mice receiving 0·5% Span 60 was not supported by any treatment-related increase in female mice, nor was there any evidence of a treatment-related effect in males. Moreover, amyloidosis was reported by Dunn (1965) to be widespread in many strains of mice, so this finding is not considered to reflect an effect of the emulsifier.

The incidence of tumours among mice in this study was, in general, similar to that to be expected in old mice (Cloudman, 1956; Tucker & Baker, 1967). The liver-cell carcinoma found in a female mouse fed 4%

Span 60 without comparable incidence in other female groups probably represents a spontaneous occurrence. The incidence of spontaneous hepatocellular neoplasms is generally 2–4 times higher in male mice than in females (Grasso & Hardy, 1975), so that the finding of three such tumours in the males did not suggest any compound-mediated effect, particularly since two of these occurred in the control and lowest-treatment groups.

In female mice, generalized lymphosarcoma showed a higher incidence in the Span 60-treated animals than in the controls. However, there was no direct relationship between the occurrence of this tumour and dose level, the group given 0·5% Span 60 showing the greatest incidence. Moreover, no similar tumours were found in males on the two highest dietary levels of Span 60. In another 80-wk carcinogenicity study (Phillips, Butterworth, Gaunt, Evans & Grasso, 1979) carried out in the same strain of mice in these laboratories at the same time as the present study, the spontaneous incidence of lymphosarcoma in the untreated animals was 7% (8/86). In view of these observations and the fact that there was no statistically significant difference between the incidence in the female controls and the individual Span 60-treated groups, or between the controls and the total incidence in the treated groups, it seems reasonable to conclude that the lymphosarcomas occurring in the Span 60-treated female mice did not represent a carcinogenic effect.

This study provides no evidence of a carcinogenic effect on the part of Span 60 administered to mice at up to 4% of the diet for 80 wk. Feeding of the 4% dietary level resulted in mean kidney weights higher than those of the controls and an increased incidence of nephrosis in both sexes, and in female mice of this group there was a reduced count of total leucocytes throughout the study. The no-untoward-effect level in mice was 2% of the diet, providing an intake of approximately 2600 mg/kg/day. Reported no-untoward-effect levels in the diet of rats range from 5% (J. C. Krantz, Jr., unpublished data 1947, *loc. cit.*; Fitzhugh *et al.* 1959) to 10% (Oser & Oser, 1957b) equivalent to 2500–5000 mg/kg/day.

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Fd Cosmet. Toxicol. Vol. 16, pp. 535–542

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SHORT-TERM TOXICITY STUDY OF SORBITAN MONO-OLEATE (SPAN 80) IN RATS

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Abstract—Groups of 15 male and 15 female rats were maintained on a diet containing 0, 2·5, 5 or 10% Span 80 or 5% oleic acid for 16 wk and groups of five rats of each sex were given the same treatments (except the 2·5% Span 80 diet) for 2 or 6 wk. In addition, two groups of ten male rats were pair-fed with 0 or 10% Span 80 for 17 wk. Treatment with each level of Span 80 for 16 wk resulted in significantly increased kidney weights, associated in the female groups given 5 or 10% Span 80 with tubular changes of uncertain pathological significance. In addition, liver enlargement was found in the males and females on the highest dietary level. In the females this effect was associated with periportal fatty change. Reduced weight gain was observed in groups given 5 or 10% Span 80, but this was due largely to the unpalatability of the diet. Minor changes in the haematological findings could not definitely be attributed to treatment. In view of the increased kidney weights at all treatment levels, it was not possible to establish a no-untoward-effect level for Span 80 in this study.

INTRODUCTION

Span 80® (sorbitan mono-oleate) is a non-ionic surface-active agent, used extensively in cosmetics and topically applied pharmaceutical preparations and to a lesser extent as a food additive. In 1972 the Food Additives and Contaminants Committee re-examined the requirements for the further testing of sorbitan esters and requested for Span 80 a short-term study in a second species. Although the Joint FAO/WHO Expert Committee on Food Additives (1974) considered data only on the monoesters of palmitic and stearic acids and on the triesters of the latter, the estimated acceptable daily intake of 0–25 mg/kg was for total sorbitan esters and therefore, by implication, covered the use of Span 80. Subsequently a Directive of the European Economic Community (1974) approved the use of Span 80 in foodstuffs for a 5-yr period provided justification for its use was submitted. Following this it was confirmed as a permitted emulsifier in the UK (Statutory Instrument 1975, no. 1486).

The ip and iv LD_{50} values for Span 80 in rats have been reported to be 10 ml/kg and 0·27 g/kg, respectively, and in a long-term feeding study, a dietary level of 5% Span 80 fed to 30 male rats for 2 yr failed to produce any significant changes in growth, survival, haematology or pathology (J. C. Krantz, Jr., unpublished data 1945, cited from Joint FAO/WHO Expert Committee on Food Additives, 1974; *Food and Cosmetics Toxicology*, 1970). In a 68-day feeding study in hamsters, however, growth retardation occurred at the 5 and 15% dietary levels (Harris, Sherman & Jetter, 1951).

Although there are no data on the metabolism of Span 80, some idea of its breakdown products can be deduced from a consideration of other Spans. Elworthy & Treon (1967) concluded that the ester bond of fatty acid esters of sorbitan is hydrolysed in the gastro-intestinal tract, the fatty acid being metabolized in the normal way while the sorbitan moiety is absorbed and excreted largely unchanged in the urine. Hence, if the fatty acid has no toxic effects and the ester cleavage is rapid and complete, any toxic effects following administration by the oral route are likely to be due to the sorbitan moiety.

The permitted use levels of sorbitan esters in food are based on a no-untoward-effect level of 5% in the diet established by the studies of J. C. Krantz, Jr. (unpublished data 1945, *loc. cit.*). The work described here re-examined the oral toxicity of Span 80 at concentrations around the supposed no-untoward-effect level.

EXPERIMENTAL

Materials. Span 80 and oleic acid were supplied by Honeywill Atlas Ltd., Mill Lane, Carshalton, Surrey. The Span 80 was prepared by the esterification of sorbitol with edible oleic acid and consisted of approximately 95% of a mixture of the partial esters of sorbitol and its mono- and di-anhydrides with oleic acid. It conformed to the requirements of the *British Pharmaceutical Codex, 1973* and the Emulsifiers and Stabilisers in Food Regulations 1975 (Statutory Instrument 1975, no. 1486) and complied with the following specification: Amber-coloured oily liquid at 25°C; specific gravity at 25°C, approx. 1; acid number, 5·0–8·0; saponification value, 149–160; hydroxyl number, 193–210; water content, max 1·0%; sulphated ash, max 0·25%; arsenic, max 3 ppm; lead, max 10 ppm; copper, max 50 ppm; zinc, max 25 ppm.

The oleic acid complied with the following specification: Amber-coloured oily liquid at 25°C; acid number, 195–205; saponification value, 197–206; water content, max 0·5%; titre, max 9°C; unsaponifiable matter, max 0·5%; iodine value, 85–90; arsenic, max 3 ppm; lead, max 10 ppm.

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Animals and maintenance. Rats of a Wistar strain were obtained from a specified-pathogen-free colony (A. Tuck & Son, Rayleigh, Essex) and given tap-water *ad lib.* and ground Spiller's Laboratory Small Animal Diet. The animal room was maintained at 20 ± 1°C with a relative humidity of 50–70%.

Short-term feeding study. Groups of 15 male and 15 female rats were housed five to a cage and fed *ad lib.* diets containing 0 (control), 2·5, 5 or 10% Span 80 or 5% oleic acid for 16 wk. The 5% oleic acid diet served as an additional control as this approximated to the amount of oleic acid likely to be released in the gastro-intestinal tract from 10% Span 80. In addition, groups of five male and five female rats were fed diets containing 0, 5 or 10% Span 80 or 5% oleic acid for 2 or 6 wk. All rats were weighed individually at the start of treatment, after 1, 2, 6, 9 and 14 days and then at weekly intervals up to 105 days. Food and water intakes by each cage of rats were measured for the 24 hr prior to body-weight determinations.

Urine samples were collected during the last 2 days of each treatment and examined for appearance, microscopic constituents, glucose, ketones, bile salts and blood. A renal concentration test was also carried out by measuring the specific gravity and volume of urine following a 6-hr period of water deprivation, during the 2 hr following a water load of 25 ml/kg and in the 4-hr period commencing after 16 hr without water. The number of cells in the urine was determined in the sample collected for 2 hr after the water load.

At the end of wk 2, 6 or 16 the appropriate rats were fasted for 24 hr and then killed by exsanguination under barbiturate anaesthesia. Any macroscopic abnormalities were noted at autopsy 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 the lungs, lymph nodes, salivary glands, trachea, oesophagus, aorta, thymus, urinary bladder, colon, rectum, pancreas, uterus and skeletal muscle were fixed in buffered formalin. Paraffin-wax sections for histological examination were cut from all organs of half of the rats fed the control diet and of all of the rats fed 10% Span 80 or 5% oleic acid. In rats given the 2·5 and 5% dietary levels of Span 80, the histological examination was confined to the liver, spleen, kidneys, heart and lungs. The sections were stained with haematoxylin and eosin for histopathological examination, or with Oil Red 0 where appropriate.

Blood taken at autopsy was used for haematological examination and serum analyses. The blood was examined for haemoglobin concentration, packed cell volume and counts of erythrocytes and leucocytes. Slides were prepared for reticulocyte counts. The serum was analysed for urea, glucose, total protein and albumin, as well as for the activities of glutamic–oxalacetic transaminase, glutamic–pyruvic transaminase and lactic dehydrogenase.

Paired-feeding study. Two groups of ten male rats were caged individually and given either a diet containing 10% Span 80 or the control diet for 17 wk. Each rat given the test diet was paired with a littermate given control diet and each day the control rat was given a quantity of food equal to that consumed the previous day by its pair on the test diet. Body-weight, organ-weight and haematological measurements were carried out as described for the main study.

RESULTS

Short-term feeding study

No abnormalities in the appearance of the rats were noted during treatment. There were significantly lower body weights in both sexes given 10% Span 80 and in the males given 5% Span 80 in the diet (Table 1). This reduction in weight gain was apparent by day 1 of treatment and was accompanied by a dose-related reduction in food consumption (Table 1),

Table 1. *Mean values for body weight and food and water consumption of rats fed diets containing 0–10% Span 80 or 5% oleic acid for 16 wk*

Diet	Dietary level of test material (%)	Body weight (g) at day				Food consumption (g/rat/day) at day				Mean food consumption (g/rat/day)	Mean water consumption (ml/rat/day)
		0†	35	71	105	0†	35	71	105		
Males											
Control	0	94	307	397	444	13·0	21·6	22·3	23·7	20·6	30·2
Span 80	2·5	89	298	393	441	12·4	23·3	24·5	23·2	20·8	27·6
	5	89	282**	376	424	12·2	18·1	20·7	20·8	18·3	24·1**
	10	91	231***	298***	340***	13·5	14·7	16·5	17·7	15·1**	23·4**
Oleic acid	5	94	315	417	468	13·5	20·9	23·5	23·2	20·6	28·4
Females											
Control	0	90	193	230	249	11·9	14·9	18·3	19·2	15·7	25·7
Span 80	2·5	91	186	233	256	13·2	14·1	19·1	18·9	15·9	29·0
	5	91	188	228	252	13·3	13·8	18·0	18·7	14·9	27·9
	10	91	164***	197***	223***	12·9	9·7	15·9	16·8	12·1**	25·3
Oleic acid	5	90	198	247	266	12·1	13·3	20·7	17·8	15·6	25·5

†Pretreatment value on day 1 of study.

Body-weight values are means for 15 animals and food- and water-consumption values are means for three cages of five animals. Those marked with asterisks differ significantly (Student's *t* test for body weights and the ranking test of Kruskal & Wallis (Wilcoxon & Wilcox, 1964) for food and water intakes) from the control values: $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$.

Table 2. *Mean haematological values of rats fed on diets containing 0–10% Span 80 or 5% oleic acid for 16 wk*

Diet	Dietary level of test material (%)	Hb (g/100 ml)	PCV (%)	RBC (10^6/mm^3)	Retics (% of RBC)	Total leucocytes (10^3/mm^3)
			Males			
Control	0	15·2	46	7·97	1·0	5·5
Span 80	2·5	15·9	45	7·96	—	4·8
	5	15·0	44	7·72	—	5·1
	10	14·4	41***	7·46	0·8	4·0*
Oleic acid	5	15·7	45	8·16	0·7	4·3
			Females			
Control	0	14·8	45	7·12	0·6	3·0
Span 80	2·5	14·3	45	6·84	—	3·5
	5	15·5	43**	6·78	—	3·6
	10	13·1**	39***	6·48**	0·5	3·2
Oleic acid	5	15·4	42**	7·58	0·5	2·9

Hb = Haemoglobin PCV = Packed cell volume RBC = Red blood cells
Retics = Reticulocytes
Values are means for groups of 15 rats and those marked with asterisks differ significantly (Student's *t* test) from the control values: *$P < 0.05$: **$P < 0.01$: ***$P < 0.001$.

which was apparent from day 1 onwards. In the male rats the mean intakes of Span 80, calculated from body-weight and food-consumption data, were 1·84, 3·38 and 6·75 g/kg/day, whereas in the females the intakes were 2·08, 3·95 and 7·25 g/kg/day. For oleic acid the mean intakes were 3·51 g/kg/day for the males and 3·95 g/kg/day for the females. A significant reduction in mean water consumption during the treatment period was evident in the male groups fed 5 or 10% Span 80, but there were no significant differences in the female groups (Table 1).

The only significantly different figures for haemoglobin concentrations compared with the controls were a higher mean value (15·0 *v.* 14·1, $P < 0.05$) at wk 2 in the females given oleic acid and a lower value at wk 16 in the females fed 10% Span 80 (Table 2). The packed cell volumes were comparable with the control values in all treated rats at wk 2 and 6. However, at wk 16 the values were lower than the control values in both sexes given diet containing 10% Span 80. Slightly smaller reductions were found at the same time in females given 5% Span 80 or oleic acid. The markedly lower values for haemoglobin and packed cell volume in females on 10% Span 80 were accompanied by a lower mean erythrocyte count. The only difference between the treated and control rats in the number of leucocytes was a lower value in males fed 10% Span 80 for 16 wk. Although there were random statistically significant variations in the serum analyses there were no clear differences between the test and control rats (Table 3).

The urine collected from the rats at wk 2, 6 or 16 was free from bile, blood, glucose and ketones, while the concentrations of albumin were similar in all groups. The renal concentration and dilution tests showed no consistent differences between the treated and control animals, nor were there any significant increases in cell excretion rates (Table 4).

There were a number of significant differences between the organ weights of the treated animals and the controls. Most of these were lower values in rats on the highest dietary level of Span 80. These included lower values for the heart in both sexes at wk 2, for the spleen in males at wk 2, and for the caecum in both sexes at wk 16 and in males at wk 2. The last value was accompanied by a lower value in the males given 5% Span 80. In the case of the stomach, the values for males given 10% Span 80 for 16 wk were lower and those for males given 5% Span 80 or oleic acid for 6 wk were higher than those of the controls. The mean liver weight was lower than the control value in the males given 10% Span 80 for 16 wk while the corresponding value in the females was higher, as was that for the females given 5% oleic acid.

When the organ weights were expressed relative to body weight (Table 5) many values were higher than those of the controls. These included the relative brain weights in rats given 10% Span 80 for 2, 6 or 16 wk, although the differences were statistically significant only in the males. The relative heart weights of both sexes given 10% Span 80 for 16 wk were higher than the controls, as were those of the females given 5% Span 80. The relative liver weights were higher in groups given 10% Span 80 than in controls throughout the study except in females at wk 2, and high values were seen also at wk 6 in females given 5% Span 80 and at wk 16 in females given oleic acid. The only differences in relative spleen weight were higher values for all treated females at wk 6, with no comparable differences in the males. Span-treated animals showed a trend towards higher values for relative stomach and small-intestine weights throughout the experiment although even in the 10% Span 80 group not all of the differences from the controls were statistically significant. The most obvious differences among the relative organ weights were those for the kidneys. All of the values for groups given Span 80 were higher then the controls, usually to a statistically significant extent. There were no differences from the control values in the relative weights of the kidneys of rats given oleic acid. There were

Table 3. *Values of serum analyses for rats fed diets containing 0–10% Span 80 or 5% oleic acid for 2, 6 or 16 wk*

Diet	Dietary level of test material (%)	No. of rats examined	GOT (IU)	GPT (IU)	LDH (IU)	Glucose (mg/100 ml)	Urea (mg/100 ml)	Protein (g/100 ml)	Albumin (g/100 ml)
					Wk 2				
Male									
Control	0	5	42	8	978	—	15	4·0	2·8
Span 80	5	5	36	9	1020	—	15	3·2*	2·1**
	10	5	41	7	1148	—	15	3·8	2·8
Oleic acid	5	5	37	9	930	—	20	4·1*	3·1**
Female									
Control	0	5	40	5	1216	—	17	4·4	3·5
Span 80	5	3	43	7	893	—	18	4·4	3·5
	10	3	31	5	1193	—	20	4·4	3·5
Oleic acid	5	4	37	7	939*	—	19*	4·4	3·6*
					Wk 6				
Male									
Control	0	5	36	6	—	—	17	4·1	3·5
Span 80	5	5	41	7	—	—	20	3·9*	2·7**
	10	5	37	5	—	—	24**	3·9*	3·2*
Oleic acid	5	5	33**	6	—	—	16*	4·0*	2·5
Female									
Control	0	2	—	—	—	175	19	7·2	4·4
Span 80	5	1	—	—	—	180	17	7·0	4·0
	10	5	—	—	—	186	17	6·2	4·9
Oleic acid	5	3	—	—	—	173	26	6·9	4·7
					Wk 16				
Male									
Control	0	14	35	9	710	176	15	6·7	2·9
Span 80	2·5	15	33*	7	722	160	19	6·7	2·8
	5	14	34	8	780	146	17	6·7	2·7
	10	15	32	7	733	160	17	5·6	2·0
Oleic acid	5	15	32	8	800	157	19	6·4	2·6
Female									
Control	0	15	34	8	898	123	24	6·2	2·3
Span 80	2·5	14	35	7	852	142	15	6·4	2·6*
	5	14	33	7	992	138	18	6·6	2·2
	10	13	33	6	965	131	13**	6·3	2·5
Oleic acid	5	12	33	7	770	159	13	6·3	2·6

GOT = Glutamic–oxalacetic transaminase GPT = Glutamic–pyruvic transaminase
LDH = Lactic dehydrogenase IU = International units
The figures are means for the numbers of rats shown and values marked with asterisks differ significantly (White, 1952) from those of the appropriate controls: *$P < 0.05$: **$P < 0.01$.

isolated high values for the relative weights of the adrenals, gonads and pituitary among the groups given the 10% dietary level of Span 80.

Most of the histological findings were similar in incidence and severity in the control and treated animals. However, at wk 16, renal tubular damage was evident in the females given 5 or 10% Span 80 in the diet, and the latter group also showed periportal fatty change in the liver. The kidney tubule alterations consisted of proximal tubule dilation with rounded vesicles of cytoplasmic debris, apparently 'budded off' from the cells lining the tubules, lying in the lumen. Extensive vacuolation was observed in the proximal tubular cells in four of the seven affected females in the 10% group. Oil Red O staining was negative.

Paired-feeding study

In this study the mean body weight of the rats given 10% Span 80 was 3% lower than that of the control group at the start of feeding (Table 6) due to differences in the weight of litter mates. This difference became more significant in the first half of the treatment period, amounting to some 7% of the mean control weight by wk 4–8. In the second half of the treatment period the difference between the two groups decreased again, to below 3% by wk 17.

The packed cell volume and haemoglobin concentration of the rats given 10% Span 80 were significantly lower than those of their pair-fed controls (Table 7), but the serum analyses showed no significant differences. Values significantly higher than the control figures were recorded in the Span 80-fed rats for both kidney weight (3·28 *v.* 2·11 g, $P < 0.001$) and relative kidney weight (Table 8), whereas the liver showed only a significantly higher relative weight.

DISCUSSION

The reduced weight gain associated with the higher dietary levels of Span 80 seemed to be due largely

Table 4. *Results of renal concentration and dilution tests and urinary cell counts for rats fed diets containing 0–10% Span 80 or 5% oleic acid for 2, 6 or 16 wk*

Diet	Dietary level of test material (%)	Cells (10^3/hr)	Concentration test: Specific gravity 6 hr	Concentration test: Specific gravity 16–20 hr	Concentration test: Volume (ml) 6 hr	Concentration test: Volume (ml) 16–20 hr	Dilution test (2 hr): Specific gravity	Dilution test (2 hr): Volume (ml)
Wk 2								
Male								
Control	0	1·3	1·040	1·091	1·8	0·1	1·006	3·4
Span 80	5	1·2	1·045	1·086	0·8**	0·2	1·015**	2·6
	10	2·3	1·033	1·080	1·5	0·1	1·015*	2·4
Oleic acid	5	0·9	1·023*	1·082	1·8	0·4	1·006	4·6*
Female								
Control	0	2·1	1·056	—	1·3	—	1·009	2·3
Span 80	5	2·1	1·052	—	1·2	—	1·009	2·4
	10	1·2	1·037	—	1·6	—	1·014*	1·4**
Oleic acid	5	4·0	1·057	—	0·7	—	1·012	1·1**
Wk 6								
Male								
Control	0	5·5	1·037	1·080	2·5	0·3	1·007	6·0
Span 80	5	4·5	1·036	1·080	2·1	0·1	1·003**	9·4**
	10	3·6	1·051*	1·083	1·8*	0·1	1·009	5·1
Oleic acid	5	3·2*	1·056*	1·080	2·0	0·3	1·005**	7·6*
Female								
Control	0	2·7	1·064	1·070	0·7	0·2	1·009	3·1
Span 80	5	1·7	1·060	1·081	1·6**	0·1	1·006**	4·1*
	10	2·4	1·046**	1·076	1·2**	0·3*	1·007*	4·0*
Oleic acid	5	1·5	1·062	1·093**	0·7**	0·2	1·008	3·1
Wk 16								
Male								
Control	0	3·5	1·048	1·073	2·4	0·2	1·007	6·2
Span 80	2·5	4·4	1·057	1·077	1·4*	0·2	1·007	5·2*
	5	2·1	1·058	1·083***	1·7*	0·2	1·007	5·1**
	10	3·4	1·053	1·084***	1·8	0·2	1·007	4·3**
Oleic acid	5	2·2*	1·058*	1·069	1·4*	0·2	1·018	3·1***
Female								
Control	0	1·7	1·062	1·083	1·0	0·1	1·007	2·5
Span 80	2·5	2·1	1·058	1·085	1·3	0·2	1·006	3·1
	5	1·5	1·061	1·075	0·8	0·1*	1·010	3·4*
	10	3·1*	1·060	1·081	1·2	0·1	1·011	2·6
Oleic acid	5	1·4	1·060	1·081	1·1	0·1	1·006	3·3*

Figures are means for groups of five rats at wk 2 and 6 and of 15 at wk 16 and values marked with asterisks differ significantly (White, 1952) from those of the appropriate controls: $*P < 0.05$: $**P < 0.01$: $***P < 0.001$.

to the unpalatability of the diet. This was indicated by the early onset of a reduced food intake in these groups. In the paired-feeding study, the significantly lower body weights in the Span 80 group half-way through the study were probably due to the difference in initial weight, since the difference was not significant at the end of the study. The reduced rate of body-weight gain found in the rats given 10% Span 80 in the main study was in accordance with the reduced rate of weight gain in hamsters given 5 or 15% Span 80 or Span 20 (sorbitan monolaurate) in the diet (J. C. Krantz, Jr., unpublished data 1945, *loc. cit.*).

The reduced water consumption in the males given 5 or 10% Span 80 was associated with a lower body weight and a lower food consumption. As both Strominger (1947) and Cizek & Nocenti (1965) have demonstrated that water consumption is proportional to food consumption, this lower water intake is unlikely to have been due to treatment.

The reduced packed cell volumes at wk 16 in the rats of both sexes given 10% Span 80 and the accompanying reduction in the red cell count and haemoglobin level in the females were not associated with increased reticulocyte counts. That these effects were not due to weight differences is indicated by the demonstration of a similar reduction in packed cell volume but no reduction in body weight in the Span 80 group compared with the controls at wk 17 of the paired-feeding experiment. Since the reduction of packed cell volume was less in the females given oleic acid than in those given 10% Span 80 and was not found at all in the oleic acid-fed males, it would seem that the effect of Span 80 on the packed cell volume was not due to the oleic acid moiety.

Many of the differences in organ weights consisted of lower values in animals with low body weights and were either eliminated or reversed when expressed relative to body weight. This pattern of normal or low organ weights with higher relative values

Table 5. *Mean relative organ weights of rats fed diets containing 0, 10% Span 80 or 5% oleic acid for 6 or 16 wk*

Diet	Dietary level of test material (%)	Relative organ weights (g/100 g body weight)												Terminal body weight (g)
		Brain	Heart	Liver	Spleen	Kidneys	Stomach	Small intestine	Caecum	Adrenals†	Gonads‡	Pituitary†	Thyroid†	
Wk 6														
Male														
Control	0	0·59	0·31	2·88	0·19	0·65	0·43	2·69	0·32	19·7	1·11	2·8	4·02	295
Span 80	5	0·56	0·29	2·95	0·21	0·75**	0·46	2·88	0·30	19·6	1·10	2·5	4·31	320
	10	0·69***	0·33	3·33**	0·22	1·01***	0·56***	3·45***	0·35	21·4	1·30*	2·3	5·04	243**
Oleic acid	5	0·53	0·31	3·10	0·21	0·69	0·47	2·73	0·33	18·4	1·01	2·7	4·81	323
Female														
Control	0	0·82	0·32	2·64	0·22	0·68	0·58	2·86	0·45	37·9	65·2	4·5	7·19	206
Span 80	5	0·88	0·40	2·96**	0·27*	0·91***	0·71	3·30	0·44	38·7	75·0	4·5	9·04	172**
	10	0·95	0·39	3·46***	0·28*	1·02***	0·70	3·98	0·41	44·1	90·0	5·3	8·64	160***
Oleic acid	5	0·82	0·37	2·90	0·29**	0·70	0·67	3·17	0·43	40·8	82·2	5·1	7·04	200
Wk 16														
Male														
Control	0	0·44	0·26	2·49	0·15	0·53	0·42	1·84	0·28	12·7	0·83	2·4	—	437
Span 80	2·5	0·44	0·26	2·41	0·17	0·57**	0·43	1·97	0·30	13·0	0·85	2·3	—	434
	5	0·44	0·28	2·64	0·16	0·67***	0·45	1·97	0·28	14·7	0·85	2·5	—	417
	10	0·56***	0·32***	2·74*	0·19	0·88***	0·48**	2·36***	0·28	16·2*	1·10***	2·7**	—	335***
Oleic acid	5	0·42	0·26	2·47	0·16	0·55	0·40	1·67*	0·27	13·4	0·82	2·2	—	458
Female														
Control	0	0·72	0·31	2·27	0·20	0·56	0·56	2·56	0·40	30·1	60·9	4·9	—	245
Span 80	2·5	0·72	0·33	2·42	0·21	0·67***	0·56	2·45	0·41	29·3	61·3	5·0	—	246
	5	0·75	0·34*	2·50	0·20	0·82***	0·60	2·55	0·38	30·5	63·9	5·4	—	243
	10	0·80	0·36***	3·02***	0·20	1·16***	0·67	3·02**	0·37	30·3	61·6	5·1	—	217
Oleic acid	5	0·70	0·32	2·54**	0·21	0·61	0·54	2·43	0·35	28·3	56·6	4·8	—	259

†Weights of this organ are expressed in mg/100 g body weight.
‡Weights of female gonads are expressed in mg/100 g body weight.
Figures are means for groups of five rats at wk 6 and of 15 at wk 16. Values marked with asterisks differ significantly (Student's *t* test) from those of controls: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table 6. *Mean body weights of male rats pair-fed with diet containing 0 or 10% Span 80 for 17 wk*

Duration of feeding (wk)	Mean body weights (g) of rats fed dietary level (%) of	
	0	10
0†	100	97*
2	152	145
4	227	210***
6	278	260
8	311	288***
10	334	316
12	342	327*
14	371	350
17	389	378

†Pretreatment value on day 1 of study.
Values are means for groups of ten animals. Those marked with asterisks differ significantly (Student's *t* test) from the values for the pair-fed controls: $*P < 0.05$; $***P < 0.001$.

for organs such as the brain, heart, intestine, adrenal gland and testis has been found in rats given a standard diet in an amount restricted to 90% of the normal intake (M. Sharratt, unpublished data 1970), and is therefore not considered to be a direct reflection of treatment. This argument does not apply to the liver and kidney in which increased absolute values were encountered both in the main study and in the pair-fed animals.

Liver enlargement was found consistently only in the 10% Span 80 groups and its greater enlargement in the females accorded with the finding of periportal fatty change in the females given the highest dietary

Table 7. *Mean haematology and serum-analysis data for male rats pair-fed with diet containing 0 or 10% Span 80 for 17 wk*

Parameter	Values for rats fed a dietary level (%) of	
	0	10
Hb (g/100 ml)	15·5	14·4**
PCV (%)	46·3	43·8*
RBC (10^6/mm^3)	7·75	7·70
Retics (% of RBC)	0·7	0·5
Total leucocytes (10^3/mm^3)	4·5	3·7
GOT (IU)	34	33
GPT (IU)	9	7
LDH (IU)	825	888
Glucose (mg/100 ml)	153	158
Urea (mg/100 ml)	16	20
Protein (g/100 ml)	6·6	6·9
Albumin (g/100 ml)	2·7	2·7

Hb = Haemoglobin PCV = Packed cell volume
RBC = Red blood cells Retics = Reticulocytes
GOT = Glutamic-oxalacetic transaminase
GPT = Glutamic-pyruvic transaminase
LDH = Lactic dehydrogenase
IU = International units
Values are means for groups of ten rats and those marked with asterisks differ significantly (Student's *t* test for haematological data and White (1952) test for serum analyses) from the values for the pair-fed controls: $*P < 0.05$: $**P < 0.01$.

Table 8. *Relative organ weights of male rats pair-fed with diet containing 0 or 10% Span 80 for 17 wk*

Organ	Relative organ weights (g/100 g body weight) for rats fed a dietary level (%) of	
	0	10
Terminal body weight...	386	369
Brain	0·47	0·49
Heart	0·26	0·28
Liver	2·56	2·85**
Spleen	0·18	0·19
Kidneys	0·54	0·88***
Stomach	0·54	0·51
Small intestine	2·01	2·19
Caecum	0·33	0·33
Adrenals†	15·26	18·27
Testes	0·88	0·96
Pituitary†	2·51	2·55

†Relative weights of these organs are expressed in mg/100 g body weight. Values are means for groups of ten rats and those marked with asterisks differ significantly (Student's *t* test) from the control values: $**P < 0.01$; $***P < 0.001$.

level. This finding is consistent with the report of fatty change in the liver of rats fed 15–25% Span 20 by Fitzhugh, Schouboe & Nelson (1960). In addition there were some increases in liver weight in rats given oleic acid suggesting that this effect may have been due to the fatty acid.

Kidney enlargement occurred with all dietary levels of Span 80 in both sexes. The greater enlargement in the female groups was associated with histological changes in the kidney tubules only in the two female groups given 5 and 10% Span 80. Chatelanot & Simon (1969) have stated that changes similar to those described here are of doubtful pathological significance and are seldom seen in untreated animals. The "fraying of the free edges of cells" described by Harris *et al.* (1951) after the feeding of 15% Span 20 to hamsters appears to be similar to the tubule changes found in this study. Tubular changes in the kidney were also reported by Fitzhugh *et al.* (1960) following administration of 15–25% Span 20. The failure of J. C. Krantz, Jr., (unpublished data 1945, *loc. cit.*) to detect a kidney effect with 5% Span 80 in rats could have stemmed partly from his use of male rats only and partly from a failure to weigh the organs. It appears, therefore, that mild histological changes of the type seen in the present study are common with sorbitan esters. The kidney-weight data showing increased relative values in all groups given Span 80 but no increase in the groups given the fatty acid suggests that the renal effects must be attributed either to the activity of the unchanged sorbitan ester or to the excretion of large quantities of the sorbitan moiety in the urine following ester cleavage (Elworthy & Treon, 1967).

General conclusions

The pattern of response observed in this test accords with that found in previous studies with sor-

bitan esters, but effects were found at lower dose levels than had previously been reported. Of particular importance was the finding of increased kidney weight, which was highly significant at wk 16 in the rats given 2·5% Span 80 in the diet and was found as early as wk 2 when 5% Span 80 was fed. Although the kidney enlargement may have been a functional hypertrophy, it cannot be ignored since tubular changes of uncertain pathological significance were found in the females given dietary levels of 5 or 10%. Unless further work is undertaken to investigate the biological significance of the kidney changes, it must be presumed that these represent an untoward effect of Span 80. As such changes were observed at the 2·5% dietary level, a reconsideration of the present safety-in-use level would be inevitable in the absence of a further clarification of the nature of these changes.

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TRANSFER OF POLYCHLORINATED BIPHENYLS FROM MOTHERS TO FOETUSES AND INFANTS

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Abstract—Blood, milk, adipose tissue and other tissues of mothers, foetuses and infants were collected at delivery, stillbirth or lactation, and analysed for polychlorinated biphenyls (PCBs). The PCB levels in the maternal blood were significantly higher than those in the corresponding cord blood at delivery, but lower than those in the corresponding infants' blood. A positive but weak correlation was observed between the PCB levels of maternal and cord blood. However, there was no significant correlation between the PCB levels of maternal and infant blood. The PCB levels in the adipose tissue, liver and adrenals of the foetus were much lower than the corresponding values for the adult. These findings suggest that the transfer of PCBs via the milk is much more significant than is placental transfer and that there may be a placental barrier against PCBs.

INTRODUCTION

Accidental ingestion of polychlorinated biphenyls (PCBs) with rice oil caused more than 1500 cases of Yusho in Western Japan in 1968 (Katsuki, 1969). Foetuses and infants from affected mothers showed some of the characteristic signs of Yusho (Kikuchi & Masuda, 1976; Taki, Hisanaga & Amagase, 1969; Yamaguchi, Yoshimura & Kuratsune, 1971; Yoshimura, 1974). This indicates that PCBs are apparently transferred to foetuses and infants through the placenta and by breast feeding. Similar transfer from dams to foetuses and offspring has been confirmed in the mouse (Masuda, Kagawa, Tokudome & Kuratsune, 1978), rat (Takagi, Otake, Kataoka, Murata, Aburada, Akasaka, Hashimoto, Uda & Kitaura, 1976), and monkey (Barsotti, Marlar & Allen, 1976). In the studies with mice and rats most transfer of PCBs to offspring took place through the milk, while relatively small amounts reached the foetus via the placenta. It is important to discover whether this transfer pattern applies to man. Transfer of PCBs in man has not been examined thoroughly except in a few studies on maternal and infant blood (Akiyama, Ohi, Fujitani & Yagyu, 1975; Kodama & Ohta, 1977) and on foetal tissues (Shiota, 1976). The study described here was undertaken to obtain further human data on the transfer of PCBs through the placenta and milk.

EXPERIMENTAL

Paired samples of maternal blood and cord blood were collected at normal delivery, and sets of specimens of maternal adipose tissue, maternal blood and cord blood were obtained at Caesarean births. These samples were obtained at large public hospitals in Fukuoka from December 1973 to April 1974.

In a separate study healthy babies who had been breast-fed were selected from visitors to the Department of Paediatrics, Kurume University School of Medicine, from May 1974 to June 1975. The infants were 2–3 months old, had been nourished only with their mother's milk and had not been weaned at the time of sampling. Blood from these babies and blood and milk from their mothers were collected as pairs or sets of samples. The second group of babies used were 4–12 months old and had been nourished with their mother's milk for several months but were partly weaned by the time of sampling. Blood from the babies and both blood and milk from their mothers were also examined.

Specimens of the adipose tissue, liver, and adrenal were obtained from stillborn foetuses after more than 7 months of gestation. The foetuses were autopsied within 24 hr of delivery. These specimens were collected from February 1973 to June 1975. All the samples were kept in glass bottles that had been cleaned with *n*-hexane and were stored at $-20^{\circ}C$ until analysed.

The analytical procedure used for the PCBs was essentially the same as the method described in our previous studies (Masuda, Kagawa & Kuratsune, 1974a; Masuda, Kagawa, Shimamura, Takada & Kuratsune, 1974b). The blood, or *n*-hexane extract of tissue or milk, was refluxed in 1 N-sodium hydroxide–

Table 1. *Concentration of PCBs in maternal adipose tissue, maternal blood and cord blood*

Source	No. of samples	PCB levels (ppb†) Mean ± SEM	Range
Maternal adipose tissue			
In fat only	30	780 ± 46	270–1360
Total tissue	30	600 ± 44	210–1350
Maternal blood	60	2·5 ± 0·14	0·4–5·7
Cord blood	60	0·61 ± 0·05*	0·2–2·5

† b = 10^9.
The value marked with an asterisk is significantly lower than the value for maternal blood ($P < 0.001$).

ethanol solution for 1 hr. The ethanolic solution was extracted with *n*-hexane. The *n*-hexane solution was washed with distilled water and chromatographed on silica gel eluted with *n*-hexane. The eluate was concentrated to a small volume and subjected to gas chromatography in a Shimadzu GC-4BM fitted with an electron-capture detector of Nickel 63. A glass column (3 mm × 2 m) packed with 5% SE-30 on Chromosorb W AW DMCS was used. Quantitative estimates of PCBs were made by comparing the total heights of gas-chromatographic peaks with those of a mixture of Kanechlor 500 and 600 (1:1).

RESULTS AND DISCUSSION

The PCB contents of maternal blood, cord blood and maternal adipose tissue are summarized in Table 1. The level of PCBs in maternal blood was about four times higher than that in cord blood. These levels were very similar to those of people living in Tokyo, reported by Akiyama *et al.* (1975). A positive but weak correlation ($r = 0.244$) was observed between the PCB levels of 60 paired maternal and cord blood samples as shown in Fig. 1 and Table 2. In a similar analysis, Kodama & Ohta (1977) noted a positive correlation ($r = 0.445$), while Akiyama *et al.* (1975) failed to do so ($r = 0.018$). The correlation coefficient between the levels of PCBs in pairs of maternal blood samples and maternal adipose tissue was statistically significant both when based on whole adipose tissue or when based on the fat content alone. However, the correlation coefficient between paired maternal adipose tissue and cord blood samples was not significant (Table 2).

Gas-chromatographic patterns of PCBs in the maternal adipose tissue, maternal blood and cord blood were alike, suggesting that the transplacental passage of PCBs was not a function of differences in the chemical composition of the PCBs within the three media.

Table 3 shows the PCB concentrations of the samples of milk, maternal blood and infants' blood collected in Kurume. The levels of PCBs in milk were considerably lower than the average levels of 30 ppb previously reported by us for people living in Fukuoka (Masuda *et al.* 1974a) and obtained in a nationwide survey by the Ministry of Health and Welfare (Tatsukawa, 1976). The levels of PCBs in maternal blood samples were also lower than those in the maternal blood collected at the time of delivery in Fukuoka.

The levels of PCBs in the blood samples from infants were significantly higher than those in the maternal blood. When the babies were divided into two groups by age, one consisting of babies 2–3 months old fed only their mother's milk, and another

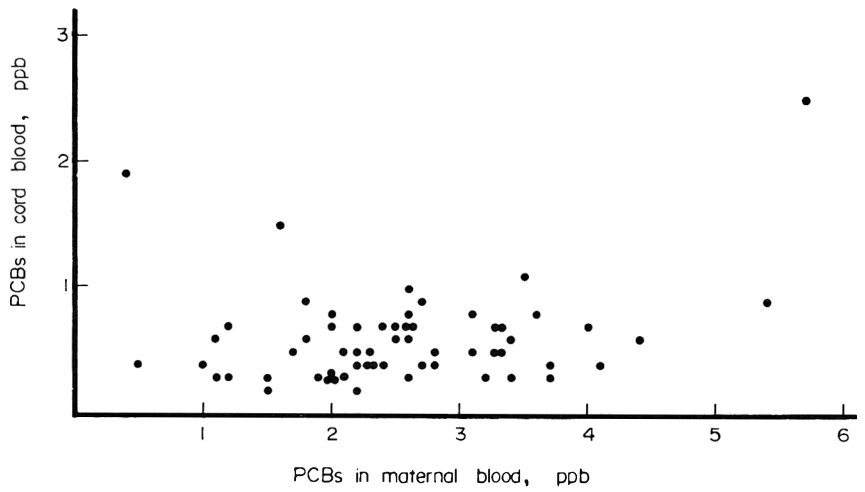

Fig. 1. Correlation of PCB concentrations in cord and maternal blood (60 pairs; b = 10^9)

Table 2. *Correlation coefficients between the levels of PCBs in maternal, infant and cord blood, milk and maternal adipose tissue*

Materials tested for correlation	Number of pairs	Correlation coefficient
Maternal adipose tissue (whole basis) Maternal blood	30	0·444**
Maternal adipose tissue (fat basis) Maternal blood	30	0·451**
Maternal adipose tissue (whole basis) Cord blood	30	0·065
Maternal adipose tissue (fat basis) Cord blood	30	0·039
Maternal blood Cord blood	60	0·244*
Maternal milk (whole basis) Maternal blood	52	0·140
Maternal milk (fat basis) Maternal blood	52	0·289**
Maternal milk (whole basis) Infant blood	38	0·026
Maternal milk (fat basis) Infant blood	38	0·069
Maternal blood Infant blood	42	0·154

Values marked with asterisks show significant correlation (*$P < 0·1$; **$P < 0·05$)

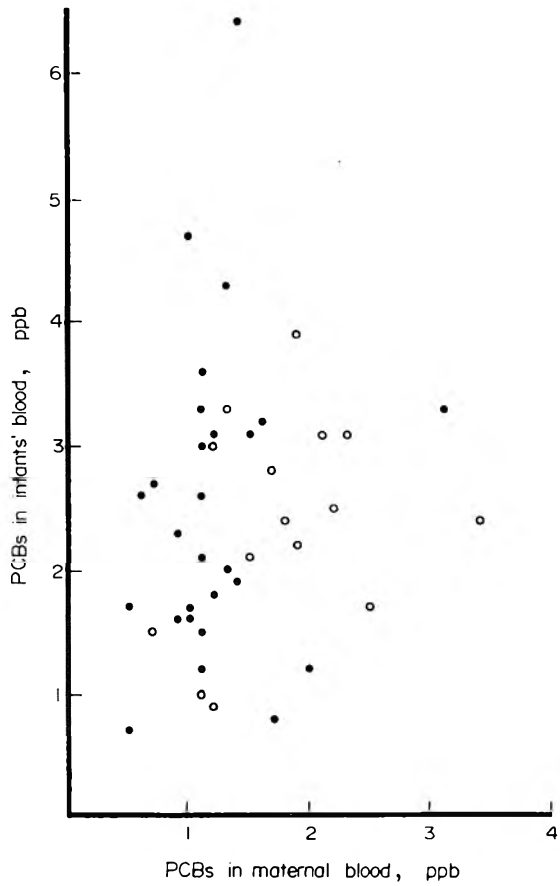

Fig. 2. Correlation of PCB concentrations in infant and maternal blood (42 pairs) at lactation (○, 2–3 months old infant: ●, 4–12 months old infant: b = 10^9)

consisting of babies 4–12 months old taking maternal milk and baby foods, both groups showed significantly higher blood levels of PCBs than the maternal level, as shown in Table 3.

It was demonstrated that the PCB concentrations in the maternal blood samples correlated significantly with those in the fat in the maternal milk, but not significantly with those calculated on the basis of whole milk (Table 2). These findings are similar to the observations by Hamano, Yakushiji, Inoue, Watanabe, Murata, Yoshida, Hasegawa & Kunita (1974). No significant correlation was observed either between the levels of PCBs in baby blood and maternal milk or between those in baby blood and maternal blood as shown in Table 2 and Fig. 2. This con-

Table 3. *Concentration of PCBs in maternal milk and blood and in infants' blood*

Source	No. of samples	PCB levels (ppb†) Mean ± SEM	Range
Maternal milk			
In fat only	52	350 ± 25	30–870
Total milk	52	13 ± 1·2	1–36
Maternal blood			
Mothers of 2–3-month-old infants	15	1·8 ± 0·67	0·7–3·4
Mothers of 4–12-month-old infants	27	1·3 ± 0·64	0·5–3·1
Total	56	1·4 ± 0·08	0·5–3·4
Infant blood			
2–3-month-old	15	2·4 ± 0·22*	0·9–3·9
4–12-month-old	27	2·5 ± 0·24**	0·7–6·2
Total	42	2·5 ± 0·17**	0·7–6·2

†b = 10^9.

Values marked with an asterisk differ significantly from those of the maternal blood (*$P < 0·05$, **$P < 0·001$)

Table 4. *Concentration of PCBs in adipose tissue, liver and adrenal of foetus at 7–10 months old*

Source	No. of samples	PCB levels (ppb*) Mean ± SEM	Range
Adipose tissue			
In fat only	14	470 ± 55	270–960
Total tissue	14	150 ± 29	1–380
Liver	16	7·3 ± 1·3	0·3–21
Adrenal	16	26 ± 4·8	6–80

*b = 10^9.

flicts with the findings of Kodama & Ohta (1977) who noted significant correlations between the levels in infant blood and those in maternal blood or milk.

Table 4 shows the PCB concentrations in the adipose tissue, liver and adrenals from stillborn babies. These values were less than one tenth of the corresponding values reported for adults which were on average 1·54, 0·16 and 0·55 ppm for the adipose tissue, liver and adrenals, respectively (Suzuki, Yamanobe, Ohsawa, Koizumi & Totani, 1972). Shiota (1976) analysed various tissues of human foetuses for PCBs, finding that an average concentration in the foetal liver was 0·034 ppm, much higher than our results (0·007 ppm).

This study demonstrated a higher PCB level in the blood of infants but a lower level in the cord blood than that in maternal blood. This suggests that the transfer of PCBs via the milk is much more significant than placental transfer. Our previous study (Masuda *et al.* 1978) using mice supports this interpretation. Furthermore, foetal tissues were demonstrated to be much lower in PCBs than the corresponding adult tissues. This also correlates with our results from animal experiments that showed that PCB concentrations were much higher in dams than in foetuses. A placental barrier against PCBs seems to exist.

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TERATOGENICITY OF ACRYLONITRILE GIVEN TO RATS BY GAVAGE OR BY INHALATION

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Abstract—The teratogenic potential of ingested or inhaled acrylonitrile (AN), a monomer used in the production of various plastics and fibres, was evaluated in Sprague-Dawley rats. Pregnant rats were given 0, 10, 25 or 65 mg AN/kg/day by gavage from day 6 to 15 of gestation. Additional rats were exposed for 6 hr/day to 0, 40 or 80 ppm AN by inhalation from day 6 to 15 of gestation. Oral administration of 65 mg AN/kg/day, a maternally toxic level, resulted in significant embryotoxicity, including an increased incidence of foetal malformations (short tail, short trunk, missing vertebrae and right-sided aortic arch). Findings suggestive of a teratogenic effect were noted at 25 mg AN/kg/day by gavage and at 80 ppm AN by inhalation. No evidence of embryotoxicity or teratogenicity was discerned in rats given 10 mg AN/kg/day orally or in those inhaling 40 ppm AN.

INTRODUCTION

Acrylonitrile ($CH_2:CH\cdot CN$; vinyl cyanide; AN; VCN), copolymerized with other monomers, is widely used in the production of various plastics and fibres. AN is a highly toxic chemical, affecting primarily the central nervous, gastro-intestinal, respiratory and peripheral blood systems. Interim results of chronic toxicity studies conducted in this laboratory in rats indicate that, under the conditions of the studies, AN was a carcinogen (J. F. Quast, R. M. Enriquez, C. E. Wade, C. G. Humiston and B. A. Schwetz, unpublished data 1977); the types of tumours seen at an increased incidence included brain tumours (microglioma) and tumours of the stomach. Initial findings of an epidemiological study conducted by E.I. duPont DeNemours & Co., Inc., have suggested higher incidences of lung and intestinal cancer among workers exposed to AN in a fibre-manufacturing operation (M. J. O'Berg, unpublished data 1977); however, these are preliminary findings and do not provide definitive evidence that AN is carcinogenic in man.

A review of the scientific literature on AN revealed a lack of information about its teratogenic potential. The purpose of the study reported here was to assess the potential teratogenicity of AN in rats given the compound by gavage or by inhalation. The oral route of administration was chosen because trace amounts of unreacted AN monomer may migrate into food that is packaged in containers made of AN copolymers. The inhalation route was selected because it is a likely route of exposure in the workplace.

EXPERIMENTAL

Test material. Technical-grade AN (Lot No. 7363, purity >99%) was supplied by E.I. duPont DeNemours & Co., Inc., Memphis, TN. The test material contained 35–40 ppm monomethyl ether of hydroquinone (MEHQ) to inhibit polymerization.

Animals. Adult female Sprague-Dawley rats (Spartan Research Animals, Haslett, MI) were mated by the supplier and transported to the laboratory within 4 days of mating. The day on which sperm were found in the vaginal smear was considered day 0 of pregnancy. Rats were housed individually in wire-bottomed cages in rooms at a controlled temperature (21°C), humidity (45%), and light cycle (12/12 hr light/dark). The animals were maintained on commercially available laboratory chow (Ralston Purina Company, St. Louis, MO) and tap-water *ad lib.*

Experimental design. Groups of 29–39 mated rats were given 10, 25 or 65 mg AN/kg/day by gavage on days 6–15 of gestation. Acrylonitrile was administered as an aqueous solution; the dose volume was 2 ml/kg body weight. A group of 43 mated rats received 2 ml water/kg body weight on the same days of gestation to serve as vehicle controls. The volume of material administered was adjusted daily according to each animal's body weight.

The dose levels of AN used in the gavage study were selected on the basis of a preliminary study in which groups of three to five mated rats were given 0, 10, 30, 65 or 100 mg AN/kg/day by gavage from day 6 to 15 of gestation. At 100 mg AN/kg/day, three of four rats died; signs of toxicity noted at that dose included salivation, hyperexcitability, lethargy, convulsions, dyspnoea, and perforating gastric ulcers. At 65 mg AN/kg/day, decreased maternal body-weight gain and thickening of the non-glandular portion of the stomach were observed. The only evidence of toxicity at the lower dose levels was a slight gastric thickening at 30 mg AN/kg/day.

For the inhalation study, groups of 30 pregnant rats were each exposed to 0, 40 or 80 ppm AN for 6 hr/day from day 6 to 15 of gestation. These levels were selected in view of the TLV (20 ppm) and the preliminary results of a long-term inhalation study.

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The control rats were exposed to filtered room air in a chamber identical to those used for AN exposure. Rats did not have access to food or water while in the exposure chambers.

More rats were later used in the inhalation study for the following reason. When they were killed (day 21 of gestation), 13 rats mated on the same day produced several litters with unusually low foetal body weights; these litters were distributed evenly between the control and AN-exposed groups. Among 13 rats bred 1 day later, a female from each group delivered a litter on the morning of day 21 of gestation, approximately 24 hr earlier than would normally be expected. This combination of events strongly suggested that some of these two groups of rats may have been interchanged. To compensate for this likely error in the timing of pregnancy, additional groups of ten, eight and ten mated female rats were exposed to 0, 40 and 80 ppm AN, respectively. The 26 rats bred on the 2 days in question were not used to calculate the maternal body weights, food and water consumptions, foetal body measurements or the incidence of minor skeletal variants, since these parameters are highly dependent on the exact day of gestation. Data from these animals were used in the calculations of the incidence of pregnancy, the average litter size, the incidence of resorptions and the incidence of foetal malformations.

Inhalation conditions. Pregnant animals were exposed in stainless-steel and glass Rochester-type chambers of 4·3 m^3 volume under dynamic airflow conditions. The chamber atmosphere was generated by metering AN at a known rate into the airstream being drawn into the exposure chamber. The concentration of AN in the chambers was analysed three times/day by gas–liquid chromatography. Analytical concentrations (time-weighted mean) of AN in the chambers for the 40 and 80 ppm exposures were 40 ± 2 and 77 ± 8 ppm (mean ± SD), respectively.

Maternal and foetal observations. Animals were observed daily, beginning on day 6 of gestation, and weighed on days 6, 10, 16 and 21. Food and water consumptions were measured at 3-day intervals beginning on day 6 of gestation. On day 21 the females were killed by carbon inhalation, and the maternal liver weight was recorded. The numbers and positions of live, dead and resorbed foetuses were noted. The uteri of apparently non-pregnant animals were stained with a 10% solution of sodium sulphide and were examined for evidence of implantation sites (Kopf, Lorenz & Salewski, 1964). This procedure was conducted to determine the incidence of pregnancy; implantation sites that were detected in this way were not included in calculations of the incidence of foetal resorptions.

After being weighed, measured (crown–rump length) and sexed, all foetuses were examined for external abnormalities and cleft palate. One third of the foetuses of each litter, selected by a table of random numbers, was examined immediately for evidence of visceral malformations by dissection under a stereo-microscope (Staples, 1974). The head of each rat foetus that was examined for soft-tissue abnormalities was placed in Bouin's solution and subsequently examined by the razor-section technique of Wilson (1965). All remaining foetuses were cleared with KOH, stained with Alizarin Red-S (Dawson, 1926) and examined for skeletal alterations.

Statistical evaluation. Foetal body weights and body measurements, maternal body weights and liver weights, and food and water comsumption data were analysed by one-way analysis of variance and Dunnett's test (Steel & Torrie, 1960). The Wilcoxon test, as modified by Haseman & Hoel (1974), was used to evaluate the incidence of foetal alterations and resorptions. The incidence of pregnancy was analysed by the Fisher exact probability test (Siegel, 1956). The level of significance chosen for all cases was $P < 0.05$.

RESULTS

Maternal toxicity

Rats given 65 mg AN/kg/day by gavage exhibited hyperexcitability and excessive salivation. One dam given 65 mg/kg/day died on the first day of treatment, and another dam delivered her litter on day 20 of gestation, i.e. 1 day before the scheduled day for killing. No maternal deaths or early deliveries were seen in the gavage study at the lower dose levels. Sialodacryadenitis, as diagnosed by the presence of swollen salivary glands, was observed in the gavage study among most of the rats in each group, including the control group. Thickening of the non-glandular portion of the stomach was seen in the majority of the rats given 65 mg AN/kg/day and in three rats given 25 mg AN/kg/day.

The incidence of pregnancy was significantly decreased among the rats given 65 mg AN/kg/day (Table 1). After the uterus of each non-pregnant rat was stained with sodium sulphide, four additional dams with implantation sites were revealed at the 65 mg/kg/day level. No effects on the incidence of pregnancy or on the proportion of pregnancies detected by staining were seen in rats given the lower doses of AN.

The weight gain of the dams receiving 65 mg AN/kg/day by gavage was significantly decreased between days 6 and 9, and days 10 and 15 compared to that of the control dams (Table 1); no effect on body weight gain was observed at the lower dose levels. The maternal liver weight, expressed as absolute and relative weight, was significantly increased among dams given 65 mg AN/kg/day, but not among those receiving the lower dose levels. On the first few days of administration of AN, the amount of food consumed by rats given 65 or 25 mg AN/kg/day was significantly decreased compared to that of the control group. Water consumption was significantly increased from day 6 to 20 of gestation (as measured at 3-day intervals) at 65 mg AN/kg/day, but not at lower doses.

In the inhalation study, no deaths, gastric thickening or changes in appearance were observed among the dams inhaling 40 or 80 ppm AN. Sialodacryadenitis did not occur in rats used in the inhalation study.

The incidence of pregnancy was not affected adversely by inhalation of AN (Table 1). Staining the uterus of apparently non-pregnant females for evidence of implantation sites revealed three additional pregnancies at 80 ppm; no additional pregnancies were detected in either the control or the 40 ppm-exposed groups.

Table 1. *Maternal toxicity among rats given acrylonitrile (AN)*

Parameter	AN given by gavage (mg/kg body weight/day)				AN given by inhalation (ppm)		
	0	10	25	65	0	40	80
No. of deaths/no. of females	0/43	0/39	0/33	1/29	0/40	0/38	0/40
Percentage (no.) pregnant	88 (38)	90 (35)	89 (29)	69 (20)*	88 (35)	97 (37)	90 (36)
Additional pregnancies detected by stain	0	0	0	4	0	0	3
Body-weight gain (g)† of dams from gestation days							
6 to 9	18 ± 8	17 ± 7	16 ± 10	2 ± 9*	19 ± 5	1 ± 6*	−5 ± 10*
10 to 15	43 ± 11	42 ± 11	39 ± 12	31 ± 12*	43 ± 8	32 ± 14*	31 ± 17*
16 to 20	83 ± 17	76 ± 14	74 ± 24	79 ± 30	82 ± 12	84 ± 22	92 ± 15
Liver weight on gestation day 21							
Absolute (g)†	15·8 ± 1·9	15·6 ± 1·9	15·8 ± 2·2	17·5 ± 2·2*	16·0 ± 1·8	15·9 ± 1·8	15·3 ± 1·6
Relative (g/kg body weight)†	39·3 ± 4·4	38·8 ± 4·4	40·9 ± 4·6	45·5 ± 2·7	38·6 ± 2·9	41·3 ± 3·1	40·3 ± 4·3

†Mean ± SD
Values marked with an asterisk differ significantly from the control value (*$P < 0.05$).

The maternal body-weight gain of the dams exposed to 40 or 80 ppm AN was significantly decreased compared to that of the control group on days 6–9 and 10–15 of gestation (Table 1). The maternal liver weight was unaffected by exposure to AN. The food consumption of the dams inhaling 40 or 80 ppm AN was significantly decreased on days 6–8 of gestation, but not during subsequent intervals. The amount of water consumed by the pregnant rats inhaling 40 or 80 ppm AN was significantly greater than that of the controls on days 9 to 20 of gestation (as measured at 3-day intervals).

Embryo- and foetotoxicity

Administration of AN by gavage did not have a statistically significant effect on the average numbers of implanations, live foetuses/litter or resorptions/litter (Table 2). Compared to the control group, foetal body weight and crown–rump length were significantly decreased at 65 mg AN/kg/day but not at the lower doses.

In the inhalation study, mean numbers of implantations, live foetuses and resorptions were not significantly altered by exposure to 40 or 80 ppm AN (Table 2). No effect on foetal body measurements was evident among the offspring of rats that inhaled AN.

Foetal malformations

Short tail occurred significantly more often among the litters of rats given 65 mg AN/kg/day than among the control litters (Table 3). The skeletal examination revealed that each short-tailed foetus was also missing vertebrae. This defect ranged in severity from missing one lumbar vertebra to missing all sacral, lumbar and most thoracic vertebrae; when thoracic vertebrae were missing, corresponding pairs of ribs also were absent. Several of the short-tailed foetuses at 65 mg AN/kg/day exhibited additional malformations, including short trunk (probably resulting from the missing vertebrae), imperforate anus, right-sided aortic arch, missing kidney and anteriorly-displaced ovaries. At 25 mg AN/kg/day no malformation occurred at an incidence statistically significantly different from that of the control group. A number of the same malformations seen at 65 mg AN/kg/day were also observed at 25 mg AN/kg/day, including one right-

Table 2. *Litter data of pregnant rats given acrylonitrile (AN)*

Parameter	AN given by gavage (mg/kg body weight/day)				AN given by inhalation (ppm)		
	0	10	25	65	0	40	80
No. of litters	38	35	29	18	33	36	35
Implantations/dam†	12 ± 3	12 ± 3	11 ± 4	12 ± 3	13 ± 2	13 ± 2	12 ± 3
Live foetuses/litter†	12 ± 3	11 ± 3	11 ± 4	12 ± 3	13 ± 2	12 ± 2	12 ± 3
Resorptions/litter†	0·7 ± 0·9	0·6 ± 0.8	0·4 ± 0·6	0·6 ± 0·7	0·6 ± 0·7	0·7 ± 1·1	0·5 ± 0·6
Foetal body weight, g†	5·68 ± 0·28	5·78 ± 0·25	5·80 ± 0·33	5·26 ± 0·32*	5·79 ± 0·33	5·72 ± 0·42	5·90 ± 0·25
Foetal crown–rump length, mm†	44·4 ± 1·0	44·5 ± 1·3	45·0 ± 1·2	43·6 ± 1·2*	43·9 ± 2·1	43·5 ± 2·2	43·7 ± 2·2

†Mean ± SD
Values marked with an asterisk differ significantly from the control value ($P < 0.05$).

Table 3. *Incidence of foetal malformations among litters of rats given acrylonitrile (AN)*

Type of malformation	AN given by gavage (mg/kg body weight/day)				AN given by inhalation (ppm)		
	0	10	25	65	0	40	80
	No. foetuses/No. litters examined						
External and skeletal malformations	443/38	388/35	312/29	212/17	421/33	441/36	406/35
Visceral malformations	154/38	135/35	111/29	71/17	140/33	148/36	136/35
	No. of foetuses (litters) affected						
External malformations							
Short tail	1 (1)	0 (0)	2 (2)	8 (6)*	0 (0)	0 (0)	2 (2)
Short trunk†	0 (0)	0 (0)	0 (0)	3 (3)*	0 (0)	0 (0)	1 (1)
Imperforate anus†	0 (0)	0 (0)	0 (0)	2 (2)	0 (0)	0 (0)	0 (0)
Omphalocele	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	1 (1)
Visceral malformations							
Right-sided aortic arch	0 (0)	0 (0)	1 (1)	1 (1)	0 (0)	0 (0)	0 (0)
Missing kidney, unilateral	1 (1)	0 (0)	0 (0)	1 (1)	0 (0)	0 (0)	0 (0)
Anteriorly-displaced ovaries†	0 (0)	0 (0)	1 (1)	1 (1)	0 (0)	0 (0)	1 (1)
Skeletal malformations							
Missing vertebrae (associated with short tail)†	1 (1)	0 (0)	2 (2)	8 (6)*	0 (0)	0 (0)	2 (2)
Missing two vertebrae and a pair of ribs	7 (1)	0 (0)	7 (2)	0 (0)	8 (1)	2 (1)	7 (2)
Hemivertebra	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)
Total malformed	8 (2)	0 (0)	10 (4)	8 (6)*	8 (1)	3 (2)	11 (6)**

†This malformation was observed only in foetuses with short tail.
Values marked with asterisks differ significantly from the control value (*$P < 0.05$; **$P = 0.06$).

sided aortic arch and two cases of short-tail associated with missing vertebrae. At 10 mg AN/kg/day, no malformations were observed.

In the inhalation study, no single major malformations occurred among the offspring of the AN-exposed dams at an incidence significantly different from that of the controls (Table 3). The incidence of total major malformations, when considered collectively, was slightly increased ($P = 0.06$) at 80 ppm compared to the control incidence. The types of malformations observed at the highest level of exposure included short tail, missing vertebrae, short trunk, omphalocele and hemivertebra. At 40 ppm, the overall incidence of malformations was comparable with that seen in the control group.

Minor skeletal variants

Several minor skeletal variants occurred significantly more often among the offspring of rats given 65 mg AN/kg/day by gavage; these included delayed ossification of sternebrae, split sternebrae and delayed ossification of centra of cervical vertebrae. No significant effect on the incidence of skeletal variants was noted in rats given lower doses of AN by gavage. Inhalation of AN resulted in a statistically significant decrease in the incidence of delayed ossification of skull bones at 80 ppm but not at 40 ppm.

DISCUSSION

The results of these studies indicate a potential for AN to interfere with embryonal and foetal development. Oral administration of 65 mg AN/kg/day, a maternally toxic dose, was teratogenic, as indicated by a significant increase in the incidence of foetuses with a short tail and missing vertebrae. The incidence of this malformation at 65 mg AN/kg/day was 4% (8/212) among foetuses and 35% (6/17) among litters compared to a historical control incidence of 0·2% (13/6192) among foetuses and 2% (11/538) among litters. Other malformations observed among the short-tailed offspring of rats given 65 mg AN/kg/day included short trunk, anteriorly-displaced ovaries, imperforate anus, missing kidney and right-sided aortic arch. Most of these defects have been seen in short-tailed foetuses in control groups from earlier studies; however, right-sided aortic arch has never been seen in over 1000 litters of rats examined in this laboratory before this study. At 25 mg AN/kg/day by gavage, less maternal toxicity was observed, but a low incidence of the same malformations seen at 65 mg AN/kg/day was noted. At 10 mg AN/kg/day, there was no evidence of toxicity to the dams or to their offspring. Inhalation exposure to 40 or 80 ppm AN for 6 hr/day did not result in a statistically significant increase in the occurrence of any single malformation. However, the total incidence of major malformations, considered collectively was marginally increased at 80 ppm ($P = 0.06$). Also at 80 ppm, two foetuses from different litters had a short tail and missing vertebrae; this defect was not observed in either the control or 40 ppm-exposed groups.

Findings suggestive of a teratogenic effect were noted both at 25 mg AN/kg/day by gavage and at 80 ppm AN by inhalation. Estimates of the total amount of AN and its metabolites in the blood of rats (on the basis of the area under the curve of blood level versus time) indicate that a single 6-hr inhalation exposure to 80 ppm AN is equivalent to a single gavage administration of 23 mg/kg body weight (J. D. Young, personal communication 1978). No toxicity to the embryo or foetus was seen in rats given 10 mg AN/kg/day by gavage or in those inhaling 40 ppm AN. A single 6-hr 40 ppm inhalation exposure

was estimated to be equivalent to a single gavage administration of 14 mg/kg.

Signs of AN-induced maternal toxicity were similar in the inhalation and gavage studies. Both routes of exposure produced decreased body-weight gain, decreased food consumption and increased water consumption. However, the gastric thickening seen in the dams given the higher levels of AN by gavage was not observed in those given AN by inhalation; thus it is likely that the gastric thickening was due to the direct deposition of the test material in the stomach. Also, signs of maternal hyperexcitability or salivation, which were observed among the dams given 65 mg AN/kg/day by gavage, were not observed in the inhalation study.

It is not known what role maternal toxicity played in the aetiology of the foetal malformations seen in these studies. However, it is unlikely that the malformations were caused by maternal toxicity alone since there was no apparent correlation between the degree of toxicity seen in the individual dams and the occurrence of malformations in their offspring, and the types of malformations seen in this study have not occurred at an increased incidence in previous studies in this laboratory in which rats were stressed to an even greater degree. The presence of sialodacryadenitis, presumably of viral origin, in most of the rats in the gavage study was not believed to affect significantly the outcome of the study, since the disease occurred with equal frequency among all groups and since the control group did not appear to be different from past control groups in any respect other than the signs of the disease.

In conclusion, AN was teratogenic in rats given 65 mg/kg/day by gavage. Findings suggesting a teratogenic effect were noted at 25 mg AN/kg/day by gavage and at 80 ppm AN by inhalation. No evidence of embryotoxicity or teratogenicity was discerned in rats given 10 mg AN/kg/day orally or exposed to 40 ppm AN by inhalation.

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DISPOSITION OF ZINC PYRITHIONE IN THE RAT*

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Abstract—The pharmacokinetic profile of orally administered [2,6-^{14}C]-labelled zinc pyrithione (ZPT) was defined in male and female rats and was used to predict the blood concentrations of ^{14}C at steady state correlating with the skeletal-muscle weakness observed in long-term feeding tests and mechanism studies. The ^{14}C concentration was determined in blood, urine and faeces as a function of time following oral administration of doses of 0·5, 1·25 and 12·5 mg [^{14}C]ZPT/kg. The major route of elimination from the body was the urine, in which all the ZPT appeared as metabolites. Mass-spectroscopic analysis of the urine indicated that a minor metabolite was 2-mercaptopyridine-*N*-oxide and the major metabolites were *S*-glucuronides of 2-mercaptopyridine-*N*-oxide. Radioassay of tissues indicated that ZPT was rapidly excreted and was not retained in the body to any significant degree. A two-compartment open model was used to describe the dynamics of ZPT in males and females given 0·5, 1·25 or 12·5 mg/kg. Kinetic constants for the same dose indicated sex differences in plasma elimination, renal clearance, time required for attainment of maximum blood concentration and rate of absorption. The ratio for ^{14}C binding in red blood cells and plasma, respectively, was 5:1 at 96 hr and increased to $\geqslant$ 45:1 at 240 hr, a probable reason for the low renal clearance rates. No sex difference in the concentration of ^{14}C in blood at steady-state levels (maximum and minimum) was seen with the 0·5 mg/kg dose, but a significant difference was apparent at 1·25 mg/kg, the concentration being higher in the females. The significantly lower blood concentration of ^{14}C in the males, in conjunction with the apparent higher rate of metabolism in the male, provide a reasonable explanation for the demonstrated sex difference in the dietary level of ZPT capable of producing an effect.

INTRODUCTION

Zinc pyrithione (zinc bis-(1-hydroxy-2(1*H*)-pyridinethionate; ZPT), a broad spectrum antimicrobial compound†, is incorporated in hairdressing formulations and shampoos as an antiseborrhoeic agent. Safety evaluation studies with ZPT have been performed in various species by different routes of administration (Adams, Wedig, Jordan, Smith, Henderson & Borzelleca, 1976; Delahunt, Stebbins, Anderson & Bailey, 1962; Howes & Black, 1975; Klaassen, 1976; Moe, Kirpan & Linegar, 1960; Nolen, Patrick & Dierckman, 1975; Okamoto, Ito, Hasegawa & Urakubo, 1967; Snyder, Buehler & Winek, 1965; Wedig, Goldhamer & Henderson, 1974; Wedig, Kennedy, Jenkins, Henderson & Keplinger, 1976; Winek & Buehler, 1966; Ziller, 1977). Signs of skeletal-muscle wasting and weakness initially affecting the hindquarters were noted in rats given ZPT in the diet. In some cases this progressed until the animal was unable to use its hindquarters to stand or walk. Snyder *et al.* (1965) demonstrated the reversibility of this effect by observing the change in posture and gait of rats given a diet containing 100 ppm ZPT followed by untreated diet for three consecutive cycles. Winek & Buehler (1966) reported that skeletal-muscle weakness was not produced in rats by the daily oral intubation of a shampoo formulation containing an amount of ZPT comparable to that given in the diet by Snyder *et al.* (1965). A 2-yr feeding study in rats using dietary levels of 2, 5, 10, 25 and 50 ppm ZPT indicated a sex difference with respect to the dose required to elicit muscle weakness (P.S. Larson, unpublished data 1958). the dietary no-effect level in female and male rats being 10 and 25 ppm, respectively.

A neurological evaluation was conducted in male rats fed 250 ppm ZPT in the diet (equivalent to a dose of 12·5 mg/kg/day) to investigate any possible impairment in transmission in the sciatic nerve that might be responsible for this effect (Snyder, Gralla, Coleman & Wedig, 1977). The absence of any decrease in gross nerve-conduction velocities or of any

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†See Olin Technical Bulletin AD1372-667, *Zinc and Sodium Compounds of Omadine®*, Olin Corp., New Haven, CT.

light-microscopically detectable morphological lesion that could be directly related to this effect suggested a biochemical lesion.

Additional neurophysiological (sural nerve) studies conducted in rats fed 250 ppm ZPT indicated that the velocity of sensory-nerve conduction remained normal, but the potential amplitude and duration were significantly decreased, indicating axonal disease (deJesus, Towfighi & Snyder, 1978). Following nerve teasing, Wallerian degeneration has been observed in some mixed nerves (sciatic, lateral and medial plantar, and posterior tibial) of rats fed 166 ppm ZPT in the diet (Sahenk & Mendell, 1977). The apparent absence of pathological changes of this sort in the sural nerve requires further study.

The study reported here was performed to investigate the metabolism and pharmacokinetic profile of [^{14}C]ZPT following oral administration to male and female rats in order to provide a possible explanation for the sex difference in dietary levels required for the induction of skeletal-muscle weakness and to derive equations for calculating the expected blood levels of ^{14}C after the nth daily dose and for relating this to the muscular weakness.

EXPERIMENTAL

Animals. Before dosing was started, age-matched male (390–500 g) and female (240–280 g) Sprague–Dawley rats (Food and Drug Research Laboratories, Inc., Waverly, NY) were acclimatized in their cages for 3 days in a room maintained at 70 ± 2°F with a 12-hr light-dark cycle. All animals were given free access to food (Charles River Chow) and water except in the balance study, in which they were fasted overnight before dosing and for 12 hr before they were killed.

Materials and dosage. For these studies, [2,6-^{14}C]ZPT (Fig. 1), 32·7 MCi/mmol, with a radiochemical purity greater than 99% was obtained from New England Nuclear, Boston, MA. Technical-grade ZPT (98·7% pure) was supplied by the Olin Corporation, New Haven, CT. Corn oil (Mazola®) suspensions were prepared so that each rat received 15 μCi of [^{14}C]ZPT/kg plus technical-grade material to provide a final dose of 0·50, 1·25 or 12·50 mg/kg at a constant volume of 1 ml/250 g body weight. The specific activity of each solution was determined by liquid scintillation counting. The Olin Corporation provided analytical standards of the sodium salt of 2-mercaptopyridine-*N*-oxide (2-MPO), 2-mercaptopyridine, 2,2′-(pyridyl-*N*-oxide) disulphide, 2,2′-(pyridyl) disulphide, 2-(pyridyl-*N*-oxide) sulphonic acid, zinc pyrithione and 2-MPO, for use in metabolite identification.

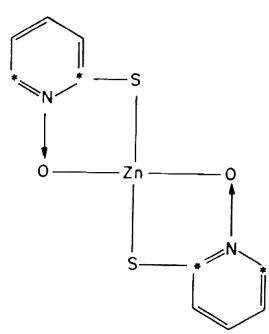

Fig. 1. [2,6-^{14}C]Zinc pyrithione, with asterisks denoting the positions of the ^{14}C label.

Measurement of radioactivity. Radioactivity was measured with a Beckman 355 liquid scintillation counter, using the external standard channel ratios method to correct for sample quench. Urine samples were treated with a quaternary ammonium solubilizer (NCS, Amersham/Searle, Arlington Heights, IL) and were assayed by direct counting in ACS liquid scintillation cocktail (Amersham/Searle). Tissue, tissue homogenates and blood were combusted in a Packard Model 306 sample oxidizer. Faeces were air dried, ground with a mortar and pestle and then oxidized. Carcasses were ground in a meat grinder and representative aliquots were oxidized. An ethanolamine–ethylene glycol monomethyl ether solution was used to trap expired CO_2 from the Roth-type metabolism cages. 2-ml aliquots being mixed with 10 ml of a liquid scintillation cocktail (NEN-Aquasol-2) and then radioassayed.

Expired air. A single oral dose of 12·5 mg ZPT/kg was given to each of two male and two female rats housed individually in modified Roth-type metabolism cages. Solutions from the CO_2 traps were collected at 4-hr intervals for 12 hr from one rat of each sex and for 16 hr from the other two animals. Since less than 0·04% of the 12·5 mg/kg dose was expired as CO_2, additional balance studies were performed in metal metabolism cages.

Blood profile. Groups of three male and three female rats, housed individually in wire rat-holding cages, were orally intubated with 0·5, 1·25 or 12·5 mg ZPT/kg, suspended in corn oil. Samples of whole blood (100 μl) were obtained by orbital-sinus puncture, using heparinized capillary tubes, at approximately 0·08, 0·17, 0·33, 0·5, 0·75, 1, 2, 4, 8, 12, 18, 24, 36, 48, 60 and 72 hr and daily thereafter up to 10 days after dosing. On day 10, the animals were anaesthetized with chloroform and blood was obtained by heart puncture for determination of red blood cell (RBC)–plasma distribution.

Balance study. The animals (three males and three females/group) were housed individually in metal metabolism cages and given a single oral dose of 0·5, 1·25 or 12·5 mg ZPT/kg, suspended in corn oil, by gastric intubation. Urine and faeces were collected at 24-hr intervals for 11 days. Each cage was washed and the washings were analysed for ^{14}C by scintillation counting. Eleven days after dosing, blood samples were obtained by orbital-sinus puncture using heparinized capillary tubes, for haematocrit determinations and radioassay. The rats were killed with chloroform and tissue samples were taken for analysis. The remaining carcass was ground in a meat grinder and an aliquot was assayed for ^{14}C.

Plasma-protein binding. To determine the fraction of plasma ^{14}C that was bound to protein, an equilibrium dialysis experiment was conducted. Male and female rats were intubated orally with 0·5, 1·25 or 12·5 mg ZPT/kg and exsanguinated 20 hr later. Whole blood was centrifuged to separate the cells from the plasma. For each dose level, 2-ml samples of plasma were pipetted into cellulose dialysis tubing tied at one end. The tubing was put into glass tubes containing 10 ml of dialysing solution (0·9% saline) and incu-

bated for 4 hr at 5°C, a time found to be adequate for equilibrium to be reached between the buffer and the plasma compartments. The ^{14}C activity in aliquots of plasma and buffer was then determined by scintillation counting. The fraction of ^{14}C bound to plasma protein was calculated by:

$$\text{Fraction bound} = \frac{[^{14}C \text{ in plasma}] - [^{14}C \text{ in buffer}]}{[^{14}C \text{ in plasma}]}$$

RBC binding. The binding of ^{14}C activity to RBCs after oral administration of [2,6-^{14}C]ZPT to rats was investigated. Whole blood samples taken from rats 96 and 240 hr after administration of 0·5, 1·25 or 12·5 mg ZPT/kg were centrifuged to separate plasma from cells. Aliquots of the RBCs, plasma and whole blood samples were assayed in a scintillation counter.

Identification of urinary metabolites. Urine excreted by six males and six females during the 24-hr period following administration of 12·5 mg ZPT/kg was pooled by sex and passed through a Dowex AG 1-2X chloride column, which was then washed with water. Elution of ^{14}C was almost quantitative with 1% formic acid. The column eluate fractions containing ^{14}C were reacted with 2,2'-dimethoxypropane (Bousquet, Christian, Knevel & Spahr, 1962) and their volume was reduced to a syrup-like residue on a rotary evaporator. The residue was dissolved in methanol and applied as a spot on 0·25 mm silica gel G plates (Brinkman Instruments, Inc., Westbury, NY), which were developed in ethanol–water–10 N-NH_4OH (90:8:2). After development, one vertical streak from each of the pooled urines was scraped off in 1·0 cm horizontal segments and counted in a scintillation counter. The segments with the greatest amount of ^{14}C activity were combined, rechromatographed (by the same procedure as before) and submitted as dry silica-gel scrapings for identification.

The scrapings were extracted with methanol, the volume of which was reduced under nitrogen to 100 μl. High-performance liquid chromatography (HPLC) was performed on a Perkin Elmer 1220 dual-syringe liquid chromatograph, using a Particil column PXS-10/25-PAC, 10 μm particle size, a flow rate of 1 ml/min and a methanol–pentane (1:1, v/v) mobile phase, connected to a Perkin Elmer Model LC 55 ultraviolet detector set at 287 nm.

Chemical ionization–mass spectroscopic analysis (CI–MS) was performed with a Finnigan 1015 mass spectrometer at 100 eV using methane or isobutane as reagent gases. Electron impact–mass spectroscopic analysis (EI–GC/MS) was conducted with a Hewlett Packard 5992 A instrument using helium (30 ml/min) as the carrier gas and a 1·3 m × 2 mm ID Dexsil 300 on 100/120 mesh Chromosorb W column. The gas chromatograph was programmed from 100 to 250°C at 10°C/min. Derivatives were prepared by reacting samples with trimethylsilylimidazole in a test tube for 15 min at 65°C before EI–MS analysis.

RESULTS

Blood levels

The [^{14}C] present in the blood of rats as a function of time following a single oral dose of 0·5, 1·25 or 12·5 mg [^{14}C]ZPT/kg is shown in Fig. 2; not all the blood samples assayed are represented. A computer programme, AUTOAN and an IBM 360/67 computer were used to determine the equation that best fitted each set of points (Sedman & Wagner, 1975). The blood concentration (C_b) and time (t) data were fitted to the equation $C_b = Ae^{-\alpha t} \pm Be^{-\beta t} \pm Ce^{-\gamma t}$ by non-linear regression using the computer and the values of A, α, B, β, C and γ were obtained from the analysis. Estimates of the mean and standard deviation of various rate constants using these equations are represented in Table 1. The blood data for the male and the female rats given 0·5, 1·25 or 12·5 mg ZPT/kg were described by a two-compartment open-system model. The existence of the rapid phase of elimination (α) from the central compartment in the two-compartment open model was definite, but the available data did not permit a reasonable estimation. The absorptive phase (*k*a) indicated that as the dose in mg/kg increased the rate constant (hr^{-1}) decreased in both male and female rats, and showed that for both sexes a shoulder was present, observed as an increase in ^{14}C blood concentrations followed first by a decrease and then by an even greater increase (the peak of this shoulder occurring approximately 30–45 min after dosing). For the males and females given the 0·5 mg/kg dose, the peak of this shoulder was the t_{max} followed by a dip, a small increase and then a final decline in ^{14}C blood concentrations. The initial part of this pattern was reversed for the 1·25 and 12·5 mg/kg dose. The shoulder may be a function of a first-pass effect due to metabolism by the liver.

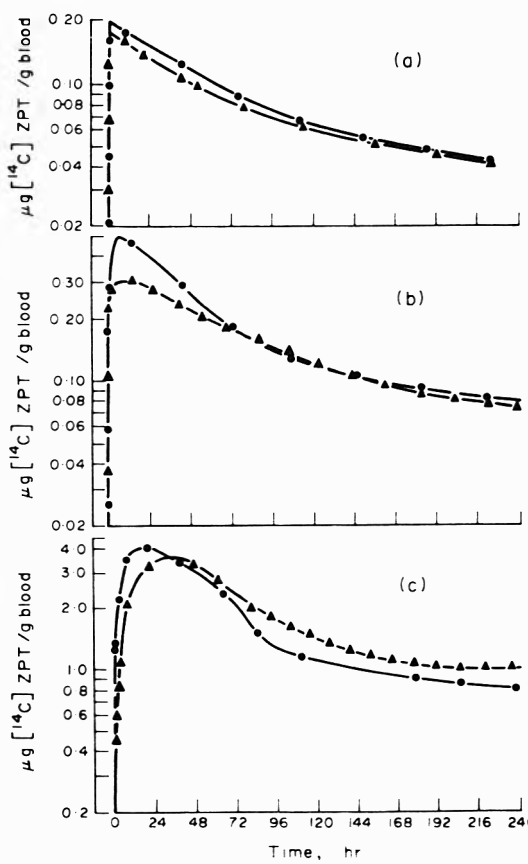

Fig. 2. Blood profiles of male (▲) and female (●) rats given an oral dose of (a) 0·5, (b) 1·25 or (c) 12·5 mg ZPT/kg including 15 μCi [2,6-^{14}C]ZPT/kg.

Table 1. *Pharmacokinetic parameters for [2,6-^{14}C]ZPT distribution and elimination in male and female rats following oral intubation*

Parameter	Values* for rats given labelled ZPT in a dose (mg/kg) of					
	0·50		1·25		12·5	
	Males	Females	Males	Females	Males	Females
Rate constants						
k_a (absorption; hr^{-1})	6·460 ± 1·327	8·501 ± 1·937	0·114 ± 0·166	5·999 ± 1·027	0·074 ± 0·015	0·191 ± 0·003
k_{12} (distribution; hr^{-1})	0·003 ± 0·002	1·235 ± 0·240	5·582 ± 0·878	0·005 ± 0·002	4·677 ± 1·968	9·452 ± 4·882
k_{21} (distribution; hr^{-1})	0·013 ± 0·006	1·820 ± 0·193	0·134 ± 0·193	0·008 ± 0·002	0·294 ± 0·079	0·710 ± 0·201
k_{el} (elimination; hr^{-1})	0·006 ± 0·001	0·013 ± 0·001	0·315 ± 0·511	0·007 ± 0·002	0·142 ± 0·058	0·153 ± 0·071
$t^{1/2}$ (β) urine (hr)	37·87 ± 11·51	41·70 ± 12·00	38·90 ± 1·20	39·23 ± 5·41	38·57 ± 2·14	29·06 ± 10·51
(β) blood (hr)	115·50 ± 0·00	117·70 ± 16·24	112·91 ± 12·84	142·45 ± 23·73	115·50 ± 0·00	141·16 ± 65·43
Clearance (ml/min)						
Cl_R (renal)	0·123 ± 0·001	0·075 ± 0·005	0·143 ± 0·005	0·065 ± 0·004	0·120 ± 0·001	0·052 ± 0·012
Blood						
AUC (μg/ml/hr)	27·01 ± 4·19	23·27 ± 2·50	59·09 ± 12·24	60·45 ± 8·64	566·83 ± 42·01	680·52 ± 230·23
t_{max} (hr)	1·07	0·30	11·96	1·11	29·12	13·26
b_{max} (μg/ml)	0·16	0·21	0·28	0·44	3·03	3·51

ZPT = Zinc pyrithione AUC = Area under blood curve

*Means or means ± SD for groups of three rats each given a single dose of technical ZPT containing 15 μCi [^{14}C]ZPT/kg. The ^{14}C measurements were made over 0–240 hr. The data are described by a two-compartment open system model:

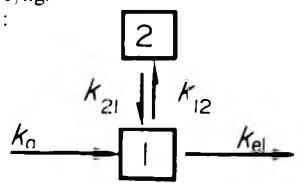

Table 2. *Excretion routes and recovery of ^{14}C from rats given a single oral dose of [2,6-^{14}C]ZPT*

	Values (% of ^{14}C administered*) for rats given labelled ZPT in a dose (mg/kg) of					
	0·50		1·25		12·5	
Tissues/excreta	Males	Females	Males	Females	Males	Females
Urine†	76·27 ± 2·49	84·20 ± 1·84	77·01 ± 0·97	76·55 ± 4·66	82·35 ± 2·68	74·27 ± 3·78
Faeces†	20·29 ± 2·26	16·11 ± 5·02	19·71 ± 2·51	20·13 ± 3·39	13·91 ± 2·94	21·81 ± 2·79
Cage washings	0·73 ± 0·19	0·25 ± 0·06	0·39 ± 0·24	0·27 ± 0·10	0·34 ± 0·14	0·44 ± 0·07
Carcass	0·839 ± 0·189	0·579 ± 0·073	0·780 ± 0·078	0·515 ± 0·068	0·647 ± 0·056	0·655 ± 0·076
Tissues						
Total‡	0·136 ± 0·017	0·112 ± 0·007	0·125 ± 0·011	0·105 ± 0·014	0·144 ± 0·025	0·109 ± 0·002
Kidney§	0·017 ± 0·000	0·013 ± 0·001	0·015 ± 0·000	0·012 ± 0·002	0·014 ± 0·000	0·015 ± 0·000
Liver§	0·079 ± 0·016	0·054 ± 0·004	0·073 ± 0·008	0·048 ± 0·007	0·090 ± 0·019	0·056 ± 0·004
Lung§	0·009 ± 0·001	0·011 ± 0·002	0·008 ± 0·001	0·009 ± 0·002	0·001 ± 0·001	0·008 ± 0·001
Total recovery....	98·28 ± 2·47	101·25 ± 5·13	98·03 ± 1·55	97·58 ± 1·27	97·39 ± 0·69	97·29 ± 0·85

ZPT = Zinc pyrithione

*Means ± SD for groups of three rats killed 11 days after administration of a single dose of technical ZPT containing 15 μCi [^{14}C]ZPT/kg.

†Collected for 11 days after treatment.

‡Includes thyroid, spleen, kidney, liver, lung, heart, adrenals, pancreas, urinary bladder, brain, skeletal muscle, bone marrow, ovaries, uterus, testes, stomach, duodenum, jejunum, ileum, colon and caecum.

§These organs, the only ones containing more than 0·009% of the dose, are included in total tissues.

The time required for the maximum blood level (t_{max}) to be reached was obtained by taking the first derivative of the blood curve equation, setting it equal to 0 and then solving for t, using an iterative non-linear least squares programme (Wagner, 1975). For all three dose levels, the t_{max} was consistently lower in the females than in the males given equivalent doses. For each sex, the t_{max} increased significantly as the dose increased. The maximum blood concentration (b_{max}) was obtained by placing the t_{max} value in the exponential blood equation and solving for C_b. The b_{max} of radioactivity in the blood of female rats was consistently greater than that of male rats for equivalent doses. The area under the blood curve (AUC) in terms of μg/ml × hr from 0 to ∞ was calculated (Wagner, 1975) by:

$$AUC_{0\to\infty} = A/\alpha + B/\beta + \ldots.$$

The AUC (μg/ml × hr) did not differ significantly between males and females given the same dose (Table 1).

Mean renal clearance (Cl_R) was calculated, to determine how the kidney handles ZPT and /or its metabolites, by dividing the total amount of ^{14}C (in μg) excreted in the urine by the area under the blood curve during that interval (Wagner, 1975). The Cl_R showed a significant difference between males and females at each dose level of ZPT (Table 1).

Balance study

Table 2 shows the excretion of ^{14}C activity (expressed as a percentage of the total dose) over 11 days in the urine and faeces of males and females given a single oral dose of 0·5, 1·25 or 12·5 mg ZPT/kg. The major route of excretion was via the urine and most of the radioactivity was excreted within the first 48 hr of administration. The ^{14}C excretion pattern in the faeces suggest enterohepatic circulation, since faecal ^{14}C elimination appeared to be slow and steady rather than in a large bolus within 24 hr of administration. There was significantly more faecal excretion of ^{14}C by the females (21·8%) than by the males (13·9%) at the highest dose level.

The urinary excretion rate was calculated using a nonlinear least squares regression equation and the coordinates dU/dt *v.* time. No significant difference in the rate of ^{14}C urinary excretion (hr^{-1}) was noted between male and female rats at the dose levels administered. A summary of the total recovery of radioactivity from groups of male or female rats given a single oral dose of 0·5, 1·25 or 12·5 mg ZPT/kg is shown in Table 2. The mean total recovery ±SD for all animals was 98·3 ± 2·9% of the dose. No target tissue was found in the rat.

Plasma-protein and RBC binding

Reversible binding of ZPT to plasma protein and/or RBCs can affect the compound's movement across tissues and its excretion. Dialysis studies conducted at equilibrium indicated that the minimum and maximum plasma-protein binding of ^{14}C from the 0·5 and 12·5 mg/kg doses was 5·9 and 12·2%, respectively. If a significant amount of ^{14}C binds to the RBC, this could be called a compartment, as was demonstrated by Hinderling, Bres & Garrett (1974) for a metabolite of disopyramide. The RBC/plasma ^{14}C ratio was approximately 5:1 at 96 hr after dosing and increased to ≥45:1 at 240 hr after dosing (Table 3). This indicates a marked affinity for the RBC as plasma levels decline and indicates that the RBC can be denoted as a compartment or subcompartment.

Urinary metabolites

HPLC analysis of the ^{14}C spots from TLC of the urine samples indicated one major and several minor components at R_F 0·09 and 0·30, and four minor components at R_F 0·20. Analysis of the HPLC and TLC eluates by CI–MS and EI–GC/MS demonstrated the presence of three urinary metabolites (Fig. 3), identified as 2-MPO (minor; R_F 0·09) and the *S*-glucuronides (free acid R_F 0·09 and the ethyl ester R_F 0·30) of 2-MPO (major). The spot with an R_F of 0·20 contained minor amounts of the *S*-glucuronides. Some ^{14}C-labelled material with an R_F of 0·09 or 0·20 could not be identified by mass spectroscopy because of the

Table 3. *RBC and plasma concentrations of ^{14}C following oral intubation of rats with [2,6-^{14}C]ZPT*

Dose* (mg/kg)	RBC/plasma distribution of ^{14}C at 96 hr: ^{14}C (% of dose × 10^2/ml) in RBC†	Plasma†	Mean RBC/plasma ratio‡	240 hr: ^{14}C (% of dose × 10^2/ml) in RBC†	Plasma†	Mean RBC/plasma ratio‡
			Males			
0·50	2·34	0·37	6·32	4·52	0·10	45·20
1·25	2·65	0·41	6·46	3·63	0·06	60·50
12·50	3·01	0·97	3·09	5·14	0·09	57·11
			Females			
0·50	3·04	0·64	4·75	6·81	0·15	45·40
1·25	4·37	0·79	5·53	5·98	0·09	66·44
12·50	4·46	1·21	3·68	5·91	0·13	45·46

ZPT = Zinc pyrithione

* Technical ZPT containing 15 μCi [^{14}C]ZPT/kg.
† Values × 10^{-2} are means for groups of three rats.
‡ Calculated on the basis of % of dose/g tissue.

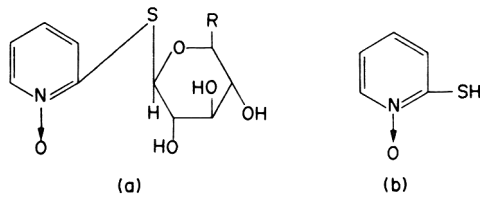

Fig. 3. Urinary metabolites of zinc pyrithione: (a) the *S*-glucuronide of 2-mercaptopyridine-*N*-oxide as the free acid (R = COOH) and ethyl ester (R = $COOC_2H_5$) and (b) 2-mercaptopyridine-*N*-oxide.

presence of contaminants. The quantity of the *S*-glucuronide metabolite differed between the male and female urines, the males excreting more of the *S*-glucuronides than the females. The quantity of 2-MPO excreted in the urine was difficult to determine because of its extreme instability. The 2-MPO *S*-glucuronides—the lactone and methyl and butyl esters, as well as the free acid (C. Mitoma and J. H. Wedig, unpublished observation 1977)—have been identified by mass spectroscopy as urinary metabolites in swine given iv doses of ZPT (Wedig, Mitoma, Howd & Thomas, 1978) and indirectly, by ultraviolet absorption, in the urine from rabbits given an iv dose of sodium pyrithione (Kabacoff, Fairchild & Burnett, 1971).

DISCUSSION

The results of this study have indicated differences in the pharmacokinetics between male and female rats given [^{14}C]ZPT orally. As the dose was increased from 0·5 to 1·25 and then to 12·5 mg/kg, the hourly rate of absorption for the males decreased more rapidly than that for the females as measured by ^{14}C appearance in the blood. Detailed examination of the blood curves revealed a shoulder during the absorptive phase which suggested a first-pass effect in the liver. When the ^{14}C concentration in the liver of the males was compared with that of the females 12, 16, 96 and 240 hr after administration of a dose of 12·5 mg/kg, a significantly greater percentage (approximately twice as high) was found in the males. ZPT has been shown to pass into the enterohepatic circulation in the rat following oral dosing (Ziller, 1977). Our findings suggest that a significantly greater percentage of the dose is retained by the liver (and possibly undergoes enterohepatic circulation) in the male. This would correlate with the longer time (t_{max}) required to reach the maximum blood level of ^{14}C in the males and with the fact that this maximum was consistently lower in the males than females, the hepatic retention and/or enterohepatic circulation resulting in a high portal/peripheral plasma gradient.

The significant amount of ^{14}C binding to the RBCs is one reason for the long blood half-life (β) and also the significant reduction noted in renal clearance. The amount of ^{14}C bound to RBCs was greater in the female than in the male at each dose level. Possibly, the *S*-glucuronide link at the thiol site on the pyridine-*N*-oxide blocks the active thiol site on this molecule for binding to the RBC. The mean renal clearance (Cl_R) differed significantly between males (0·129 ml/min) and females (0·065 ml/min) at all dose levels. In both sexes the Cl_R of ^{14}C was significantly lower than the normal glomerular filtration rate in the rat of 1·2 ml/200 g body weight/min (Renkin & Gilmore, 1973), being only about 10 and 5% of this figure in the males and females respectively. Thus, ^{14}C was either extensively reabsorbed by the kidney tubule or a large fraction was strongly bound to the RBC so that it was not filtered. Evidence supports RBC binding since the RBC/plasma ratio for ^{14}C binding was large at 96 hr and was even greater at 240 hr dosing.

Retention of ^{14}C in the blood in preference to uptake by tissues other than the liver appears to be associated with a tenacious binding of ^{14}C to the RBC. When concentrations of ^{14}C in plasma are very low, RBC binding appears relatively greater, possibly because of preferential binding to the more limited but higher affinity sites, with a consequent reduction in renal clearance. Thus, RBC binding as well as retention by the liver may be the cause of the excretion in the urine of small amounts of ^{14}C with a very long half-life. It must be emphasized that the major part of the dose was eliminated rapidly, and only a small fraction remained when the slower phase of elimination was observed.

The quantities of 2-MPO (a very unstable metabolite) in the urines of male and female rats were estimated to be approximately equal. To our knowledge, this is the first published report demonstrating the isolation and positive confirmation of 2-MPO as a metabolite following the administration of this class of pyridine-2-thiol-1-oxide compounds to animals. However, the fact that 2-MPO is very unstable presented a major difficulty in its identification. Upon exposure to ultraviolet (UV) light, especially when dissolved in an organic solvent, 2-MPO rapidly undergoes a UV-catalysed oxidative dimerization (Fig. 4) to 2,2′-(pyridyl-*N*-oxide) disulphide and then to the stable 2,2′-pyridyl disulphide and sulphur dioxide (Olin Corporation, 1960). 2-MPO appears to be more stable in urine than when the pure compound is dissolved in an organic solvent. This phenomenon has been demonstrated for drug metabolites such as those from phenylbutazone.

Since a difference in pharmacotoxicity (skeletal muscle weakness) was noted between male and female rats at a dietary level of 25 ppm (equivalent to a dose of 1·25 mg/kg/day) in a 2-yr feeding study (P. S. Larson, unpublished data 1958), the predicted maximum

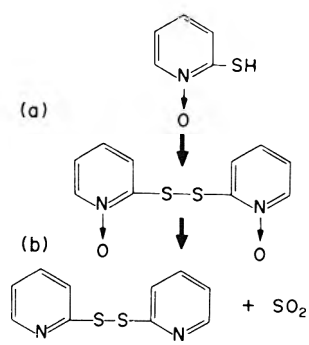

Fig. 4. UV oxidation of 2-mercaptopyridine-*N*-oxide, yielding (a) 2,2′-(pyridyl-*N*-oxide) disulphide and (b) 2,2′-pyridyl disulphide.

Table 4. *Predicted steady-state blood concentrations of ^{14}C in male and female rats after ingestion of [2,6-^{14}C]ZPT*

Parameter	Values for rats given labelled ZPT in a dose (mg/kg) of		
	0·5	1·25	12·5
Males			
C_x^{max} (μg ^{14}C/ml)	1·26	2·04	23·70
C_x^{min} (μg ^{14}C/ml)	1·09	1·45	22·37
t_{max} (hr)	0·66	0·87	5·80
Females			
C_x^{max} (μg ^{14}C/ml)	1·08	2·62	21·29
C_x^{min} (μg ^{14}C/ml)	0·84	2·28	19·04
t_{max} (hr)	0·29	0·86	5·70

ZPT = Zinc pyrithione

* Maximum and minimum steady-state concentrations of ^{14}C in blood calculated from data for groups of three rats, each given a dose of technical ZPT containing 15 μCi [^{14}C]ZPT/kg. and the time required for attainment of the maximum concentration.

and minimum ^{14}C steady-state blood levels after the nth dose were calculated (Wagner, 1975) in order to determine whether a difference existed. The minimum steady-state blood level $C_x^{min.}$ was calculated using the following equation: $C_x^{min.} = Ae^{-\alpha\psi}/1 - e^{-\alpha\psi} \pm Be^{-\beta\psi}/1 - e^{-\beta\psi} \pm \ldots$, where A and B are the coefficients and α and β the exponents from the exponential equation for the blood curves from a single oral dose and ψ is the dosing interval, 24 hr. The maximum steady-state blood concentration was calculated with the following equation:

$$C_x^{max} = Ae^{-\alpha t_{max}}/1 - e^{\alpha\psi} \pm Be^{-\beta t_{max}}/1 - e^{-\beta\psi} \pm \ldots$$

where t_{max} is the time of the maximum concentration at steady state (Wagner, 1975). Table 4 shows the results of these calculations. Following doses of 1·25 mg/kg/day, the steady-state ^{14}C blood concentration in the female would be higher than that in the male assuming that no hepatic enzyme induction occurred.

If one were to administer smaller doses six times a day, the maximum and minimum steady-state ^{14}C concentrations should approach an average level about midway between the 24-hr dosing interval, i.e. the range should decrease as the dose is being distributed more evenly over 24 hr. This would result in an average steady-state ^{14}C blood level in the male of 1·74 μg/ml (a no-effect level) and a ^{14}C blood level of 2·45 μg/ml (an effect level) in the female at a dietary concentration of 25 ppm ZPT. The data viewed in this manner resemble more closely the actual food-consumption pattern of the rat.

This projected divergence in ^{14}C blood concentrations, in conjunction with a greater metabolic activity in the male, may be considered to be an explanation for the sex difference in the levels eliciting the pharmacotoxic sign of skeletal muscle weakness observed in the rat.

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EFFECT OF NITRILOTRIACETATE (NTA) ON CATION BALANCE IN THE RAT

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Abstract—Weanling rats were fed various forms of nitrilotriacetate (NTA) to ascertain whether the cations associated with NTA influence renal toxicity expressed as kidney/body weight ratio. The effect of NTA on cation balance after 4 wk of NTA ingestion was also determined. The results demonstrate that the renal toxicity caused by trisodium NTA ($Na_3NTA.H_2O$) ingestion cannot be attributed to the increased Na intake alone since an equivalent intake of Na as sodium acetate did not cause comparable renal toxicity. Ingestion of the mixed K and Na salts of NTA caused as great an increase in kidney/body weight ratio as $Na_3NTA.H_2O$ showing that the renal effect is not a result of monovalent cation imbalance. Further, the increased kidney/body weight ratio is not a simple function of systemic load of NTA since H_3NTA and $Na_3NTA.H_2O$ resulted in the same urinary output of NTA but the $Na_3NTA.H_2O$ caused a much greater increase in kidney/body weight ratio than H_3NTA. All forms of NTA studied (except Zn(K/Na)NTA) increased the faecal excretion of the major monovalent (Na and K) and divalent (Ca and Mg) cations but reduced faecal Zn. The only cation that displayed an NTA-associated decrease in apparent carcass retention during a 3-day balance period was Mg. Weight gain was proportional to and kidney/body weight ratio inversely proportional to carcass Mg retention.

INTRODUCTION

Nitrilotriacetate (NTA) is nephrotoxic to rats, and its nephrotoxicity is a function of the quantity ingested (Mahaffey & Goyer, 1972; Nixon, 1971; Nixon, Buehler & Niewenhuis, 1972). When it is fed at extreme levels (0·75–2% of the diet) it is associated with neoplasms of the urinary tract (National Cancer Institute, 1976).

The nephrotoxicity of NTA has been described as a "hydropic degeneration" (Nixon *et al.* 1972) and is easily characterized by an increase in the kidney/body weight ratio (g/100 g). A detailed description of the morphological changes induced by NTA after short-term ingestion will be the subject of a separate report.

This nephrotoxicity may result from physical changes that occur when high concentrations of NTA are being processed, or it may result from NTA's ability to sequester metals and make them unavailable for essential biological activities. The nephrotoxicity cannot be attributed to any metabolic product of NTA, for it is absorbed from the gastro-intestinal tract and excreted in the urine without undergoing any chemical change beyond the possible replacement of the metal ions with which it forms salts or chelates (Budny, 1972; Budny & Arnold, 1973; Budny, Niewenhuis, Buehler & Goldenthal, 1973; Michael & Wakim, 1971). The nephrotoxicity cannot be attributed to an interaction of NTA with genetic material for NTA is neither teratogenic (Nolen, Buhler, Geil & Goldenthal, 1972; Nolen, Klusman, Back & Buehler, 1971a; Scharpf, Hill, Wright, Plank, Keplinger, Moreno & Calandra, 1972; Tjälve, 1972) nor mutagenic (Kramers, 1976).

The study described here was undertaken to determine whether the biological response to NTA is influenced by the cation associated with it, with particular attention being paid to Zn, since NTA ingestion markedly increases the excretion of Zn in the urine (Budny *et al.* 1973; Michael & Wakim, 1971; Nixon *et al.* 1972). The effect of NTA ingestion on cation balance was also determined.

EXPERIMENTAL

For 5 wk, groups of seven or ten weanling, male, Charles River rats were housed individually and fed *ad lib.* with diets consisting of ground Purina Laboratory Chow or of ground Chow to which a supplement had been added. One of the following five supplements was used: 2% $Na_3NTA.H_2O$, 1·5% H_3NTA, 1·5% $ZnKNTA.H_2O$ + 0·8% $ZnNaNTA.H_2O$, 1·5% $K_3NTA.H_2O$ + 0·8% $Na_3NTA.H_2O$ or 2% sodium acetate. Each supplement was added at a concentration to provide a dosage of NTA equivalent to 2% $Na_3NTA.H_2O$. The mixed Na and K salts were added to yield the same K/Na ratio as in Chow. The rats were allowed distilled water *ad lib.* Feed consumption and body weights were recorded each week.

All the urine voided by the animals during 4 days of each week was collected under toluene and its volume was recorded. It was assayed with Multistix (Ames Company, Elkhart, Illinois) for haemoglobin, glucose and protein. The pH was measured on a pH meter.

During wk 4 five animals of each group were fitted with anal cups. The animals were given 2 days to adjust to the cups, then their total urine and faeces were collected separately for 3 days. The faeces were weighed before and after drying in a vacuum oven at approx. 30°C. The urine was analysed for NTA by isotope dilution (Budny *et al.* 1973). The urine, the dried faeces and samples of each diet were analysed by atomic absorption spectrometry for Ca, Cu, Zn, Fe, Na, K, Mg and Mn.

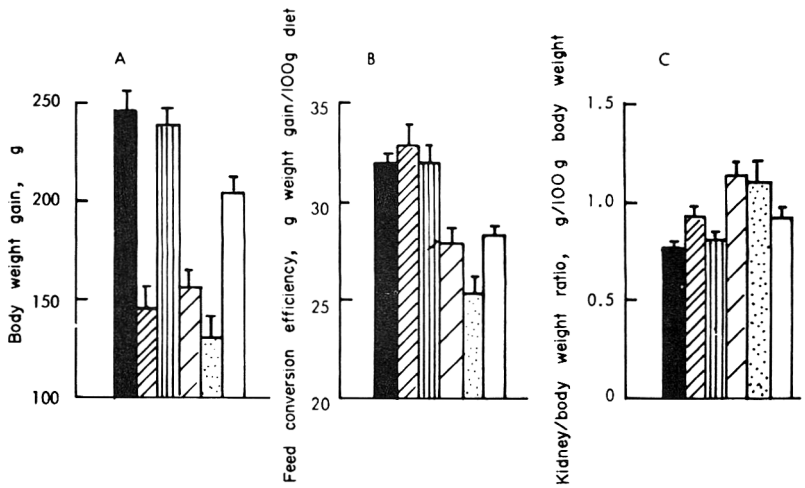

Fig. 1. Effect of dietary supplements of various forms of NTA and of sodium acetate on (A) weight gain, (B) feed efficiency and (C) kidney/body weight ratios of rats during a 5-wk *ad lib.* feeding period. The initial weight of the animals was 66 ± 1 g. Each value is the mean ± SEM for the number of animals indicated in brackets. Control, ■ (7 in A and B, 4 in C); H_3NTA, ▨ (9 in A and B, 6 in C); Na acetate, ▥ (9 in A and B, 6 in C); Na_3NTA ▨ (10 in A and B, 7 in C); K/Na NTA, ▦ (8 in A and B, 7 in C); Zn(K/Na)NTA, □ (10 in A and B, 7 in C).

After 5 wk, the animals were anaesthetized with Nembutal (50 mg/kg), and then both kidneys were excised and weighed.

RESULTS

Growth and feed efficiency

All of the NTA supplements reduced the animals' growth (Fig. 1A), but the zinc chelate reduced growth less than the other forms of NTA. Sodium acetate did not reduce growth. The growth reduction was caused by NTA ingestion rather than by excessive monovalent cation consumption, since H_3NTA reduced growth but sodium acetate did not.

The growth reduction was a consequence of both reduced food intake and less efficient food use (Fig. 1B), except in the case of H_3NTA, which did not reduce feed conversion-efficiency. Since some of the animals scattered and lost some of their feed during the early days of the study, the reduced feed conversion efficiency cannot be considered a very reliable finding.

All of the supplements containing NTA led to increases in the kidney/body weight ratio, a greater effect being produced by the Na and mixed K/Na salts than by H_3NTA or Zn(K/Na)NTA (Fig. 1C).

Urine quantities and analyses

Table 1 characterizes the urine collected during the feeding phase of the study. Each value is the mean for all samples collected before anal cups were used. All of the supplements that contained high levels of Na or K increased the pH of the urine, while H_3NTA made the urine more acidic. The Zn complex of NTA, a neutral material, did not affect urinary pH.

None of the treatments caused a detectable proteinuria greater than that seen in the Chow controls. All of the supplements, including sodium acetate, pro-

Table 1. *Analyses of urine from rats consuming diets supplemented with the equivalent of 2% $Na_3NTA.H_2O$ for 28 days*

Dietary treatment	No. of observations	pH of urine*	Haemoglobinuria		Glucosuria	
			Max % of animals with haemoglobinurea	Time to first detection of Hb (days)	Max % of animals with glucosuria	Time to first detection of glucose (days)
Control	84	7·0 ± 0·3	0	—	0	—
$Na_3NTA·H_2O$	120	7·7 ± 0·1	80	5	8	9
H_3NTA	108	6·2 ± 0·1	22	12	8	11
K/NaNTA	120	7·6 ± 0·1	90	4	9	18
Zn(K/Na)NTA	120	7·1 ± 0·2	50	9	0	—
NaAc	108	8·0 ± 0·1	30	17	0	0

Hb = Haemoglobin

*Mean ± SD for days 4–23 of the study. Observations on 12 separate 24 hr collections from each animal on each treatment.

Table 2. *Concentration of NTA in the urine of rats fed various forms of NTA*

Dietary treatment	Urinary NTA*		
	μmol/100 g/day	μmol/ml	% of dose
Control	0	0	—
H_3NTA	292 ± 34	42·4 ± 7·3	38 ± 5
$Na_3NTA·H_2O$	284 ± 62	26·9 ± 5·6	39 ± 8
Zn(K/Na)NTA	104 ± 27	15·3 ± 6·3	15 ± 3

*Mean ± SEM of five urine samples/treatment. Urine was collected during the fourth week of ingestion of diets containing various forms of NTA.

duced some haemoglobinuria. It appeared earliest and was most severe in animals that received the Na and mixed K/Na salts of NTA. All of the dietary forms of NTA except Zn(K/Na)NTA caused sporadic glucosuria, but this response was not consistent.

Urine collected during wk 4 from animals fitted with anal cups was analysed for NTA (Table 2). The control animals excreted no NTA. Animals consuming either $Na_3NTA.H_2O$ or H_5NTA excreted the same amount of NTA in their urines, but supplementation with Zn(K/Na)NTA halved the output. Thus the quantity of NTA excreted in the urine is not a simple function of the dietary concentration of NTA but markedly influenced by the associated cation.

Balance study

The separate collections of urine and faeces during wk 4 of the study made it possible to determine the effect(s) of high Na and NTA ingestion on the disposition of ingested cations. The carcass retention values are calculated as [intake − (urinary + faecal)] and thus are only apparent values, since they are not corrected for losses of cations by routes other than urine and faeces; e.g. sloughed skin and hair.

Figure 2 summarizes the animal responses during this period. Body-weight changes were erratic and did not reflect feed intake, which was fairly even among the groups (Figs 2A and 2B).

Faecal samples collected in the anal cups of rats ingesting all forms of NTA were moist, a phenomenon not noted during normal housing conditions. For this reason the faeces from all animals were dried in a vacuum oven before cation analyses. The water content of faeces from animals consuming NTA was two to three times greater than for control animals or those fed with sodium acetate (Fig. 2C).

The disposition of Cu, Fe and Mn was not appreciably influenced by NTA ingestion except that the treatments that caused haematuria showed an increase in urinary Fe. Further, the measurements of apparent carcass retention showed that the animals were all essentially at equilibrium for these cations (intake ≈ excreta output). For these reasons these cations will not be discussed further.

All forms of NTA increased faecal Na at least twofold but sodium acetate did not (Fig. 3). Urinary Na levels were proportional to Na ingestion except that Zn(K/Na)NTA ingestion resulted in a lower urinary Na level than that of Chow or H_3NTA. Carcass Na retention like the urine level was proportional to Na ingestion but H_3NTA resulted in Na retention more than twice that of the control group ingesting approximately the same amount of Na.

NTA ingestion with or without K resulted in increased faecal K levels (Fig. 4) but the K increases were not as great as those seen with Na. Urinary K was elevated only in the animals ingesting the K_3/Na_3 NTA mixed salts. The K retained in the carcass was elevated by H_3NTA ingestion and reduced by $Na_3NTA.H_2O$ ingestion but the other treatments had no effect.

Faecal Ca output was increased in the animals fed Zn(K/Na)NTA compared to the other groups (Fig. 5). In contrast, urinary Ca was increased in animals with high urinary NTA levels ($Na_3NTA.H_2O$ and H_3NTA) but not influenced by low urinary NTA

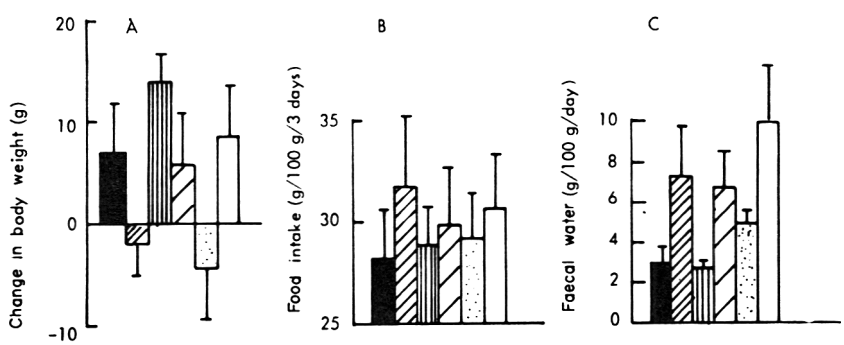

Fig. 2. Effect of dietary supplements of various forms of NTA and of sodium acetate on (A) weight gain, (B) food intake and (C) faecal moisture in rats during a 3-day balance study. Each value is the mean ± SEM for the number of animals in brackets. Control. ■ (5); H_3NTA ▨ (4); Na acetate. ▥ (4); Na_3NTA. ▨ (5); K/NaNTA. ▦ (4); Zn(K/Na)NTA. □ (5).

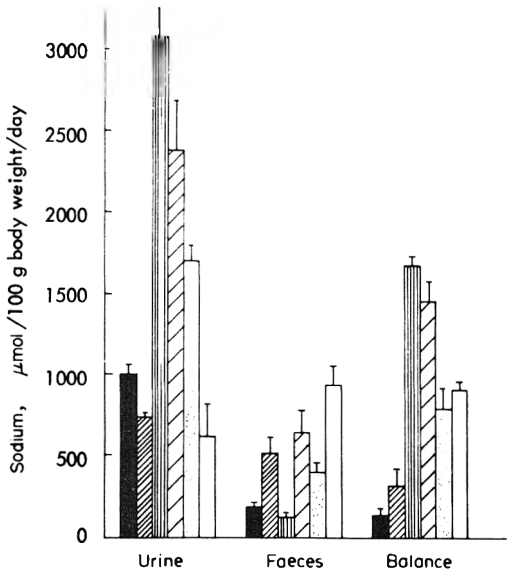

Fig. 3. Effect of dietary supplements of various forms of NTA and of sodium acetate on sodium disposition. Values are means ± SEM for four or five rats per treatment. Control. ■: H_3NTA. ▨: Na acetate. ▥: Na_3NTA. ▨: K/NaNTA. ▦: Zn(K/Na)NTA. □.

[Zn(K/Na)NTA] or high urinary Na (sodium acetate). Ca retention in the carcass was lower with diets high in either Na or Na and K and the presence of NTA in the diet had little effect on this response. Zn(K/Na)NTA resulted in the lowest Ca balance.

Mg disposition appeared to be influenced more by NTA ingestion than by high Na or K intake (Fig. 6). All forms of NTA increased faecal Mg and reduced both urinary Mg and carcass retention of Mg. The reduced carcass retention of Mg associated with NTA ingestion ranged from a 60% reduction with 2% $Na_3NTA.H_2O$ to a 40% reduction with 1·5% H_3NTA compared to the Chow controls.

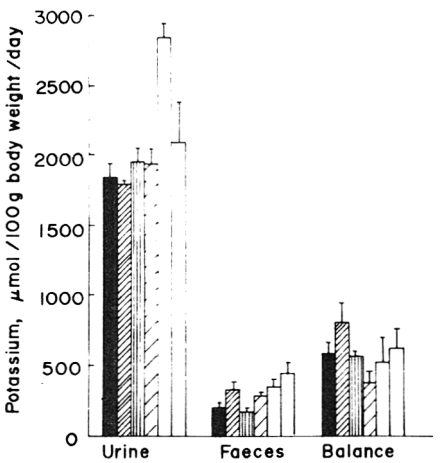

Fig. 4. Effect of dietary supplements of various forms of NTA and of sodium acetate on potassium disposition. Values are means ± SEM for four or five rats per treatment. Control. ■: H_3NTA. ▨: Na acetate. ▥: Na_3NTA. ▨: K/Na NTA. ▦: Zn(K/Na)NTA. □.

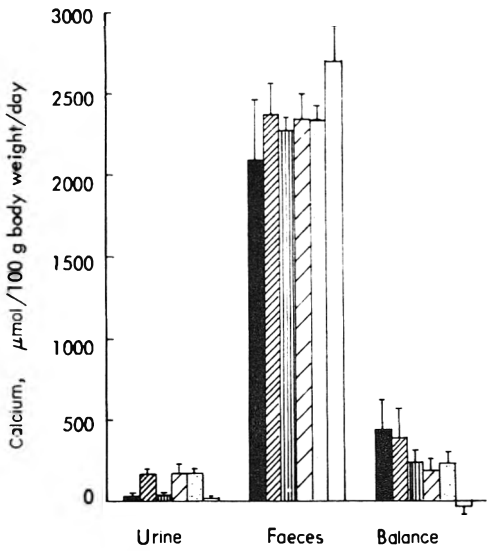

Fig. 5. Effect of dietary supplements of various forms of NTA and of sodium acetate on calcium disposition. Values are means ± SEM for four or five rats per treatment. Control. ■: H_3NTA. ▨: Na acetate. ▥: Na_3NTA. ▨: K/NA NTA. ▦: Zn(K/Na)NTA. □.

Finally, the effects of NTA on Zn disposition are presented in Table 3. In confirmation of previous work (Michael & Wakim, 1973; Nixon *et al.* 1972), NTA ingestion resulted in a marked increase in urinary Zn. The increased urinary Zn was compensated for by decreased faecal Zn, and NTA did not influence the apparent carcass retention of Zn except when the Zn complex was fed. Ingestion of the Zn complex of NTA resulted in a massive increase in carcass retention of Zn but only a doubling of urinary Zn relative to NTA feeding without Zn supplementation.

DISCUSSION

The major objective of this experiment was to learn whether ingestion of extreme doses of NTA causes alterations in cation balance that could be associated

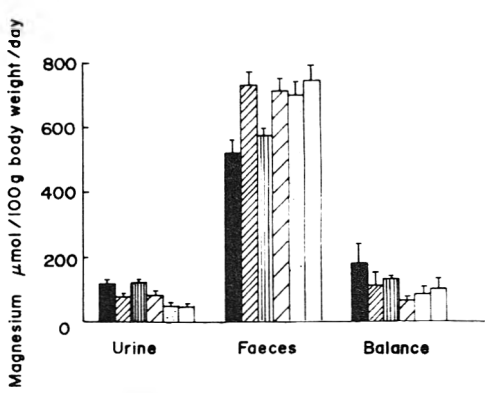

Fig. 6. Effect of dietary supplements of various forms of NTA and of sodium acetate on magnesium disposition. Values are means ± SEM for four or five rats per treatment. Control. ■: H_3NTA. ▨: Na acetate. ▥: Na_3NTA. ▨: K/NA NTA. ▦: Zn(K/Na)NTA. □.

Table 3. *Effect of NTA ingestion on Zn balance*

Dietary treatment	Zinc (μmol/100 g body weight/day)		
	Urine	Faeces	Balance retained by carcass*
Control	0·2 ± 0	8·6 ± 0·5	−1·0 ± 0·7
H_3NTA	3·3 ± 0·1	5·7 ± 0·8	−0·3 ± 0·5
NaAc	0·1 ± 0	8·4 ± 0·2	−0·6 ± 0·2
Na_3NTA	3·0 ± 0·2	6·0 ± 0·8	−1·2 ± 0·6
K/Na NTA	3·3 ± 0·2	5·5 ± 0·4	−0·9 ± 0·4
Zn(K/Na)NTA	6·9 ± 2·9	635 ± 61	64 ± 31

*Zn intake in food − (urinary + faecal) losses.

with renal toxicity. The test included several secondary objectives and these will be considered individually.

Since the ingestion of 2% $Na_3NTA.H_2O$ results in a greater than threefold increase in Na intake (Chow, 1·3 mmol Na/100 g body weight/day; 2% $Na_3NTA.H_2O$, 4·5 mmol Na/100 g body weight/day) it was desirable to ascertain the effect of ingestion of an equivalent load of Na without NTA (2% Na acetate, 4·8 mmol Na/100 g body weight/day). Increased Na load in the absence of NTA produced alkaline urine and increased volume, but did not cause such extensive haematuria as $Na_3NTA.H_2O$. Further, sodium acetate did not increase the kidney/body weight ratio. Since the apparent carcass retention of Na was comparable in the sodium acetate and $Na_3NTA.H_2O$ animals, the kidney toxicity associated with Na_3NTA ingestion cannot be attributable to Na retention.

The possibility that monovalent cation imbalance (change in K/Na ratio) was responsible for the nephrotoxicity is negated by the similarity in the response of the animals fed the mixed K/Na salts of NTA to those given the same level of $Na_3NTA.H_2O$ alone.

Ingestion of NTA resulted in some marked alterations in the K/Na ratios of the pool retained by the carcass during the three-day balance period (Table 4). It is difficult to attribute the toxic effects to this change, however, since sodium acetate caused as severe a depression in the K/Na ratio as any of the NTA treatments and yet produced no renal toxicity.

Since several experiments had demonstrated that NTA ingestion increased Zn excretion in the urine (Budny *et al.* 1973; Michael & Wakim, 1973; Nixon *et al.* 1972), the effect of NTA on Zn distribution was considered. The major effect of Zn supplementation was to reduce the urinary output of NTA, presumably by reducing its absorption. All forms of NTA produced an increase in urinary Zn whether the diet contained supplemental Zn or not. The results with Zn(K/Na)NTA showed that, even when animals retained massive amounts of Zn, urinary excretion of NTA did not effectively compete with the binding of Zn to the carcass.

Table 4. *Effect of NTA on the K/Na ratio in the pool retained by the rat carcass during the 3-day balance trial*

Dietary treatment	K/Na balance*
Control	4·3
H_3NTA	2·6
NaAc	0·3
Na_3NTA	0·3
K/Na NTA	0·7
Zn(K/Na)NTA	0·7

*Determined in the apparent pool retained in the carcass during the three-day mineral balance study.

Since the only significant reactivity of NTA is its ability to form stable complexes with divalent cations, it was important to learn the effects of this ligand on Ca and Mg balance. The results show that NTA ingestion caused reduced carcass retention of these two divalent cations, but for different reasons. The reduced carcass retention of Mg was a consequence of its increased faecal output, while the reduced retention of Ca was more a consequence of its increased urinary output. Zn(K/Na)NTA was an exception to this, in that its effect on Ca was associated with increased faecal output, not urinary load. This difference in ligand effect on Ca and Mg excretion may be the result of a difference in their intestinal transport (Wilkinson, 1976). That is, Ca absorption is a result of active transport by a specific protein which probably competes with NTA for Ca binding in the gut, while Mg absorption, which is primarily a passive process, cannot compete with NTA for Mg.

The reduction in Mg balance associated with NTA ingestion showed several significant correlations: the 5-wk weight gain was significantly correlated with Mg balance ($r = -0·83$, $P < 0·05$), the kidney/body weight ratio showed a significant negative correlation with Mg balance ($r = -0·88$, $P < 0·01$), the increase in faecal Mg was significantly correlated with faecal moisture ($r = 0·58$, $P < 0·01$) measured during the 3-day balance period, and finally, it was noted that NTA ingestion resulted in a significant increase in the Ca/Mg ratio in the minerals retained in the carcass (Ca/Mg with Chow or sodium acetate ingestion, 2·09 ± 0·39 v. NTA, 3·47 ± 0·81). it is well-documented that the Ca/Mg ratio can significantly alter metabolic pathways (Bygrave, 1976) but such an effect cannot be assessed from this experiment since we did not determine the Ca/Mg ratio in tissues. The relationship of Mg balance to NTA ingestion and renal toxicity is under further investigation.

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HYPERCALCINURIA AND CRYSTALLURIA DURING INGESTION OF DIETARY NITRILOTRIACETATE*

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Abstract—Weanling rats were maintained for 4 wk on rations containing various levels of nitrilotriacetate (NTA) as either H_3NTA or $Na_3NTA.H_2O$. Ingestion of more than 400 μmol/100 g body weight/day of either form of NTA caused dose-dependent increases in urinary Ca excretion. The urine formed by animals receiving these doses contained abundant minute crystals of CaNaNTA. Urine pH was increased by these high doses of $Na_3NTA.H_2O$ and decreased by H_3NTA. Dietary ingestion of 1000 μmol $Na_3NTA.H_2O$/100 g body weight/day caused marked haematuria.

INTRODUCTION

Trisodium nitrilotriacetate (NTA) has been proposed as a replacement for sodium tripolyphosphate in granular detergents. It is readily absorbed from the gut and is excreted in the urine without any biotransformation (Budny & Arnold, 1973; Budny, Niewenhuis, Buehler & Goldenthal, 1973; Michael & Wakim, 1971). When $Na_3NTA.H_2O$ is fed in laboratory chow at doses greater than 0·75% for 90 days, it induces kidney damage as assessed by an increase in kidney/body weight ratio (Nixon, 1971) and other criteria.

In chronic feeding studies (National Cancer Institute, 1977; Nixon, Buehler & Niewenhuis, 1972) diets containing less than 0·15% $Na_3NTA.H_2O$ did not induce any detectable kidney damage, but diets containing levels between 0·15 and 0·75% caused renal tubular damage described as "hydropic degeneration". Dietary levels of either $Na_3NTA.H_2O$ or H_3NTA ≥0·75% were associated with increased numbers of urinary-tract neoplasms (National Cancer Institute, 1977). The neoplasms in rats were predominantly tubular-cell carcinomas and transitional-cell carcinomas of the kidney, ureter and bladder. The yield of neoplasms did not attain statistical significance until the dietary concentration of NTA was 2·0% $Na_3NTA.H_2O$ or its molar equivalent, 1·5% H_3NTA.

Neoplasms associated with administration of NTA occur only in the urinary tract and only at extreme dietary levels, and NTA does not undergo metabolic transformation during passage through any of several mammalian species (Budny & Arnold, 1973; Budny *et al.* 1973; Michael & Wakim, 1971). NTA has given negative results in mutagenesis assays (Kramers, 1976; Stine & Hardigree, 1971). For these several reasons we initiated studies to determine the effects of the carcinogenic levels of NTA on the composition and characteristics of rat urine.

In a cation balance study (Anderson & Kanerva, 1978) it was noted that 2% $Na_3NTA.H_2O$ and 1·5% H_3NTA caused an approximately fivefold increase in urinary calcium output. The present report relates the concentration of NTA in the diet to the urinary output of calcium and to the precipitation of crystalline CaNaNTA in the urine.

EXPERIMENTAL

The $Na_3NTA.H_2O$ used in these studies was a commercial sample (supplied by Monsanto, St. Louis, MO) and the H_3NTA was prepared from it by HCl precipitation. Elemental and X-ray diffraction analyses were consistent with the structures. Weanling or 250-g Charles River rats were fed Ground Purina Laboratory Chow (Ralston Purina Co., St. Louis, MO), with or without NTA added either as $Na_3NTA.H_2O$ or as H_3NTA, *ad lib.* for 14 or 28 days. The NTA was mixed with ground Chow before feeding in concentrations of 0·5, 0·75 and 1·5% for both H_3NTA and $Na_3NTA.H_2O$ and 2% for $Na_3NTA.H_2O$, roughly corresponding to NTA ingestion levels of up to 1000 μmol/100 g body weight/day. The animals were housed individually in stainless-steel metabolism cages designed to separate urine and faeces and kept at a controlled temperature and humidity with a 12-hr light/dark cycle. In all studies the animals were given distilled water *ad lib.*

Urine analyses were conducted on 24-hr total urine collections. In some studies the urines were collected under toluene and in others they were not. The samples collected under toluene did not differ detectably from those that were exposed to air.

Urine volume and pH analyses were conducted on all samples. The urine samples from control and treated animals were centrifuged at about 700 *g* for 5 min on a Clay Adams Inc. (Parsippany, NJ), Dynac desk-top centrifuge. The insoluble material was washed with 2–3 ml 95% ethanol, air-dried overnight, and analysed by X-ray diffraction on a Rotaflex RU-100H High Intensity X-ray Generator (Rigaku, USA, Inc., Wakefield, MA). Some pooled urine samples were filtered through Eaton Dikeman Grade 617 filter paper under vacuum to remove feed particles before the insoluble products were harvested.

*Parts of this work were presented by R.L.A. to the Toxicology Forum, Aspen, CO, in July 1977 and by R.L.A. and R.L.K. to the Toxicology Forum, Washington, DC, in February 1978.

Urine Ca was measured using atomic absorption by a standard technique and urine NTA concentrations were determined by isotope dilution.

Weekly body-weight and feed-consumption records were maintained on all animals.

RESULTS

Figure 1 shows urines from female rats given Chow with 1·5% H_3NTA, Chow alone, or Chow with 2% $Na_3NTA.H_2O$. These urines were voided during wk 4 of ingestion of the test diets, but they did not appear to differ from urines voided after only 2–3 days on diet. The photograph shows two important characteristics: the urines from the animals consuming both forms of NTA contain considerable amounts of insoluble material and the urines voided by rats consuming a diet with 2% $Na_3NTA.H_2O$ contain easily visible amounts of haemoglobin. To show how much insoluble material these urines contained, they were photographed with back lighting (Fig. 2).

In a dose-response study it was shown that neither form of NTA at a dietary concentration of 0·5% produced visible insolubles, but at 0·75% both forms produced visible amounts of insoluble material. A dietary concentration of $Na_3NTA.H_2O$ of 1·5%, but not lower doses, resulted in visible haematuria. X-ray analyses showed that the insoluble material in the urine of rats fed NTA contained crystalline CaNaNTA. Control urine insolubles occasionally contained crystals of $MgNH_4PO_4$ but this material was not detected in the urine of animals fed NTA.

The effects of NTA on growth, urine pH and urine volume are summarized in Table 1. H_3NTA at 0·5% of the diet did not influence body weight or urine pH but did decrease the volume of urine. Higher doses decreased body weight and urine pH but had no further effect on urine volume. In contrast, all doses of $Na_3NTA.H_2O$ reduced growth and increased urine pH above that of the controls. Although $Na_3NTA.H_2O$ reduced the urine volume when fed at 0.5%, higher doses increased the volume.

Since both forms of dietary NTA induced the presence of crystalline CaNaNTA in the urine, the effect

Table 1. *Effect of concentration of H_3NTA and $Na_3NTA.H_2O$, on body weight and urine pH and volume, when ingested for 4 wk by female rats*

Treatment and dietary level (%)	Body weight* (g)	Urine	
		pH	Volume (ml 100 g day)
Control (13)	193 ± 3	7·1 ± 0·1	7·3 ± 0·4
H_3NTA			
0·5 (5)	193 ± 7	7·0 ± 0	5·2 ± 0·1
0·75 (5)	176 ± 11	6·8 ± 0·2	4·8 ± 0·5
1·5 (9)	158 ± 6	6·6 ± 0·1	5·6 ± 0·5
$Na_3NTA.H_2O$			
0·5 (5)	170 ± 9	7·6 ± 0·2	5·5 ± 0·4
0·75 (5)	188 ± 4	7·8 ± 0·1	7·8 ± 0·8
1·5 (5)	165 ± 11	7·8 ± 0·1	9·4 ± 1·5
2·0 (9)	140 ± 10	8·2 ± 0·3	11·1 ± 1·3

*Initial weight 59 ± 3 g.
Each value is the mean ± SEM for the number of animals indicated in brackets.

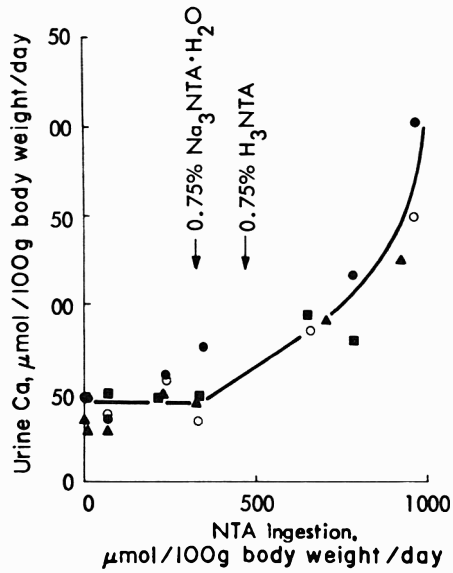


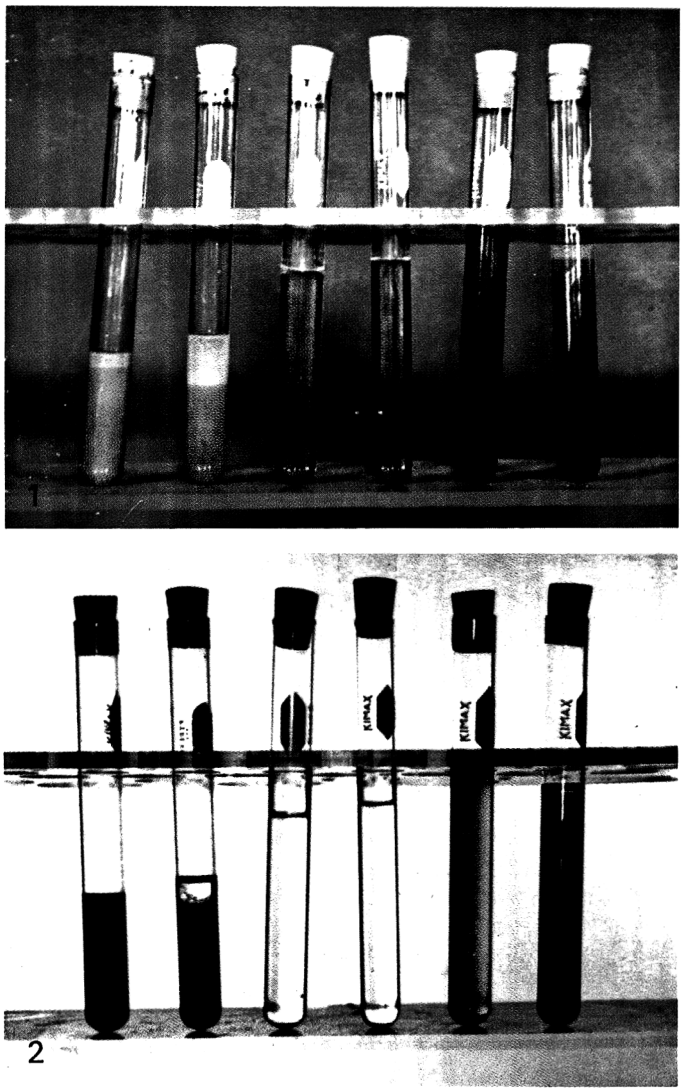
Fig. 3. Urinary Ca level as a function of NTA ingestion level in female rats fed H_3NTA (●) or $Na_3NTA.H_2O$ (○) and male rats fed H_3NTA (▲) or $Na_3NTA.H_2O$ (■). Each value is the mean of samples from five animals.

of NTA dose on urinary Ca output was measured as a function of the NTA ingestion rate. For this study we expanded the range of NTA doses fed and used both male and female rats. The urinary Ca output (μmol/100 g body weight/day) is shown (Fig. 3) as a function of the NTA ingestion (μmol/100 g body weight/day), which was calculated from measured diet disappearance during wk 3 of the study, when the urinary Ca was determined. At NTA ingestion rates below 400 μmol/100 g/day the urinary Ca levels were very similar to those of the control animals. NTA intakes of 600 μmol/100 g body weight/day or more resulted in a dose-dependent increase in urinary Ca output. Also indicated in Fig. 3 are the NTA ingestion levels of animals consuming 0·75% $Na_3NTA.H_2O$ or 0·75% H_3NTA, the lowest levels that resulted in crystalluria. Finally, the increased urinary Ca output associated with NTA ingestion was the same for male and female rats and for $Na_3NTA.H_2O$ and H_3NTA at comparable levels of intake.

Studies in which both urinary Ca and NTA were measured are summarized in Fig. 4. All of the data were obtained on male rats weighing about 250 g and fed diets containing various forms of NTA for 2 wk before the urinary Ca and NTA levels were measured. When the urinary NTA levels were less than 100 μmol/100 g body weight/day, urinary Ca did not differ from that of control animals. When urinary NTA exceeded 100 μmol/100 g/day, it was associated with a linear increase in urinary Ca output until urinary NTA was approximately 170 μmol/100 g/day. Since urinary NTA levels greater than 170 μmol/100 g/day further increased urinary Ca levels only slightly, the increase in urinary Ca by urinary NTA may be a saturable process.

DISCUSSION

No attempt was made in these studies to measure the quantity of crystalline CaNaNTA in the urine



. Urines voided by female Charles River rats fed, from left to right, 1·5% H_3NTA (two samples), ɔl diet (two samples), 2·0% $Na_3NTA.H_2O$ (two samples). Each sample is a 24-hr urine collection an animal on the experiment for 4 wk.

Photograph of urines shown in Fig. 1 but with back lighting.

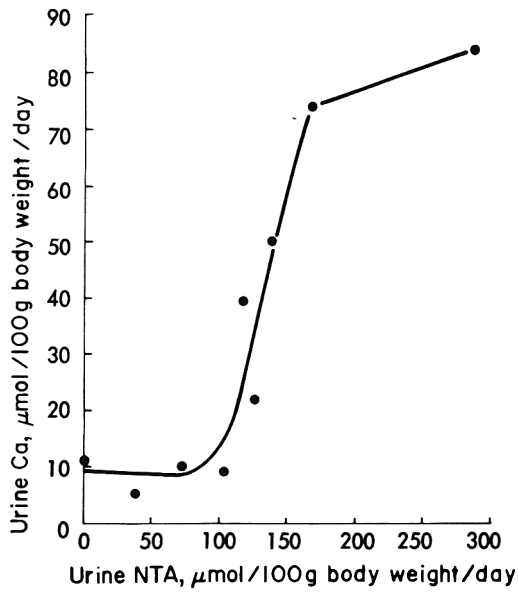


Fig. 4. Urinary Ca as a function of urinary NTA level in male rats after 14 days of NTA ingestion. Each data point is the mean for at least three animals fed specific levels of NTA.

since the intact complex cannot be isolated free of contaminating insolubles. However, none of the samples contained enough Ca to have all of the NTA present as a CaNTA complex, and the highest Ca/NTA ratio noted (in female rats fed 2% $Na_3NTA.H_2O$) was 0·7.

In two separate studies it was demonstrated that increased urinary Ca is not a simple linear function of NTA intake (Fig. 3) or NTA load in the urine (Fig. 4) but shows a marked threshold. That is, the urinary excretion of Ca did not increase except when NTA intake exceeded 300 μmol/100 g body weight/day or when urine NTA exceeded 100 μmol/100 g/day. Feeding studies with [^{14}C]NTA showed that when blood ^{14}C had reached equilibrium (5–10 days), about 30% of the ingested NTA was excreted in the urine. This relationship held over a dose range of 0·02 to 2·0% (R. A. Hiles, personal communication 1978). Thus it is not surprising that an ingestion rate of about 300 μmol/100 g/day gives a Ca response equivalent to a urinary output of about 100 μmol NTA/100 g/day.

The observation that 0·75% NTA (either H_3- or Na_3-) resulted in the appearance of visible crystals of CaNaNTA in the urine is particularly significant in view of the carcinogenicity study with NTA reported by the National Cancer Institute (1977). In that study no NTA-associated urinary-tract neoplasms were found at NTA doses below 0·75% H_3NTA or $Na_3NTA.H_2O$. When the dietary concentration of NTA was 2% $Na_3NTA.H_2O$ or its equivalent 1·5% H_3NTA, there was a marked increase in the neoplasms of the kidney, ureter and bladder. The most frequent neoplasms were of the transitional epithelium. Thus all of the transitional-cell neoplasms noted in the National Cancer Institute's bioassay of NTA were associated with doses of NTA that caused crystalluria. A similar association between crystalluria and urinary-tract neoplasms has been noted with several other compounds, including xylitol (Schiffrin, 1977), terephthalic acid (Gross, 1977) and 4-ethylsulphonylnaphthalene-1-sulphonamide (Stanley, 1977).

The crystalluria can be accounted for by some observations on the behaviour of CaNaNTA in the laboratory. CaNaNTA does not readily dissolve in water; distilled water dissolves only enough to form solutions of about 0·003 M. When the complex is formed by bringing a solution of H_3NTA and CaO to pH 7 with NaOH, it forms a metastable solution at concentrations up to 0·025 M. These metastable solutions spontaneously yield crystalline CaNaNTA after 48 hr at ambient temperature (*c.* 20°C). If they are seeded with crystalline CaNaNTA, crystallization occurs immediately. The metastable solutions of CaNaNTA are more stable at pH 6 and less stable at pH 8 than at pH 7. We believe that the following observations are significant in relation to the *in vivo* crystalluria:

(i) Consumption of a diet containing 0·75% NTA leads to the production of urine that is approximately 0·025 M in NTA. This urine is metastable with respect to the crystallization of CaNaNTA;

(ii) The presence of crystalline CaNaNTA in the urine would provide a source of seed crystals to destabilize newly formed urine;

(iii) Once the urine contains crystalline CaNaNTA, conditions to dissolve such crystals would not exist *in situ*;

(iv) It requires a greater molar concentration of dietary H_3NTA (mol wt = 191), which produces an acidic urine, than of $Na_3NTA.H_2O$ (mol wt = 275), which produces an alkaline urine, to cause crystalluria.

We conclude that NTA ingestion increases urinary calcium output when the systemic load of NTA exceeds 100 μmol/100 g body weight/day and that such loads exceed the solubility of CaNaNTA in the urine, resulting in the precipitation of crystals of this material. Further, the dose required to cause crystalluria corresponds almost exactly to the dose found to cause neoplasms of the urinary tract in chronic feeding studies, suggesting a causative relationship between crystalluria and urinary-tract neoplasms.

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INFLAMMATION EXPERIMENTALE ET TAUX PROTEIQUE ALIMENTAIRE CHEZ DES RATS INTOXIQUES SUBCHRONIQUEMENT AU NABAME

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Résumé—Dans le but de rechercher de nouveaux tests d'intoxication, nous avons tenté de mesurer chez le rat la réponse à une agression de type inflammatoire. A cet effet, des rats ont été alimentés avec des régimes à taux protéique variable (0, 3.5, 9, 26 et 81% de caséine) et par un régime standard de laboratoire. Les doses de Nabame (*N,N'*-éthylènebis(dithiocarbamate) disodique) dans l'alimentation sont de 0, 10, 50, 100, 500, 1000 et 2000 ppm. La réaction inflammatoire a été objectivée par la détermination du taux des divers constituants d'un granulome expérimental—mucopolysaccharides totaux, mucopolysaccharides à acide hexuronique, ceux à acide *N*-acétylneuraminique, collagène total, collagène neutro-soluble, collagène acido-soluble, collagène labile à 60°C et collagène insoluble. Chez des animaux normalement nutris l'administration de Nabame diminue le poids frais des granulomes et le taux de ces différents constituants et ceci proportionnellement à la dose de toxique administrée. Lorsque la teneur en protéines du régime diminue, la réaction inflammatoire diminue de façon corrélative, le Nabame accentue nettement l'effet du régime, plus le taux protéique est bas plus la dose efficace de Nabame diminue. L'effet du Nabame affecte principalement le poids frais des granulomes exprimé par rapport au poids corporel, le taux de mucopolysaccharides et en particulier celui des fractions solubles et le taux ainsi que la distribution du collagène. Le test de l'inflammation expérimentale s'avère sensible pour déceler précocement des intoxications insidieuses et pour mesurer les possibilités de défense de l'organisme selon l'état nutritionnel de l'individu.

Abstract—In research on new toxicity tests, attempts have been made to measure the response to an inflammatory type of attack. Groups of rats were fed diets containing various levels of protein (0, 3·5, 9, 26 and 81% casein) or a standard laboratory chow diet, and each group was further divided into subgroups given Nabam, disodium *N,N'*-ethylenebis(dithiocarbamate), at a dietary level of 0, 10, 50, 100, 500, 1000 or 2000 ppm. The inflammatory response was followed in these animals by determining the levels of total mucopolysaccharides, mucopolysaccharides containing hexuronic acid, mucopolysaccharides containing *N*-acetylneuraminic acid, total collagen and neutrosoluble, acid-soluble, labile (at 60°C) and insoluble collagen fractions in an experimental granuloma. In animals on a normal diet, Nabam administration reduced both the fresh weight of the granuloma and the levels of these different constituents, in a dose-related manner. When the protein level of the diet was reduced, the inflammatory reaction showed a corresponding decrease, an effect clearly accentuated by Nabam; the lower the protein level, the lower the effective dose of Nabam. Nabam affected mainly the fresh weight of the granuloma expressed in relation to body weight, the levels of mucopolysaccharides, particularly the soluble fractions, and the level and distribution of collagen. This test of experimental inflammation is a sensitive method for the early detection of a toxic effect and for relating an organism's defensive capacity to its nutritional state.

INTRODUCTION

Dès 1966 à la demande le l'OMS des travaux ont été entrepris pour déterminer l'incidence du taux protéique alimentaire sur la toxicité des pesticides afin de savoir si leur risque d'emploi n'était pas accru dans les pays où la teneur en protéines de la ration alimentaire est normalement basse. Les travaux de Boyd (1972) ont apporté de nombreuses données concernant la toxicité aiguë de pesticides organochlorés et organophosphorés et la teneur protéique alimentaire mais à notre connaissance peu de travaux traitent de ce problème dans les cas d'intoxication chronique ou subchronique. En effet, les résultats obtenus dans ce type d'expérimentation sont difficiles à apprécier (Suschetet et Causeret, 1974) car les procédés classiques d'investigation ne fournissent que peu de renseignements surtout lorsque les composés sont administrés à faible dose; d'où l'utilisation de techniques d'exploration plus fines comme, par exemple, la mesure de la réaction de défense de l'organisme contre une agression externe. Cette mesure a fait l'objet d'un test très utilisé en pharmacologie pour l'étude des substances anti-inflammatoires; il s'agit d'injecter par voie sous-cutanée une substance irritante non diffusible qui s'entoure d'éléments fibreux formant ainsi le granulome lequel est essentiellement constitué de mucopolysaccharides, de collagène et de protéines non fibrillaires.

L'application de ce test dans les cas d'intoxication à moyen et long terme d'un animal soumis à certaines

distorsions nutritionnelles présente plusieurs intérêts: (i) l'administration continue d'un toxique pourrait déterminer des modifications de la réaction inflammatoire donc une modification du 'terrain' de l'organisme, (ii) la sensibilité de ce test pourrait permettre de déceler une intoxication insidieuse précoce, et (iii) l'influence du régime alimentaire, et en particulier de sa teneur protéique, sur les processus de défense de l'organisme pourrait éventuellement potentialiser les effets toxiques de certaines substances.

Le composé que nous avons choisi d'étudier est un fongicide de la série des dithiocarbamates, série très importante du point de vue phytosanitaire car elle comprend à la fois des insecticides, des herbicides et surtout des fongicides. Le Nabame (*N,N'*-éthylène-bis(dithiocarbamate) disodique) est utilisé contre de nombreux parasites des fruits, des feuillages, des légumes, du tabac, du maïs et des plantes ornementales. Il est absorbé facilement au niveau du tractus digestif des animaux et métabolisé en monosulfure de thiurame éthylénique, en thiourée éthylénique, en sulfure d'hydrogène et en sulfure de carbone (Falk, Thompson et Kotin, 1965). La biotransformation du Nabame ainsi que celle d'autres dithiocarbamates, leur action sur les systèmes réticuloendothélial et hématopoïétique, sur la fonction thyroïdienne et sur la physiologie de la reproduction et sa potentialité cancérogène sont très étudiés (Engst et Schnaak, 1970; Vonk et Kaars-Sijpestein, 1970). La DL_{50} du Nabame administré per os est de 395 mg/kg chez le rat (Blackwell-Smith, Finnegan, Larson, Sahyoun, Dreyfuss et Haag, 1953) et de 580 mg/kg chez la souris (Kligman et Rosensweig, 1948); la concentration ne provoquant pas d'effets toxiques à long terme (*no-effect level*) dans l'alimentation n'a pas été déterminée et la dose journalière acceptable provisoire pour l'homme a été fixée à 0,025 mg/kg de poids corporel pour les composés initiaux et pour la somme de tous les fongicides de la série des dithiocarbamates quand il y en a plus d'un (FAO/OMS, 1971).

Nous avons donc étudié la réaction inflammatoire au travers de certains constituants chimiques du tissu enflammé chez des rats traités subchroniquement avec différentes doses de Nabame et placés dans des conditions de malnutrition protéique.

METHODES EXPERIMENTALES

Animaux et régimes alimentaires. Nous avons utilisé des rats mâles albinos de souche CD, d'origine Sprague-Dawley (Charles Rivers, St-Aubin-les-Elbeuf 76410) âgés de 18 à 20 jours (fin de sevrage) et pesant 54 g. Ils sont placés en cage individuelle dans une animalerie éclairée de 7.00 à 19.00 hr, maintenue à une température ambiante de 23 ± 1°C, avec une hygrométrie relative de 50% et ventilée à raison de 12 cycles par jour.

A leur arrivée au laboratoire les rats sont divisés en six groupes (I–VI) puis en sept sous-groupes qui ingèrent une nourriture additionnée respectivement de 0, 10, 50, 100, 500, 1000 et 2000 ppm de Nabame. Les régimes alimentaires donnés aux animaux des groupes I–VI ne diffèrent entre eux que par leurs taux respectifs de glucides et de protéines: ce sont des régimes semi-synthétiques isocaloriques équilibrés en vitamines (1% d'un mélange vitaminique complet) et en sels minéraux (6% d'un mélange salin complet) tous contiennent 8% d'un mélange lipidique (huile de maïs 3%, saindoux 5%). La composition détaillée des mélanges salin et vitaminique a été indiquée dans un travail antérieur (Périquet et Derache, 1976). Le tableau 1 montre la composition glucidique et protidique des régimes donnés aux rats des groupes I–V) l'aliment donné aux rats du groupe VI est une provende pour animaux de laboratoire (U.A.R., Villemoisson s/Orge 91) qui pourrait permettre de savoir si, en plus du taux, la nature protéique influence l'inflammation expérimentale car dans les autres régimes la source protéique est la caséine. Les taux respectifs de 26 et 59% de caséine et d'amidon de blé (groupe IV) permettent une bonne croissance des animaux (Hegsted et Chang, 1965); l'amidon de blé a été retenu comme source d'hydrate de carbone de préférence au glucose et au saccharose (Boyd et Liu, 1968) qui peuvent entraîner la mort par déshydratation (Boyd et Carsky, 1967) et potentialiser l'action toxique de certaines substances (Boyd, Covert et Pitman, 1966).

Protocole expérimental. Les régimes alimentaires et l'eau de boisson sont fournis *ad lib.* Après 28 jours nous procédons à l'implantation de deux granulomes par injection sous-cutanée dans la région inter-scapulaire de 0,25 ml d'essence de térébenthine selon le procédé de Bazin et Delaunay (1964). Dix jours après les granulomes sont extraits, pesés et regroupés par lots puis divisés en trois fractions servant à déterminer les teneurs en eau, mucopolysaccharides et collagène.

Techniques de dosage. Le contenu en eau est déterminé selon le procédé de Johnson (1967). Les granulomes destinés au dosage des mucopolysaccharides sont broyés à trois reprises (Ultra-Turrax) dans dix volumes de tampon phosphate (0,1 N, pH 7,8): le culot insoluble déshydraté et délipidé, ainsi que la fraction soluble sont soumis à une digestion pancréatique de 12 hr à 37°C (pancréatine de titre 200, à raison de 5 mg/g de tissu frais): après 24 hr de dialyse contre de l'eau distillée les liquides de digestion sont lyophylysés. Les mucopolysaccharides totaux sont déterminés par la technique d'Elson et Morgan (1933) modifiée par Immers et Vasseur (1952), les mucopolysaccharides avec acide hexuronique sont mesurés selon le procédé de Tollens (1908) modifié par Deichmann (1943): le dosage des mucopolysaccharides par

Tableau 1. *Composition protidique et glucidique des régimes alimentaires*

Groupe	Constituant (% du régime)	
	Caséine	Amidon de blé
I	0	85
II	3,5	81,5
III	9	76
IV	26*	59
V	81	4
VI	22†	63

* Teneur protéique optimale d'un régime alimentaire équilibré selon Hegsted et Chang (1965).

† Mélange de protéines d'origine animale (farine de poisson et de lait) et d'origine végétale (tourteaux et levure).

la réaction de Dische (1930) modifié par Ayala, Moore et Hess (1951) et par Coburn, Moore et Haninger (1953) a nécessité une purification de l'extrait par passage sur colonne (1 cm^2 × 20 cm) de résine échangeuse d'ions Dowex 1 × 100, 200–400 mesh. Du lot destiné au dosage du collagène nous avons extrait, selon la technique de Bazin et Delaunay (1970), quatre fractions: collagène neutro-soluble (NaCl 0,45 M, pH 7,8 à +4°C), collagène acido-soluble (tampon citrate 0,1 M, pH 3,6 à +4°C), collagène labile à 60°C (tampon phosphate 0,2 M, pH 6,6, NaCl 0,15 M), et collagène insoluble traité au TCA 0,3 M à 90°C (Fitch, Harkness et Harkness, 1955).

Le taux de collagène de chaque fraction est déterminé par titrage de l'hydroxyproline selon le procédé de Stegeman (1967), les valeurs en hydroxyproline sont multipliées par 7,6 pour être exprimées en valeur de collagène.

RESULTATS

Croissance des animaux et poids des granulomes

Chez les rats du groupe IV (26% de caséine), considéré comme groupe de référence (Hegsted et Chang, 1965), c'est la dose de 1000 ppm de Nabame dans l'alimentation qui provoque un ralentissement de la croissance; il en est de même dans le groupe alimenté par le régime standard de laboratoire (Tableau 2). Cette dose de 1000 ppm dans le régime est inférieure à celle établie par Seifter et Ehrich (1948), qui ont montré que la courbe de croissance des rats était modifiée a partir de 1500 ppm de Nabame dans le régime. Chez les animaux alimentés par les régimes hypoprotéiques les doses de Nabame provoquant un ralentissement de la croissance sont plus faibles que chez les animaux normalement nutris; elles sont respectivement de 10, 50 et 500 ppm chez les animaux des groupes I, II et III (Tableau 2). Dans le groupe V (81% de caséine) cette dose est de 1000 ppm. Le comportement des rats est différent selon les teneurs alimentaires des régimes: les rats placés au régime hyperprotéique présentent les signes d'intoxication à la caséine décrits par Boyd, Krijnen et Peters (1967), ceux alimentés avec les régimes hypoprotéiques présentent les signes cliniques du Kwashiorkor et du marasme (Boyd et De Castro, 1970), et les animaux du groupe protéiprive recevant les doses de 500, 1000 et 2000 ppm sont morts et ce d'autant plus rapidement que la dose de Nabame est élevée.

Le poids frais des granulomes est d'autant plus faible que la teneur protéique alimentaire est basse; la dose de Nabame qui produit un abaissement du poids frais des granulomes est de 50 ppm pour tous les groupes de rats, le pourcentage de diminution dans chaque catégorie alimentaire est dose-dépendant, et ces pourcentages paraissent plus élevés chez les animaux alimentés avec les régimes hypoprotéiques (Tableau 2). Par contre, si on exprime le poids frais des granulomes par 100 g de poids corporel les doses de Nabame réellement efficaces sont variables: elles sont toujours de 50 ppm chez les animaux des groupes à 0, 3,5 et 9% de caséine, de 100 ppm chez le groupe à 26% de caséine et chez les rats placés au régime standard de laboratoire, et de 1000 ppm chez les rats dont le régime est hyperprotéique. Le tableau 2 montre aussi que les pourcentages de diminution du poids des granulomes, exprimés en valeurs absolue et relative, par rapport au poids des granulomes témoins sont en général plus forts chez les animaux malnutris que chez les animaux correctement alimentés, les différences sont également plus significatives. Nous n'avons pas fait figurer les valeurs des poids secs, des teneur en eau et en lipides des granulomes car les variations des poids secs sont identiques à celle des poids frais; la teneur en eau n'est pas affectée par le régime alimentaire et par le Nabame; la teneur en lipides n'est pas influencée par le Nabame (Périquet, de Saint-Blanquat et Derache, 1978a) et les variations imputables au régime alimentaire ont été décrites par ailleurs (Périquet, de Saint-Blanquat, Derache et Souqual, 1978b).

Teneur en mucopolysaccharides

Le tableau 3 montre que dans chaque groupe alimentaire la fraction soluble des mucopolysaccharides diminue lorsque la dose de Nabame augmente, ceci est surtout net pour les mucopolysaccharides totaux et avec acide hexuronique mais dans ce dernier cas les différences ne sont significatives qu'avec des doses plus fortes de Nabame; quant à la fraction soluble des mucopolysaccharides avec acide *N*-acetylneuraminique, la diminution n'est significative qu'aux doses les plus élevées de Nabame (1000 et 2000 ppm).

La dose de Nabame qui provoque une diminution du taux de mucopolysaccharides varie selon le taux protéique alimentaire; elle est, en ce qui concerne les mucopolysaccharides totaux de 50 ppm dans les groupes à 0, 3,5 et 9% de protéines et de 100 ppm dans les autres groupes (Tableau 3). Cette même remarque apparaît en considérant les taux de mucopolysaccharides avec acide hexuronique mais les doses de Nabame provoquant une diminution sont de 100 ppm dans les régimes à faible teneur protéique (groupes I, II et III) et de 1000 ppm dans les groupes à teneurs protéiques normale ou forte (groupes IV, VI et V).

La fraction soluble des mucopolysaccharides avec acide *N*-acétylneuraminique en plus de ne diminuer qu'aux plus fortes doses de Nabame ne semble pas affectée par la teneur protéique alimentaire, la diminution de sa teneur n'est significative qu'avec les doses de 1000 et 2000 ppm quelque soit le régime alimentaire (Tableau 2). Si on considère maintenant la fraction insoluble des mucopolysaccharides dans leur ensemble, il y a, dans tous les groupes alimentaires une tendance à la diminution de cette fraction insoluble lorsque la dose de Nabame dans le régime croit mais les différences ne sont significatives qu'avec les plus fortes doses de Nabame (1000 et 2000 ppm) et seulement dans les mucopolysaccharides totaux et avec acide hexuronique (Tableau 3).

L'influence du taux protéique de la ration sur la fraction insoluble des mucopolysaccharides totaux ne semble pas se faire sentir car la diminution n'est significative que pour les doses de 1000 et 2000 ppm dans tous les groupes alimentaires à l'exception du groupe à 0% de caséine où le seuil de diminution est de 50 ppm (Tableau 3); la fraction insoluble des mucopolysaccharides à acide hexuronique diminue significativement dans le groupe à 3,5% de caséine aux doses de 500 et 1000 ppm, dans les autres cas les différences ne sont pas significatives (parfois il n'a

Tableau 2. *Effet du Nabame sur le poids frais des granulomes implantés chez des rats alimentés avec des régimes à taux protéique variable*

Groupe	Taux protéique alimentaire (%)	Nombre d'animaux	Nabame dans le régime (ppm)	Poids corporel (g)		Gain ou perte de poids	Poids frais des granulomes (g)	
				Initial	Final		Valeur absolue	Valeur en g/100 g de poids corporel
I	0	44	0	48,9 ± 0,6	39,9 ± 0,4	−8,9	0,2060 ± 0,0511	0,515 ± 0,037
		40	10	50,1 ± 0,3	38,1 ± 0,3***	−12,0	0,1956 ± 0,0452	0,512 ± 0,041
		39	50	49,8 ± 0,6	36,2 ± 0,4****	−13,6	0,0815 ± 0,0142*	0,225 ± 0,039****
		41	100	51,4 ± 0,5	30,4 ± 0,2****	−21,0	0,0685 ± 0,0103***	0,225 ± 0,028****
II	3,5	37	0	56,0 ± 0,7	82,3 ± 1,4	+26,0	0,4651 ± 0,0709	0,565 ± 0,067
		38	10	54,0 ± 0,4	81,1 ± 1,6	+27,1	0,5113 ± 0,0633	0,630 ± 0,051
		39	50	50,6 ± 0,6	74,0 ± 2,0***	+23,4	0,3014 ± 0,0363*	0,406 ± 0,046*
		37	100	48,4 ± 0,5	74,5 ± 1,2****	+26,1	0,2904 ± 0,0317*	0,389 ± 0,052***
		37	500	51,3 ± 0,7	59,3 ± 0,9****	+7,9	0,2042 ± 0,0251****	0,343 ± 0,048****
		37	1000	52,4 ± 0,5	55,0 ± 0,6****	+2,5	0,1797 ± 0,0212****	0,326 ± 0,046****
		37	2000	49,6 ± 0,5	46,5 ± 0,3****	−3,1	0,0979 ± 0,0142****	0,210 ± 0,039****
III	9	37	0	46,7 ± 0,6	135,5 ± 3,0	+88,7	0,9170 ± 0,0836	0,676 ± 0,072
		39	10	47,8 ± 0,5	140,8 ± 3,4	+93,0	1,0031 ± 0,0910	0,712 ± 0,084
		39	50	46,4 ± 0,6	130,6 ± 2,9	+84,2	0,6461 ± 0,0655***	0,494 ± 0,062*
		39	100	46,8 ± 0,7	127,6 ± 3,1	+80,8	0,6119 ± 0,0549***	0,479 ± 0,055***
		39	500	47,0 ± 0,6	107,7 ± 3,6****	+60,7	0,5598 ± 0,0642****	0,519 ± 0,037****
		39	1000	45,6 ± 0,6	104,6 ± 2,0****	+58,9	0,4194 ± 0,0594****	0,400 ± 0,050****
		39	2000	45,6 ± 0,7	70,6 ± 1,5****	+25,0	0,2845 ± 0,0462****	0,402 ± 0,046****
IV	26	37	0	45,0 ± 0,3	254,0 ± 6,2	+207,2	1,3431 ± 0,1571	0,528 ± 0,066
		38	10	46,0 ± 0,4	255,0 ± 5,5	+208,9	1,1827 ± 0,1496	0,463 ± 0,059
		37	50	47,1 ± 0,6	249,6 ± 6,1	+202,4	0,9467 ± 0,0832**	0,379 ± 0,049
		37	100	45,4 ± 0,4	237,1 ± 6,1	+191,7	0,7906 ± 0,0806****	0,333 ± 0,047**
		37	500	44,8 ± 0,2	236,2 ± 6,8	+191,4	0,7449 ± 0,0832****	0,315 ± 0,049***
		37	1000	44,5 ± 0,3	206,4 ± 4,9****	+161,9	0,7179 ± 0,0747****	0,347 ± 0,040**
		39	2000	44,7 ± 0,3	147,3 ± 4,1****	+102,5	0,4464 ± 0,0552****	0,302 ± 0,045***
V	81	41	0	53,0 ± 0,7	141,2 ± 6,7	+88,2	0,8168 ± 0,0704	0,578 ± 0,068
		38	10	51,0 ± 0,8	139,4 ± 5,4	+88,4	0,8456 ± 0,0815	0,606 ± 0,077
		40	50	49,4 ± 0,5	140,5 ± 4,9	+91,0	0,6179 ± 0,0717*	0,439 ± 0,053
		37	100	50,7 ± 0,6	137,4 ± 5,1	+86,6	0,6074 ± 0,0742*	0,441 ± 0,032
		36	500	48,8 ± 0,7	127,3 ± 4,1	+78,5	0,5486 ± 0,0657***	0,430 ± 0,030
		42	1000	52,4 ± 0,6	111,5 ± 4,2****	+59,0	0,4158 ± 0,0636****	0,372 ± 0,034***
		40	2000	49,5 ± 0,7	94,1 ± 2,4****	+44,6	0,3178 ± 0,0466****	0,337 ± 0,049***
VI	22	38	0	51,0 ± 0,6	243,4 ± 6,4	+192,4	1,2422 ± 0,1396	0,510 ± 0,052
		38	10	47,0 ± 0,5	250,1 ± 7,1	+203,0	1,2644 ± 0,1529	0,505 ± 0,061
		37	50	54,1 ± 0,4	245,3 ± 6,1	+191,1	0,9842 ± 0,0812	0,401 ± 0,059
		39	100	49,9 ± 0,5	240,4 ± 6,0	+190,5	0,7762 ± 0,0917***	0,322 ± 0,041***
		37	500	51,3 ± 0,4	229,3 ± 5,4	+177,9	0,7886 ± 0,0845***	0,343 ± 0,040**
		37	1000	49,6 ± 0,6	204,5 ± 4,9****	+154,8	0,7025 ± 0,0742****	0,343 ± 0,044**
		37	2000	50,4 ± 0,5	165,3 ± 3,1****	+114,9	0,4119 ± 0,0518****	0,249 ± 0,030***

Les valeurs représentent la moyenne ± l'erreur standard pour le nombre d'animaux indiqué. Les astérisques indiquent que la différence avec le témoin est significative: *$P < 0,05$; **$P < 0,02$; ***$P < 0,01$; ****$P < 0,001$.

Tableau 3. *Effet du Nabame sur la teneur et la composition en mucopolysaccharides de granulomes implantés chez des rats alimentés avec des régimes alimentaires à taux protéique variable*

Groupe	Taux protéique alimentaire (%)	Dose de Nabame dans le régime (ppm)	Mucopolysaccharides totaux (mg de glucosamine/g de tissu sec délipidé)		Mucopolysaccharides avec acide hexuronique (mg d'acide glucuronique/g de tissu sec délipidé)		Mucopolysaccharides avec acide-*N*-acetylneuraminique (mg d'acide-*N*-acetylneuraminique/g de tissu sec délipidé)	
			Solublest	Insolublest	Solublest	Insolublest	Solublest	Insolublest
I	0	0	5,9 ± 0,4	1,3 ± 0,2	0,6 ± 0,1	tr (traces)	1,0 ± 0,2	tr
		10	6,2 ± 0,5	1,4 ± 0,2	0,7 ± 0,1	tr	0,9 ± 0,1	tr
		50	4,1 ± 0,4***	0,7 ± 0,1***	0,4 ± 0,1	tr	0,7 ± 0,06	tr
		100	3,9 ± 0,3****	0,6 ± 0,1***	0,3 ± 0,1*	tr	0,7 ± 0,05	tr
II	2,5	0	11,3 ± 0,9	3,6 ± 0,5	1,5 ± 0,2	0,5 ± 0,1	2,1 ± 0,3	0,6 ± 0,1
		10	10,7 ± 0,9	3,8 ± 0,5	1,6 ± 0,2	0,5 ± 0,1	2,0 ± 0,2	0,7 ± 0,1
		50	8,5 ± 0,6**	3,4 ± 0,4	1,2 ± 0,1	0,6 ± 0,1	1,7 ± 0,2	0,6 ± 0,1
		100	8,3 ± 0,5***	2,9 ± 0,3	1,0 ± 0,09*	0,4 ± 0,08	1,6 ± 0,1	0,4 ± 0,07
		500	8,0 ± 0,6***	2,7 ± 0,3	1,0 ± 0,1*	0,2 ± 0,06***	1,4 ± 0,1*	0,4 ± 0,08
		1000	6,4 ± 0,5****	2,3 ± 0,2	0,9 ± 0,1***	0,2 ± 0,05***	1,1 ± 0,08***	0,3 ± 0,05**
		2000	6,2 ± 0,6****	2,1 ± 0,2***	0,8 ± 0,09***	tr	1,2 ± 0,09***	tr
III	9	0	21,7 ± 1,8	7,1 ± 1,1	2,4 ± 0,3	0,42 ± 0,09	4,2 ± 0,5	0,9 ± 0,1
		10	18,4 ± 1,9	6,8 ± 0,9	2,6 ± 0,3	0,5 ± 0,1	4,0 ± 0,4	1,0 ± 0,1
		50	16,4 ± 1,5*	7,0 ± 1,0	2,0 ± 0,2	0,5 ± 0,1	3,8 ± 0,7	0,8 ± 0,1
		100	15,3 ± 1,3***	6,1 ± 0,9	1,7 ± 0,2*	0,3 ± 0,08	3,1 ± 0,5	0,8 ± 0,1
		500	15,8 ± 1,4***	5,9 ± 1,0	1,6 ± 0,2*	0,3 ± 0,06	3,2 ± 0,6	0,6 ± 0,07
		1000	13,4 ± 1,1****	4,2 ± 1,0*	1,0 ± 0,1****	0,2 ± 0,04	2,7 ± 0,4**	0,6 ± 0,09
		2000	11,5 ± 1,1****	3,8 ± 0,9**	1,1 ± 0,1****	tr	2,2 ± 0,3****	tr
IV	26	0	37,2 ± 3,6	10,4 ± 1,2	3,4 ± 0,4	0,7 ± 0,1	5,9 ± 0,5	1,2 ± 0,1
		10	35,5 ± 4,2	9,5 ± 1,3	3,1 ± 0,3	0,9 ± 0,1	5,7 ± 0,6	1,6 ± 0,2
		50	28,8 ± 3,2	9,2 ± 1,0	2,9 ± 0,3	0,8 ± 0,1	5,3 ± 0,5	1,2 ± 0,1
		100	23,4 ± 2,9***	8,7 ± 0,7	2,8 ± 0,3	0,9 ± 0,1	5,0 ± 0,4	1,1 ± 0,2
		500	19,7 ± 2,4****	8,5 ± 0,7	2,6 ± 0,3	0,6 ± 0,1	4,8 ± 0,4	0,9 ± 0,1*
		1000	19,9 ± 2,5****	7,0 ± 0,8**	2,3 ± 0,2**	0,52 ± 0,08	4,2 ± 0,4***	1,0 ± 0,1
		2000	17,3 + 2,3****	6,2 + 0,5****	2,1 ± 0,2***	0,55 ± 0,07	4,0 ± 0,3***	0,9 ± 0,1*
V	81	0	25,3 ± 2,7	7,2 ± 0,8	2,1 ± 0,4	0,6 ± 0,08	3,4 ± 0,5	0,7 ± 0,1
		10	24,4 ± 3,1	6,8 ± 0,7	2,0 ± 0,3	0,8 ± 0,1	3,3 ± 0,4	0,5 ± 0,1
		50	20,7 ± 2,7	7,3 ± 0,9	1,9 ± 0,2	0,6 ± 0,1	2,8 ± 0,4	0,6 ± 0,1
		100	18,2 ± 2,1*	6,4 ± 0,6	1,6 ± 0,1	0,5 ± 0,07	2,2 ± 0,3	0,5 ± 0,09
		500	18,4 ± 2,0*	6,8 ± 0,7	1,8 ± 0,2	0,6 ± 0,08	2,3 ± 0,4	0,5 ± 0,07
		1000	16,2 ± 1,8***	5,1 ± 0,5*	1,3 ± 0,1*	0,5 ± 0,06	1,9 ± 0,2***	0,4 ± 0,07
		2000	15,8 ± 2,0***	4,2 ± 0,5***	1,2 ± 0,1*	0,4 ± 0,05	1,4 ± 0,2****	tr
VI	22	0	39,8 ± 4,0	9,5 ± 1,1	3,5 ± 0,4	0,8 ± 0,1	7,1 ± 0,8	1,5 ± 0,1
		10	40,2 ± 5,0	8,9 ± 1,6	3,2 ± 0,4	1,0 ± 0,1	6,9 ± 0,7	1,3 ± 0,1
		50	34,1 ± 4,2	7,4 ± 1,0	3,0 ± 0,4	0,7 ± 0,1	7,4 ± 0,9	1,4 ± 0,1
		100	26,4 ± 3,7**	7,0 ± 1,0	2,8 ± 0,4	0,6 ± 0,09	6,2 ± 0,8	1,6 ± 0,1
		500	25,2 ± 3,5***	6,9 ± 0,9	2,8 ± 0,3	0,7 ± 0,08	5,3 ± 0,6	1,2 ± 0,1*
		1000	22,8 ± 3,5***	6,1 ± 0,8**	2,1 ± 0,2***	0,6 ± 0,07	4,8 ± 0,6*	1,2 ± 0,1*
		2000	20,2 ± 3,1****	5,2 ± 0,6****	1,8 ± 0,2****	0,5 ± 0,06**	4,5 ± 0,5**	1,3 ± 0,1

†Solubles ou insolubles dans un tampon phosphate (0,1 N, pH 7,8).

Les valeurs représentent la moyenne ± l'erreur standard pour le nombre d'animaux indiqué en Tableau 2. Les astérisques indiquent que la différence avec le témoin est significative: $*P < 0.05$; $**P < 0.02$; $***P < 0.01$; $****P < 0.001$.

Tableau 4. *Effect du Nabame sur la teneur et la composition du collagène de granulomes implantés chez des rats alimentés avec des régimes à taux protéique variable*

Groupe	Taux protéique alimentaire (%)	Nabame dans le régime (ppm)	Collagène total (mg)	Fractions de collagène (% du collagène total)			
				Neutro-soluble	Acido-soluble	Labile à 60 C	Insoluble
I	0	0	7,8 ± 0,83	18,3 ± 2,1	14,3 ± 1,7	21,6 ± 2,5	45,8 ± 2,4
		10	7,1 ± 0,85	17,7 ± 1,8	15,5 ± 1,9	20,7 ± 2,3	46,1 ± 2,7
		50	5,4 ± 0,64*	20,6 ± 2,9	18,4 ± 1,9	20,7 ± 2,7	40,3 ± 2,1
		100	5,1 ± 0,41***	25,5 ± 2,1**	19,5 ± 1,2**	17,3 ± 2,0	37,7 ± 3,4*
II	3,5	0	9,3 ± 1,25	11,3 ± 2,7	10,2 ± 2,2	24,6 ± 2,1	53,9 ± 2,9
		10	9,4 ± 1,17	10,7 ± 2,4	11,8 ± 1,9	25,0 ± 3,1	52,5 ± 3,1
		50	6,0 ± 0,71*	14,3 ± 1,8	11,2 ± 1,6	24,3 ± 2,5	50,2 ± 3,5
		100	6,4 ± 0,71*	14,8 ± 2,6*	13,5 ± 1,8	23,0 ± 3,0	48,7 ± 3,1
		500	6,3 ± 0,69*	18,4 ± 2,1*	16,4 ± 2,0*	19,8 ± 1,8	45,4 ± 3,7
		1000	5,1 ± 0,43***	18,9 ± 1,9*	17,2 ± 1,8**	22,8 ± 2,1	41,1 ± 3,6**
		2000	3,4 ± 0,47****	19,8 ± 2,4**	17,8 ± 1,7***	23,2 ± 3,2	39,2 ± 3,8***
III	9	0	11,3 ± 1,49	4,6 ± 0,7	7,5 ± 1,4	23,6 ± 1,9	64,3 ± 5,1
		10	12,3 ± 1,51	4,8 ± 0,6	6,9 ± 0,9	25,1 ± 2,4	63,2 ± 4,2
		50	9,3 ± 1,02	5,1 ± 0,5	8,2 ± 1,1	26,4 ± 2,1	60,3 ± 5,2
		100	7,7 ± 0,85*	5,7 ± 0,5	8,5 ± 1,2	30,4 ± 2,9	55,4 ± 4,3
		500	6,4 ± 0,81***	6,9 ± 0,8**	10,2 ± 1,4	31,7 ± 3,2*	51,2 ± 4,9
		1000	6,1 ± 0,68***	8,4 ± 0,8****	11,1 ± 1,1**	31,1 ± 3,5	49,4 ± 4,8*
		2000	5,5 ± 0,61****	8,2 ± 0,9***	12,4 ± 1,2***	32,6 ± 2,8***	46,8 ± 4,2***
IV	26	0	15,5 ± 1,99	2,7 ± 0,3	4,8 ± 0,7	26,5 ± 1,9	66,0 ± 4,8
		10	15,4 ± 2,00	2,9 ± 0,3	4,0 ± 0,5	34,1 ± 2,7**	59,0 ± 4,1
		50	13,6 ± 1,75	3,1 ± 0,3	4,8 ± 0,6	27,6 ± 2,5	64,5 ± 5,1
		100	10,4 ± 1,07*	3,8 ± 0,4*	5,2 ± 0,7	34,4 ± 3,8	56,6 ± 4,9
		500	10,6 ± 0,93*	3,6 ± 0,5	6,6 ± 0,7	38,4 ± 4,2***	51,4 ± 5,1*
		1000	8,4 ± 0,91****	4,8 ± 0,6***	7,4 ± 0,8**	38,4 ± 4,3**	49,4 ± 5,0**
		2000	6,8 ± 0,74****	5,5 ± 0,8***	8,2 ± 0,8***	38,9 ± 4,4***	47,4 ± 4,0***
V	81	0	10,3 ± 0,98	7,1 ± 1,6	5,3 ± 0,9	24,8 ± 1,7	62,8 ± 4,6
		10	9,4 ± 0,87	7,2 ± 1,8	5,8 ± 0,7	23,8 ± 1,9	63,9 ± 4,3
		50	10,4 ± 1,13	8,4 ± 2,1	6,8 ± 0,8	22,2 ± 2,0	62,6 ± 4,1
		100	7,3 ± 0,93*	10,7 ± 1,9	7,9 ± 0,8*	21,0 ± 1,6	60,4 ± 4,8
		500	6,4 ± 0,74***	12,4 ± 1,5**	7,2 ± 0,5	23,6 ± 2,0	56,8 ± 5,2
		1000	5,3 ± 0,70****	11,8 ± 1,3*	8,4 ± 0,9**	27,4 ± 2,1	52,4 ± 4,4
		2000	5,1 ± 0,57****	13,2 ± 1,9**	9,8 ± 1,7**	27,9 ± 2,2	49,1 ± 4,8*
VI	22	0	16,5 ± 2,17	1,3 ± 0,2	3,9 ± 0,5	28,1 ± 2,2	66,7 ± 4,9
		10	15,4 ± 2,26	1,4 ± 0,1	4,2 ± 0,5	28,8 ± 3,1	65,6 ± 5,1
		50	12,3 ± 1,84	1,7 ± 0,2	4,4 ± 0,6	32,7 ± 3,5	61,2 ± 4,8
		100	9,4 ± 1,18***	1,8 ± 0,2	5,1 ± 0,7	37,6 ± 4,1*	55,5 ± 4,6
		500	9,3 ± 0,94***	2,1 ± 0,3*	6,3 ± 0,6****	37,4 ± 4,6	54,2 ± 5,1
		1000	8,9 ± 0,93***	2,3 ± 0,4*	6,7 ± 0,7****	41,5 ± 5,3***	49,5 ± 4,4***
		2000	7,1 ± 0,87***	2,7 ± 0,4***	7,2 ± 0,9****	41,5 ± 5,1***	48,6 ± 4,2**

Les valeurs répresentent la moyenne ± l'erreur standard pour le nombre d'animaux indiqué en Tableau 2. Les astérisques indiquent que la différence avec le témoin est significative: *$P < 0,05$; **$P < 0,02$; ***$P < 0,01$; ****$P < 0,001$.

pas été possible d'effectuer le dosage de cette fraction). Le dosage de la fraction insoluble des mucopolysaccharides avec acide *N*-acetylneuraminique n'apporte pas de nouveaux éléments car les différences ne sont pas significatives quelles que soient la dose de Nabame et la teneur protéique alimentaire.

Taux de collagène

Le taux de collagène total, à l'intérieur de chaque groupe alimentaire, diminue de façon significative lorsque la dose de Nabame dans le régime augmente: si on considère, toujours dans chaque groupe de rat, les différentes fractions de collagène il apparaît que c'est la fraction de collagène insoluble qui diminue de façon systématique alors que les fractions de collagène neutro- et acido-soluble augmentent dans chaque cas (Tableau 4). La fraction de collagène labile à 60°C semble diminuer dans les groupes à 0 et 3.5% de caséine, mais les différences ne sont pas significatives, et par contre augmente dans les autres groupes d'animaux parfois de façon significative aux fortes doses de Nabame (groupes III, IV et VI), parfois de façon non significative (groupe V).

Nous pouvons remarquer en comparant les différents groupes entre eux que, dans les régimes hypoprotéiques (0 et 3.5% de caséine) la première dose de Nabame qui produit une diminution du taux de collagène total est la dose de 50 ppm alors que pour les autres régimes cette valeur est de 100 ppm. L'augmentation ou la diminution des différentes fractions de collagène ne semblent pas apporter d'éléments supplémentaires quant à la sensibilité d'une fraction particulière de collagène à la teneur protéique alimentaire, la dose de Nabame qui produit une diminution ou une augmentation de telle ou telle fraction est variable d'un régime à l'autre (Tableau 4).

DISCUSSION

Lorsque la teneur protéique du régime alimentaire diminue: (i) la dose de Nabame dans le régime qui produit un ralentissement de la croissance des animaux est abaissée, (ii) la dose de Nabame dans le régime qui produit un abaissement du poids frais des granulomes, exprimés en valeur absolue, est la même quel que soit le régime, mais si on exprime le poids frais des granulomes par rapport à 100 g de poids corporel la dose de Nabame produisant une diminution de ce paramètre est plus basse avec les régimes hypoprotéiques, (iii) la dose de Nabame dans le régime provoquant une diminution du taux de mucopolysaccharides des granulomes est plus basse, et c'est la fraction soluble des mucopolysaccharides totaux qui paraît la plus sensible, et (iv) la dose de Nabame dans le régime qui diminue le taux de collagène des granulomes est également abaissée, mais il n'y a pas de fraction de collagène plus particulièrement sensible.

Il apparaît donc au travers de ce test que le terrain nutritionnel de l'organisme semble modifier sa réactivité à l'agression que constitue l'introduction d'une substance supposée toxique.

Il a été montré dans un travail antérieur (Périquet *et al.* 1978b) que l'adjonction de Nabame à la dose de 100 ppm dans l'alimentation équilibrée de rats provoquait une diminution de la réaction inflammatoire. Il a également été établi par de nombreux auteurs Dipasquale et Mei, 1965; Harkness, Harkness et James, 1958; Prasad et Bose, 1974) que la malnutrition protéique ainsi que tout changement dans la croissance des animaux produisaient un retard de maturation et de réticulation des granulomes.

D'après les résultats obtenus, il semble qu'il y aurait sur la croissance et la maturation des granulomes une sommation des effets dus à la carence protéique et des effets dus à la toxicité du Nabame, en effet la croissance, la maturation et la réticulation des granulomes semblent être affectés par des doses plus faibles de Nabame lorsque les animaux sont alimentés avec des régimes à taux protéique faible.

Il est important, lorsque l'on veut étudier les effets anti-inflammatoires d'une substance, d'exprimer le poids frais des granulomes en fonction du poids corporel de l'animal pour différencier l'activité réellement anti-inflammatoire de la substance d'une action directe ou secondaire sur la croissance surtout si cette substance a des effets anorexiques ou catabolisants. Ce mode d'expression nous a permis de différencier deux 'doses-seuil' auxquelles agissait le Nabame 50 ppm avec les régimes hypoprotéiques (I, II et III) et 100 ppm avec les autres régimes, alors que si l'on avait seulement considéré le poids frais des granulomes les différences étaient significatives à partir de 50 ppm dans tous les régimes alimentaires. Il convient également de signaler à propos de la croissance des rats que les doses de Nabame comprises entre 0 et 500 ppm n'affectent pas la prise alimentaire journalière, à la dose de 1000 ppm les rats ingèrent un peu moins de nourriture mais les différences ne sont pas significatives: par contre à la dose de 2000 ppm la ration quotidienne de nourriture est diminuée. Donc l'appétit des animaux n'influence pas la croissance et n'interfère pas avec les actions du Nabame jusqu'à la dose de 1000 ppm. La convergence d'action sur les granulomes de la carence protéique et de l'intoxication au Nabame aboutit à une diminution du poids frais relatif ou absolu des granulomes, à un abaissement du taux de l'ensemble des mucopolysaccharides et du collagène avec dans ce dernier cas un déplacement des fractions insolubles vers les fractions solubles.

Les changements observés au niveau des granulomes peuvent être dus à une modification soit de leur formation, soit de leur dégradation: l'hypothèse d'une modification de la genèse des granulomes nous paraît plus vraisemblable en effet l'action de la déficience protéique se retrouve également dans la diminution de la synthèse du collagène (Paz, Blumenfeld, Rojkind, Henson, Furfine et Gallop, 1965). Il a été montré d'une part que le Nabame introduit dans une alimentation équilibrée modifie la croissance des granulomes (Périquet *et al.* 1978b) et d'autre part que la toxicité aiguë du Nabame est fortement accrue lorsque le taux protéique alimentaire diminue (Périquet et Derache, 1976): il se pourrait donc que le Nabame introduit à dose subchronique dans la nourriture ait ses effets toxiques accrus par la carence protéique comme c'est le cas lors de l'intoxication aiguë. Dans ces deux cas la malnutrition protéique diminuerait ou inhiberait la production et/ou l'activité des enzymes microsomaux hépatiques responsables des processus de détoxification (Borel, Frei et

Vannotti, 1959; Kato, Oshima et Tomizawa, 1968; Millman 1951; Wainio, Eichel, Eichel, Person, Estes et Allison, 1953).

En ce qui concerne une action plus directe du Nabame et de la carence protéique, il pourrait y avoir une atteinte hépatique déjà signalée par Griffaton, Faudemay, Rozen, Naon et Lowy (1975), qui entraînerait un changement dans la biosynthèse des glycoprotéines hépatiques dont l'augmentation est l'une des caractéristiques de l'inflammation. La carence protéique et intoxication par le Nabame pourraient également modifier la vascularisation et la perméabilité vasculaire au niveau du territoire agressé, rendant mal aisée la nutrition du granulome. Il pourrait y avoir une moindre prolifération cellulaire dans le granulome et une moindre production de mucopolysaccharides et de collagène. L'ensemble de ces perturbations pourrait être imputable à des troubles métaboliques induits par le Nabame ou par la carence protéique et en particulier au niveau enzymatique. En effet la carence protéique est responsable des faibles activités enzymatiques observées par Vasantha (1969) et en particulier de l'enzyme responsable de la réticulation du collagène (Siegel et Martin, 1970), laquelle est cuprodépendante, et il a été observé chez des enfants malnutris des taux faibles de cuivre sérique et hépatique (Lahey, 1957; MacDonald et Warren, 1961; Sandstead, Burk, Booth et Darby, 1970). Le Nabame ainsi que plusieurs dithiocarbamates de part leur structure chimique peuvent être des agents chélants (Derache et Lowy, 1974) et complexer certains oligo-éléments et en particulier le cuivre, ajoutant ainsi à la diminution du taux de cuivre hépatique et sérique observé dans les carences protéiques.

Il convient également de signaler un possible mécanisme d'action indirecte. La privation de protéines et l'intoxication par le Nabame pourrait par le moyen du stress déclencher une stimulation du cortex-surrénalien et une sécrétion de glucocorticoïdes qui ont une action anti-anabolisante sur les protéines indirectement responsable de la formation granulomateuse. Les glucocorticoïdes ont également une action anti-phlogistique qui diminue les réactions protectrices de la cellule et en particulier ils retardent la migration des leucocytes dans les tissus traumatisés; ainsi le cortisol est un agent antiphlogistique utilisé dans les maladies du collagène comme l'arthrite rhumatoïde. Cependant, il a été montré qu'après une période de quatre semaines les animaux semblent acclimatés à la carence protéique (Waterlow, 1968) et que l'intoxication subchronique pourrait également ne plus provoquer de réaction de stress et cette hypothèse semble peu probable.

Enfin, une action imputable au Nabame seul pourrait être que le Nabame produit un hyperfonctionnement thyroïdien (Ivanova, Sheytanov et Mosheva-Izmirova, 1967; Korablev, 1969; Seifter et Ehrich, 1948) et il a été montré que la thyroxine provoque une inhibition de la réaction inflammatoire (Daugherty et Berliner, 1968; Robert, Ouzilou et Robert, 1970a,b; Thieblot, Berthelay, Blaise et Laguillaume, 1963).

En ce qui concerne les effets du régime hyperprotéique et de l'intoxication par le Nabame sur la croissance des granulomes il est difficile d'en tirer des conclusions précises. Les effets observés sont probablement inputables à une action indirecte sur la croissance qu'exerce le taux excessif de caséine et ce taux excessif produirait une intoxication interférant avec celle due au Nabame.

La nature des protéines ne semble pas influencer la croissance du granulome ni avoir des effets additifs à ceux dus à l'intoxication par le Nabame; la caséine étant une protéine de bonne qualité biologique, il faudrait créer des carences précises en acides aminés (Brown et Liddy, 1971) pour préciser une influence éventuelle sur la croissance des granulomes.

Il est assez facile d'évaluer la toxicité aiguë de certaines substances en fonction du terrain nutritionnel de l'organisme car il existe une batterie de tests assez développée (doses léthales, courbe de croissance, examens anatomiques et histologiques, constantes biologiques etc.) mais ces mêmes paramètres ne fournissent que peu d'indications lorsque l'intoxication est subchronique ou chronique et lorsque les composés sont administrés à faible dose. Avec l'étude de la réaction inflammatoire au travers des constituants des granulomes nous possédons un test plus fin qui permet de déceler précocément des intoxications insidieuses et de mesurer les possibilités de défense de l'organisme selon l'état nutritionnel de l'individu.

Les paramètres utilisés au cours d'études nutritionnelles sont rarement les mêmes que ceux employés dans les investigations toxicologiques. Cependant depuis quelques années de nombreux faits établis tantôt par les toxicologues tantôt par les nutritionnistes montrent que les frontières entre les deux disciplines sont floues et nos travaux tendent à montrer que la recherche toxicologique a intérêt à introduire dans sa méthodologie une dimension nutritionnelle qui trouve naturellement sa place dans le domaine de la toxicologie alimentaire.

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Fd Cosmet. Toxicol. Vol. 16, pp. 585–589

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SHORT PAPERS

THE EFFECT OF L-TRYPTOPHAN AND CERTAIN OTHER AMINO ACIDS ON LIVER NITROSODIMETHYLAMINE DEMETHYLASE ACTIVITY

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Summary—The effect of five amino acids (L-tryptophan, L-cysteine, L-methionine, L-tyrosine and L-glycine) on liver nitrosodimethylamine-demethylase activity was studied. Each amino acid was fed at a level of 1% of the diet to weanling male Wistar rats for 12 days. Tryptophan increased the enzyme activity and cysteine had the opposite effect. The other amino acids tested had no effect. When the effect of the duration of the tryptophan feeding (3–47 days) and the age of the animals (21–51 days) were taken into account, it was shown that 6-day feeding of tryptophan had the highest activating effect on liver nitrosodimethylamine-demethylase (30% above the control values). When the 6-day feeding of tryptophan was started when the rats were 27 days old, the enzyme activity of the tryptophan-fed animals was doubled compared with that of the control animals.

Introduction

The microsomal mixed-function oxidase system of the liver has an important role in determining the rate of metabolism of foreign compounds such as drugs, pesticides and carcinogens. The activity of this enzyme system can be altered by several variables, one of which is dietary protein (Basu & Dickerson, 1974; Campbell & Hayes, 1974; Kato, Oshima & Tomizawa, 1968; Marshall & McLean, 1969).

In studies on the effect of protein on the microsomal enzyme system, casein or casein hydrolysate was used as a protein source. A linear relationship was found between the amount of casein and the activity of microsomal enzymes (Kato *et al.* 1968; Mgbodile & Campbell, 1972; Weatherholtz & Webb, 1971). When gluten was used as a protein source, drug metabolism was reduced both *in vivo* and *in vitro* (Miranda & Webb, 1973).

Nitrosodimethylamine (NDMA), like most liver carcinogens, must be activated by the microsomal enzyme, NDMA-demethylase, to produce a carcinogenic effect. Casein and casein hydrolysate apparently increased the activity of this enzyme (Venkatesan, Arcos & Argus, 1970). It became evident that more detailed information was needed about the effect of individual amino acids on drug metabolism. The amino acid tryptophan was of special interest because of its effect on liver carcinogenesis (Evarts & Brown, 1977; Kawachi, Hirata & Sugimura, 1968; Okajima, Hiramatsu, Motomiya, Iriva, Ijuin & Ito, 1971; Oyasu, Kitajima, Hopp & Sumie, 1972). Other amino acids tested for their effect on NDMA-demethylase activity were selected to represent different categories of amino acids. Since preliminary studies with L-tryptophan had shown a possible effect of the length of feeding or the age of the animals on liver NDMA-demethylase activity, these variables were also considered in the present experiment.

Experimental

In the first experiment 52 young male Wistar rats (Charles River) weighing 50 ± 0·1 g (mean ± SEM) were used. Each of the following amino acids was mixed with Wayne meal to give a 1% concentration: L-tryptophan, L-cysteine, L-methionine, L-tyrosine and L-glycine (all from ICN Pharmaceuticals, Cleveland, Ohio). Groups of eight animals were given one of these five diets for 12 days. The remaining 12 animals were used as controls. The animals were then killed by decapitation and the livers were homogenized in 5 vols(w/v) 0·25 M-sucrose and centrifuged for 20 min at 10,000 g. The supernatant was centrifuged at 100,000 g for 1 hr. The microsomal pellet was suspended in 0·1 M-potassium phosphate buffer (pH 7·4). For determination of microsomal NDMA-demethylase activity the conditions for incubation were those used by Venkatesan, Arcos & Argus (1968). The amount of formaldehyde formed was determined by the method of Nash (1953) with the modifications of Cochin & Axelrod (1959) and McLean & Day (1974). Microsomal protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

In the second experiment three groups of six male Wistar rats were given the tryptophan diet from different ages (21, 23 and 25 days) and for different

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Table 1. *Effect of dietary supplementation with five amino acids on liver NDMA-demethylase activity*

Diet	No. of animals	NDMA-demethylase (nmol HCHO/mg microsomal protein/hr)	Microsomal protein (mg/g liver)	Liver: body weight ratio (%)
Control	12	40 ± 1·7	21 ± 0·2	4·9 ± 0·1
L-Trp	8	61 ± 4·1***	22 ± 0·5	5·2 ± 0·1*
L-Cys	8	33 ± 1·9**	20 ± 0·7	4·8 ± 0·1
L-Met	8	35 ± 1·8	20 ± 0·6	5·1 ± 0·1
L-Tyr	8	38 ± 3·4	21 ± 0·6	4·6 ± 0·1
L-Gly	8	40 ± 2·3	21 ± 0·4	4·7 ± 0·1

Values are means ± SD and those marked with asterisks differ significantly from the control value by Student's *t* test: *$P < 0.05$; **$P < 0.01$%; ***$P < 0.001$%.

durations (6, 12 and 35 days, respectively). Three corresponding control groups of six animals were used. In the third experiment the effect of the length of the tryptophan feeding on the liver microsomal NDMA-demethylase activity was determined, using 48 rats. Three control rats and three rats fed tryptophan diets from 21 days of age were killed by decapitation after 3, 6, 10, 12, 16, 23, 31 or 47 days of feeding. Their body weights and liver weights were recorded and NDMA-demethylase activity of the pooled liver samples was determined in quadruplicate. To study the effect of age on the liver demethylase activity when the tryptophan diet was fed for 6 days, the tryptophan feeding was started when the animals were 21, 27, 33, 39, 45 or 51 days of age. The three livers from each test group and from each control group were pooled and NDMA-demethylase was determined in quadruplicate. For statistical evaluation, Student's *t* test was used.

Results

Of the five amino acids tested, only L-tryptophan increased liver NDMA-demethylase activity significantly above that of the control animals, whereas L-cysteine decreased the activity (Table 1). A slight but significant increase in the liver weight: body weight ratio coincided with the increase in the enzyme activity.

As shown in Table 2, L-tryptophan was effective when it was given to young rats for 6 or 12 days and the animals were killed at the age of 27 or 35 days. There was no longer any significant effect when the tryptophan diet was fed for 35 days starting at the age of 35 days and the animals were killed at the age of 10 wk.

Figure 1 shows the activation of NDMA demethylase when continuous tryptophan feeding was started at the age of 21 days and was continued for 3–47 days. The highest level of activity, compared to the control animals, was attained 6 days after the beginning of the experiment when the animals were 27 days old. There was an increase in the liver weight:body weight ratio in the rats on the tryptophan diet up to 52 days of age, but this increase was most marked at the age of 27 days.

Figure 2 shows the effect of age on the activating influence of tryptophan. The highest activation (180%) was obtained when the 6-day period of tryptophan feeding was started at the age of 27 days and the animals were killed at the age of 33 days. When feeding was started at the age of 33 days and the animals were killed 6 days later, the activity was only 23% above the control values. The highest enzyme activities were always associated with a high liver weight: body weight ratio.

Discussion

NDMA is metabolized to monomethylnitrosamine and formaldehyde in the liver, kidney and lungs (Lijinsky, Loo & Ross, 1968; Magee & Barnes, 1967). Although this reaction, catalysed by NDMA demethylase, is an activating step in the formation of the putative ultimate carcinogen, a carbonium ion, the liver is rarely the target for tumour induction by

Table 2. *Effect of an L-tryptophan-supplemented diet on liver NDMA-demethylase activity*

Age at start of feeding (days)	Length of feeding (days)	Liver weight as percentage of body weight		Liver NDMA-demethylase nmol HCHO/mg microsomal protein/hr)	
		Controls	Treated animals	Controls	Treated animals
21	6	4·0 ± 0·1	4·5 ± 0·1**	68 ± 4·7	88 ± 6·7*
23	12	3·9 ± 0·1	4·3 ± 0·1**	41 ± 3·4	64 ± 4·4**
35	35	3·5 ± 0·1	3·5 ± 0·2	30 ± 1·8	34 ± 3·3

Values are means ± SD and those marked with asterisks differ significantly from the control value by Student's *t* test: *$P < 0.05$; **$P < 0.01$.

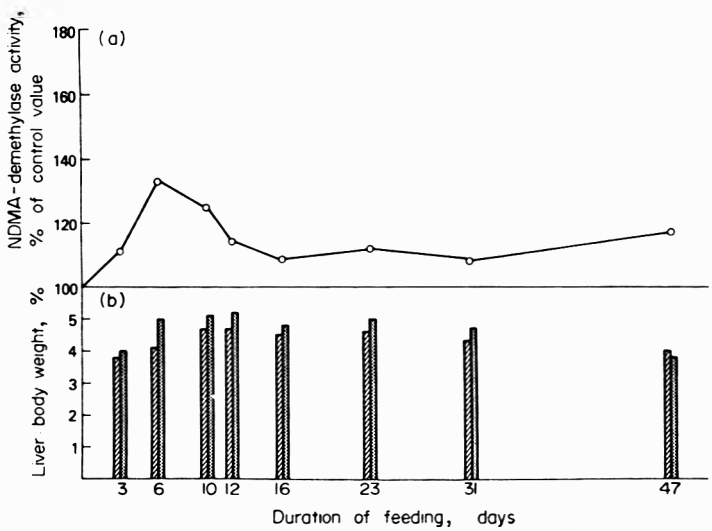

Fig. 1. The effect of the duration of tryptophan feeding (a) on the liver NDMA-demethylase activity and (b) on the liver : body weight ratio in control (▨) and tryptophan-fed (▤) animals.

a single dose of NDMA. However, the kidney, with low enzyme activity, is more sensitive and is affected by a single large dose of NDMA (Magee & Barnes, 1967).

The present experiment showed that tryptophan increased the activity of NDMA demethylase, especially among young animals. It was recently shown by Wattenberg & Loub (1978) that different naturally occurring three-substituted indole derivatives increased the activity of arylhydrocarbon hydroxylase and decreased the incidence of mammary tumours induced by 7,12-dimethylbenz[*a*]anthracene. Tryptophan (also a three-substituted indole) is known to be an inducer of enzymes like tryptophan oxygenase (Lee, 1956), tyrosine aminotransferase (Kenney & Flora, 1961), threonine hydrase (Peraino, Blake & Pitot, 1965), ornithine transaminase (Peraino *et al.* 1965) and phosphoenolpyruvate carboxykinase (Foster, Ray & Lardy, 1966).

Induction of NDMA-demethylase activity by casein feeding was first reported by Venkatesan *et al.* (1970). In general, dietary protein increases the activity of several drug-metabolizing enzymes (Basu & Dickerson, 1974; Campbell & Hayes, 1974; Kato *et al.* 1968; Marshall & McLean, 1969; Mgbodile & Campbell, 1972). In most cases casein has been used, since this protein contains sufficient amounts of all the essential amino acids. When gluten was used as a source of protein, there was a reduction in the rate of drug metabolism and liver *P*-450 content after 10 days of feeding (Miranda & Webb, 1973). The tryptophan content of corn gluten is 0·6 g/100 g protein whereas that of casein varies between 1·1 and 3·1 g/100 g protein (Block & Weiss, 1956). If gluten is used as a source of protein, growing animals soon become tryptophan deficient, since the requirement for tryptophan for the growth of rats is 1·0 g/100 g protein or 0·2% in the diet (Block & Weiss, 1956). The activating effect

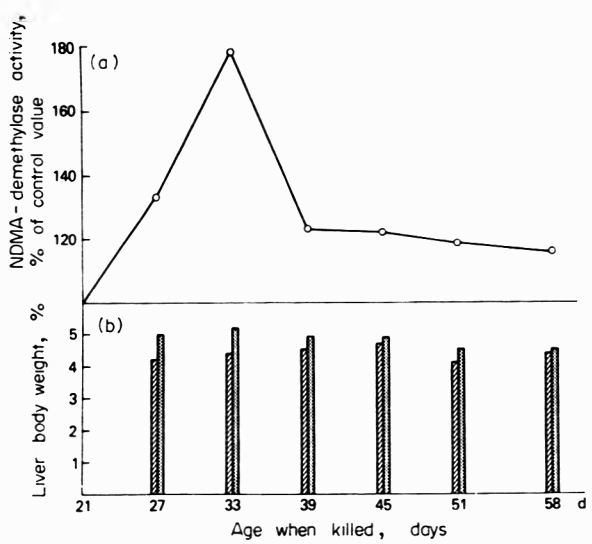

Fig. 2. The effect of age (a) on the liver NDMA-demethylase activity, and (b) on the liver:body weight ratio in control (▨) and tryptophan-fed (▤) animals following feeding of a tryptophan-supplemented diet for 6 days.

of tryptophan on NDMA demethylase may in part depend on its effect on cytochrome *P*-450 (Truex, Brattsen & Visek, 1977).

The present results on the effect of cysteine on the NDMA-demethylase activity substantiate earlier reports (Mizrahi & Emmelot, 1962 & 1963). By decreasing the demethylase activity, cysteine also protects the liver against the lethal effect of NDMA (Mizrahi & Emmelot, 1962).

Primary liver tumours in man are common only in areas where protein deficiency is also common (Berman, 1958). As mentioned, protein deficiency in experimental animals decreases the activity of microsomal drug-metabolizing enzymes. The consequences of a low-protein diet for the liver in animals exposed to a xenobiotic thus depend on the microsomal metabolism of the compound, which may be a process of activation or of detoxification. Decreased toxicity of NDMA and of heptachlor has been correlated with the decreased levels of activating enzymes in the livers of animals on a low-protein diet (McLean & Verschuuren, 1969; Weatherholtz & Webb, 1971), whereas increased toxicity has been demonstrated with several other compounds (Boyd & Krupa, 1970). The basis for increased carcinogenicity of the compounds in animals on a low-protein diet is less clear. However, a life-long regime high in protein has generally had a more consistent effect in reducing the death-rate from spontaneous tumours than a low-protein regime (Ross & Bras, 1973). Thus it seems that a low-protein diet protects against toxicity and a high-protein diet protects against carcinogenicity. The fact that tryptophan and cysteine affect the activity of microsomal enzyme systems indicates that the amino acid composition of the diet is important in determining the toxicity and carcinogenicity of foreign compounds.

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A SYNERGISTIC EFFECT OF NITROSODIMETHYLAMINE ON STERIGMATOCYSTIN CARCINOGENESIS IN RATS

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Summary—In a study of a possible syncarcinogenic effect of nitrosodimethylamine (NDMA) on sterigmatocystin (STG) carcinogenesis, male rats were fed diets containing 10 ppm STG, 10 ppm STG and 1 ppm NDMA, 1 ppm STG and 10 ppm NDMA or 10 ppm NDMA for 54 wk. Hepatic carcinomas developed in 75% of the rats fed 10 ppm STG plus 1 ppm NDMA and in 53% of those fed 10 ppm STG alone. The hepatic carcinomas developed more rapidly in the former group. About 50% of the hepatic carcinomas induced by STG or STG plus NDMA showed a tubular arrangement of tumour cells. Ultrastructurally, a mixture of the characteristic organelles of hepatocytes and ductular cells were frequently found in the same cells. Throughout the experiment, proliferation of smooth endoplasmic reticulum was observed in the hepatocytes of rats fed with NDMA. Between wk 54 and 69, Leydig-cell tumours developed in 47, 45 and 15% of the animals on the 10 ppm NDMA plus 1 ppm STG and the 1 ppm NDMA plus 10 ppm STG diets, respectively. Although, at this level, NDMA alone did not induce hepatic carcinomas, it may have a role in STG-carcinogenesis in the liver, as an inducer of carcinogen-activating enzymes.

Introduction

Sterigmatocystin (STG), a compound related to aflatoxin B_1, is a metabolite of *Aspergillus versicolor*, *A. nidulans* and Bipolaris species. Hepatocellular carcinoma and some other types of malignant tumours are induced by this mycotoxin although it is about ten times less carcinogenic than aflatoxin B_1 (Fujii, Kurata, Odashima & Hatsuda, 1976; van der Watt, 1974). In the temperate and tropical zones where it is known to be an active food contaminant, STG may be an important factor in the incidence of human hepatic carcinoma.

The widespread presence of nitroso compounds in our environment, and the fact that these compounds are taken with food as well as formed in the stomach from precursors, necessitates careful study of their potential carcinogenic hazard to man (Eisenbrand, Ivankovic, Preussmann, Schmähl & Wissler, 1975). There could be some relationship between the high incidence of hepatic carcinoma in the temperate and tropical zones and combined contamination by mycotoxins and nitroso compounds. Since the report of MacDonald, Miller, Miller & Rusch (1952), it is now well-established that several hepatic carcinogens act synergistically. Until recently, however, most of the experiments were performed on man-made compounds. The present study was undertaken to investigate the possibility of syncarcinogenic effects of nitrosodimethylamine (NDMA) and sterigmatocystin in rats, mainly from a morphological viewpoint.

Experimental

Crystalline STG used in these experiments was generously donated by Dr. Y. Hatsuda, Department of Agriculture, Tottori University, as was NDMA by Dr. M. Nakadate, National Institute of Hygienic Science, Tokyo.

From the age of 4 wk, groups of 15 or 20 male Wistar rats (supplied by Shizuoka Dobuts Nokyo, Shizuoka) were given a basal diet (CE-2 from Nihon Clea Ltd., Tokyo) containing 10 ppm STG (group 1), 10 ppm STG and 1 ppm NDMA (group 2), 1 ppm STG and 10 ppm NDMA (group 3) or 10 ppm NDMA (group 4). A further 30 rats given the basal diet alone served as controls (group 5). The experimental diets were fed for 54 wk after which basal diet was given for the remaining period. After the first 5 wk of feeding, one animal from each experimental group was killed and autopsied. All surviving animals were kept under observation for a total period of 69 wk. Autopsies were performed on all animals that died or were killed during the experiment or at termination.

For light microscopy, samples of all the major organs were fixed in 10% formalin and stained with haematoxylin and eosin or periodic acid–Schiff or by silver impregnation. For electron microscopy, pieces of the liver tissue and/or tumours were fixed in 2·5% glutaraldehyde for 12 hr, post-fixed in 1% osmium tetroxide for 2 hr and embedded in Epon 812 for sectioning. Ultrathin sections were double-stained with uranyl acetate and lead citrate.

Results

No pathological changes were detected grossly or by light microscopy in the rats killed at wk 5, but electron microscopy showed a marked proliferation of the smooth endoplasmic reticulum (Fig. 1) and an

Table 1. *Incidence of hepatic carcinomas and testicular Leydig-cell tumours in rats given sterigmatocystin and or dimethylnitrosamine*

Group no.	Carcinogens administered STG (ppm)	NDMA (ppm)	No. of rats alive at wk 1	54	No. of rats with Hepatic carcinoma	Leydig-cell tumours	Mean time to autopsy (wk)
1	10	—	15	14	8/15	0/15	60
2	10	1	20	15	15/20	3/20	57
3	1	10	20	17	1/20	9/20	69
4	—	10	15	14	0/15	7/15	69
5	—	—	30	25	0/30	0/30	69

accumulation of inter- and perichromatin granules, in the hepatocytes of the rats fed diets containing STG and/or NDMA. At a later stage, hepatic carcinoma developed in 53, 75 and 5% of the animals in groups 1 (10 ppm STG), 2 (10 ppm STG/1 ppm NDMA) and 3 (1 ppm STG/10 ppm NDMA), respectively. These were diagnosed at mean autopsy times of 60, 57 and 69 wk respectively, for these three groups. No metastases were found. Tumours in the liver were first observed after 54 wk of treatment with STG alone (group 1) and after 46 wk of treatment with STG plus NDMA (group 2). The number of rats with hepatic carcinoma increased after wk 60 in group 1 and wk 54 in group 2. By wk 69 the incidence of hepatic carcinomas was 8/14 and 15/15 in groups 1 and 2 respectively. Only one other hepatic carcinoma was detected, at wk 69, and this was in a rat given 1 ppm STG plus 10 ppm NDMA (group 3). There were no hepatic carcinomas in rats fed 10 ppm NDMA alone or in control rats.

In rats from groups 1 and 2 autopsied between wk 40 and 50 multiple gray-white nodules up to 1 mm in diameter were visible on the surface of the liver. Histological examination revealed that these nodules were clear-cell foci or neoplastic nodules. In the ensuing 12 wk they developed into hepatic carcinomas. Occasionally, circumscribed, firm, gray-white neoplastic lesions were present in every lobe of group 1, 2, and 3 rat livers and were usually from 0·5 to 2·0 cm in diameter. There was a hollow area at the surface of the neoplastic lesion in several instances. Histologically, most hepatic carcinomas were diagnosed as well- and/or poorly-differentiated trabecular type; moreover, about 50% of the hepatic carcinomas induced by STG and/or STG plus NDMA showed characteristic features (Fig. 2). These tumours showed glandular arrangements and occasional wide lumina. The lining cells were in direct contact with underlying hepatocellular carcinoma cells without intervening connective tissue. The development of hepatic carcinoma with glandular arrangement was independent of the length of time during which the animal had been receiving an experimental diet. Those tumours which showed a hollow area on the surface were always adenomatous in nature. The lumina were usually irregular, the lining cells were cuboid or flat and the cytoplasm was eosinophilic. Cells with glandular arrangement showed polarization of organelles in relation to the luminal and basal surface. With silver impregnation, they showed basement membrane and abundant granules at the luminal surface of the cytoplasm, while most of the hepatic carcinoma cells showed no such polarized distribution of the cell organelles. Occasionally the lumina of these glands were filled with eosinophilic material which did not stain with periodic acid–Schiff. Mitotic figures were frequently observed. A transitional form between lining cell and trabecular hepatic carcinoma cell was seen (Fig. 3). Clear cell foci and basophilic areas were numerous in the liver of rats treated with STG plus NDMA. The livers of rats treated with 10 ppm NDMA for 54 wk showed almost normal histological patterns.

The ultrastructure of well-differentiated hepatocellular carcinoma was similar to that reported previously (Ghadially & Parry, 1966; Ma & Webber, 1966; Ruebner, Gonzalez-Licea & Slusser, 1967; Yasutake, Nakao & Matsunaga, 1962). Lining cells both with and without basement membrane were distinguished (Figs 4 & 5). The lining cells with basement membranes usually showed dense collagen fibres and several fibroblasts under the basement membrane, while those without the membrane had no such stromal components. The ultrastructure of these two cell types was essentially similar. Most of lining cells showed a transitional form between hepatocytes and biliary cells (Figs 4 & 5). Numerous small microvilli were present on the luminal surface of the lining cells. The cells connected with neighbouring cells at complicated interdigitations. Intracytoplasmic fibrils were abundant and they anchored frequently in desmosomes. The granular endoplasmic reticulum was relatively scanty. Ribosomes attached not separately but in contact with each other on the cisternae of the granular endoplasmic reticulum and were usually only on one side of the cisternae (Fig. 5 inset). Free ribosomes and polysomes were numerous in the cytoplasm of tubular types of the hepatic carcinoma cells. Smooth endoplasmic reticulum was frequently proliferated. Membrane whorls were often present in the cytoplasm of both lining cells and underlying hepatocellular carcinoma cells (Fig. 4). Glycogen particles had decreased in number. Peroxisomes were always present. Golgi apparatus was well-developed. Mitochondria were numerous and of normal shape, though electron-dense matrix granules were more numerous than usual. Nuclei were irregular in contour and nucleoli were always prominent.

Until wk 54 no macroscopic tumours apart from those in the liver were found in any rats fed NDMA. Tumours of the testis were encountered thereafter in all groups of rats that had received NDMA. The incidence of these tumours was 15% in group 2, 45% in group 3 and 47% in group 4. The contra-lateral

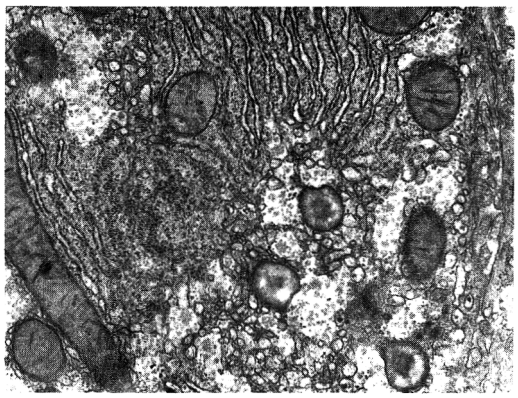

Fig. 1. Hepatocyte from a rat fed a diet containing 10 ppm STG and 1 ppm NDMA for 5 wk, showing proliferation of smooth endoplasmic reticulum in the cytoplasm. × 20,000.

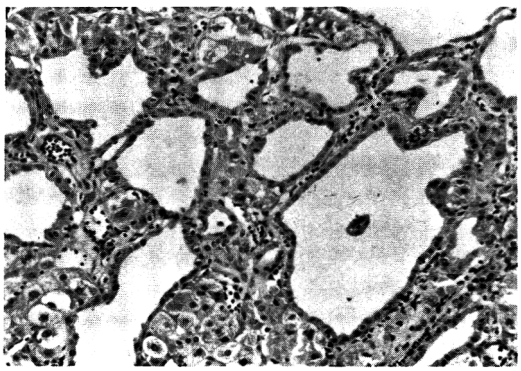

Fig. 2. Hepatic carcinoma from a rat of group 2, that received a diet containing 10 ppm STG and 1 ppm NDMA for 54 wk, showing a glandular arrangement of carcinoma cells, and lining cells in direct contact with the underlying carcinoma cells. Haematoxylin and eosin. × 200.

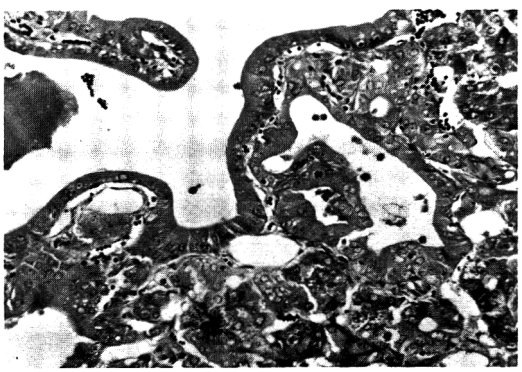

Fig. 3. Hepatic carcinoma from a rat of group 2 showing transitional form between lining cells and trabecular hepatic carcinoma cells. Haematoxylin and eosin. × 500.

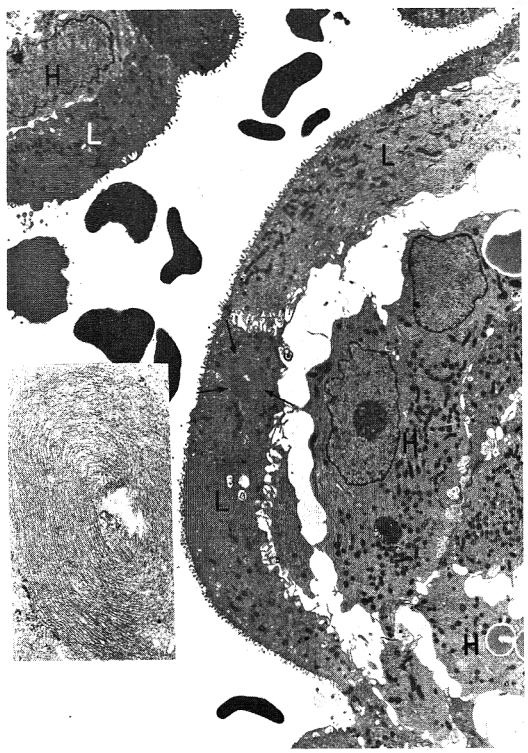

Fig. 4. Hepatic carcinoma of a group 2 rat showing lining cells (L) without basement membrane but having well-developed microvilli at the luminal surface of the cytoplasm, and a membrane whorl (arrowed, Inset). H: Malignant hepatocyte. × 3000. Inset: × 18,000.

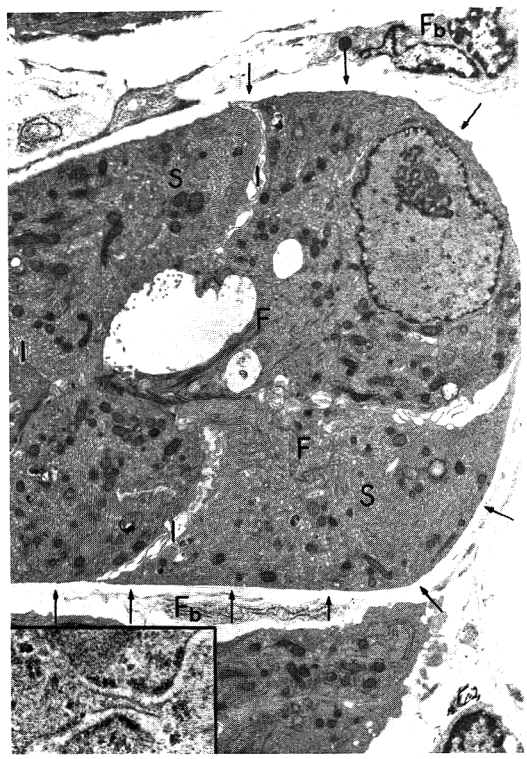

Fig. 5. Hepatic carcinoma, induced by STG, showing a distinct basement membrane (arrowed) around the carcinoma cells of glandular arrangement and several fibroblasts (Fb) under the membrane. In the cytoplasm of the tumour cells there are abundant microfibrils (F), proliferated smooth endoplasmic reticulum (S), interdigitations (I) and free ribosomes. × 5300. Inset showing an irregular arrangement of ribosomes on the cisternae of granular endoplasmic reticulum. × 60,000.

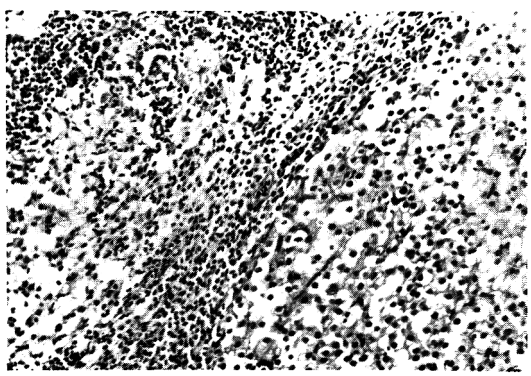

Fig. 6. Leydig-cell tumour cells in a rat fed 10 ppm NDMA for 69 wk. The tumour is composed of small cells with scanty cytoplasm and large cells with abundant eosinophilic vacuolated cytoplasm. Haematoxylin and eosin. × 200.

testis was occasionally atrophic. No mascroscopic tumours were present in the testes of rats in group 1 and the control group.

Testicular neoplasms in rats treated with NDMA or NDMA plus STG were typical Leydig-cell tumours, soft and yellowish white, and were up to 1·5 cm in diameter. Large tumours were composed of small cells with scanty cytoplasm and large cells with abundant eosinophilic vacuolated cytoplasm (Fig. 6). In the immediate vicinity of the tumour tissue, seminiferous tubules were atrophic. The proliferation of Leydig cells in the testes of older rats in all groups was frequently seen. They were detected only on microscopic examination. Their histological pattern was essentially similar to that of interstitial cell tumours.

Discussion

The data presented here demonstrated that a low concentration of NDMA may act synergistically in STG hepatocarcinogenesis. This result agrees with the previous report of the syncarcinogenic effect of minute doses of the four carcinogens: NDMA, nitrosodiethylamine, nitrosomorpholine and 4-dimethylaminoazobenzene (Schmähl, 1970). The incidence of hepatic tumours was higher in group 2 fed STG and NDMA than in those fed STG alone and the tumours developed more rapidly in the former group, although under the conditions of this experiment the level of NDMA used was apparently below the effective hepatocarcinogenic dose. No pathological changes were detected in rats fed 10 ppm NDMA continuously for 54 wk, but Magee & Barnes (1956) found that 50 ppm NDMA induced tumours in almost all the treated animals.

There is increasing evidence that minimal doses of two carcinogens elicit either a synergistic response or an inhibitory effect on liver carcinogenesis. Although there is still some disagreement about the exact factors determining either accentuation or suppression of hepatocarcinogenesis, it was confirmed recently that many carcinogens, including NDMA and aflatoxin B_1 are activated by drug-metabolizing enzymes (Nagata & Kodama, 1976). Fouts, Rogers & Gram (1966) have shown that there is a close relationship between the proliferation of smooth endoplasmic reticulum and the induction of drug-metabolizing enzymes such as *P*-450. It is interesting, therefore, that the proliferation of the smooth endoplasmic reticulum was always observed in the livers of rats fed with experimental diets containing NDMA. Although the low level of NDMA in the present experiments induced no hepatic carcinomas, it may play an important role in STG-carcinogenesis as an inducer of carcinogen-activating enzymes.

About 50% of hepatic carcinomas induced by STG or STG plus NDMA showed tubular arrangement of tumour cells. The histological pattern of this tumour is different from that of cholangiocarcinoma. Histologically, the classical cholangiocarcinoma is composed of single columnar tumour cells and stroma in which there are abundant fibroblasts, collagen fibers and capillaries. In contrast, tubularized hepatic carcinoma induced by STG or STG plus NDMA usually had only capillaries as stroma. The fine structure of these tumour cells was intermediate between ductular cells and hepatic parenchymal cells. The characteristic organelles of the hepatocyte, such as peroxisomes, proliferating agranular endoplasmic reticulum, and membrane whorls, were found in the same cells as were the typical organelles of ductular cells such as basement membrane, abundant intracytoplasmic fibrils, well-developed Golgi apparatus and free ribosomes. Glandular or tubular arrangement of hepatocytes has been observed in a variety of experimental conditions and in several human livers (Ghadially & Parry, 1966; Ma & Webber, 1966; Phillips & Steiner, 1966; Ruebner *et al.* 1967). However, little information is available about the hepatic carcinoma consisting of cells with organelles intermediate between ductular and hepatocyte types. Biliary hepatocytes, as designated by Phillips & Steiner (1966), are those hepatocytes which have a basement membrane and show distinctly a polarization of organelles toward a major biliary pole. Ruebner *et al.* (1967) reported a hepatoma with the coexistence of hepatomatous and cholangiocarcinomatous areas in the same cell. Such an observation was confirmed in rat hepatic carcinoma induced by 3′-methyl-4-dimethylaminoazobenzene (Ma & Webber, 1966). Whether these tubularized hepatic carcinomas represent ductular metaplasia of hepatocellular carcinoma cells or dedifferentiated hepatoblastoma is not certain.

The subject of Leydig-cell tumours in the testis of laboratory animal species is well documented. Spontaneous Leydig-cell tumours are occasionally found in aged rats and the incidence of the tumour shows a wide range depending on the strain. Crain (1958) reported a 9·3% incidence of spontaneous testicular interstitial tumours in Wistar rats aged between 18 and 24 months, whereas the incidence was 68% in Fischer rats aged between 16 and 30 months (Jacobs & Huseby, 1967). These tumours can be induced experimentally by several methods including the subcutaneous injection of cadmium (Gunn, Gould & Anderson, 1965), ligation of testicular vessels (Takewaki, 1962), administration of exogenous oestrogens (Hooker & Pfeiffer, 1942; Huseby, Dominguez & Samuels, 1961) and intrasplenic grafting of the testis (Twombly, Meisel & Stout, 1949). In this study, a relatively high incidence of Leydig-cell tumours was encountered only in rats receiving diets containing NDMA for a limited time. Moreover, a dose-response relationship for NDMA is indicated in the present experiments. Therefore, NDMA may accentuate the susceptibility of the testes to these tumours, although frequently spontaneous Leydig-cell tumours are encountered in the same strain. The histological features of NDMA-induced Leydig-cell tumours correspond in most respects with those previously reported for tumours of these cells (Crain, 1958; Gunn, Gould & Anderson, 1965; Hooker & Pfeifer, 1942; Jacobs & Huseby, 1967; Takewaki, 1962; Twombly *et al.* 1949).

The present results, indicating a possible syncarcinogenic effect of minute doses of naturally-occurring carcinogens such as STG and NDMA, may be of considerable significance to human health.

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Fd Cosmet. Toxicol. Vol. 16, pp. 597–600

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REORGANIZATION OF THE UK TOTAL DIET STUDY FOR MONITORING MINOR CONSTITUENTS OF FOOD

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Summary—To estimate average intakes of food constituents from total diet studies, representative amounts of key foods are obtained throughout the country. The food samples are prepared and cooked (if necessary) as for eating and then grouped for chemical analysis. The British study developed in 1966 for estimating pesticide intakes has been modified to improve its usefulness for estimating the intake of heavy metals and other food constituents, and simplified by reducing the number of foods from 78 to 68 and by eliminating most seasonal and regional differences in food purchases. The quantities of food have been updated and coverage has been extended to the whole of the United Kingdom.

Introduction

In the early 1960s there was worldwide concern about the traces of pesticides that might remain in food when it reached the consumer. As a result, it was recommended that in Britain "selective surveys [be carried out] to determine the residues of pesticides in raw and processed foods, both home-grown and imported, where pesticides have been applied in commercial practice..." (Lee, 1966). Because of the limited facilities available, it was necessary to restrict the sampling to staple foodstuffs, to foods that were important to vulnerable groups of the population such as babies, to foods eaten in fairly large quantities at certain times of the year, or to foods that were likely to be eaten without washing or other preparation that might reduce any residues present (Lee, 1966). Useful though such studies were for estimating risks in special situations, the need to determine average or background exposures of the population to pesticides soon arose. A 'total diet' study, similar to American 'market basket' surveys, was therefore set up in 1966 for this purpose, and its organization has been described in detail (Harries, Jones & Tatton, 1969).

The total diet study is still carried out, and has provided much invaluable information. But during the past decade the emphasis has shifted from pesticides (Abbott, Crisp, Tarrant & Tatton, 1970; Abbott, Holmes & Tatton, 1969; Abbott & Tatton, 1970; Department of Industry, 1976; Department of Trade and Industry, 1973) to toxic heavy metals (Ministry of Agriculture, Fisheries and Food, 1971, 1972, 1973a,b, 1975 & 1976) and has most recently included a variety of other trace elements, including nutrients, and organic constituents such as polychlorinated biphenyls (Ministry of Agriculture, Fisheries and Food, 1978; Thorn, Robertson, Buss & Bunton, 1978). The information needed for most of these constituents was not readily obtained from surveys designed to determine pesticide intakes. The piecemeal changes that were introduced to meet the new demands were eventually rationalized. Furthermore, food habits had changed during the intervening decade. The study was therefore thoroughly revised in 1975 in accordance with the recommendations of the Total Diet Study Group which reports to the Steering Group on Food Surveillance of the Ministry of Agriculture, Fisheries and Food (1978). This paper describes the changes that were made and the reasons for them, but results from the new study will be published separately.

General approach to the study

The purpose of the total diet study is to provide, as far as possible, ready estimates of the average intake of any food constituent of current concern in the UK. It can also be used to indicate which types of food require more detailed investigation before the range of possible intakes can be determined (Ministry of Agriculture, Fisheries and Food, 1978).

The general approach is the same as that of the original study (Harries *et al.* 1969) so that results can be compared with those already obtained. Its essentials are:

(i) that the number of foods included should be limited to the major components of the diet and to foods that are of special interest (eg shellfish, which contain large amounts of trace metals);

(ii) that the foods should be bought from a wide variety of retail outlets throughout the country;

(iii) that representative amounts should be prepared and cooked (if appropriate) as for eating, so that any contaminants that would normally be washed off or discarded with the inedible parts of the food would be excluded. The edible parts would then be combined into groups for analytical convenience.

It was necessary, however, to re-examine the case for the inclusion of each food and the ways in which they were grouped, and to update the quantities taken to reflect newer dietary patterns.

Foods included

The 78 different foods included in 1966/67 were combined into seven groups—namely cereals; meats

and related foods (including fish); fats and fatty foods; fruits and preserves; root vegetables; green vegetables and miscellaneous foods; and milk (Harries *et al.* 1969). The combined meat and fish group originally contained only two types of fish (fresh, frozen or cured fish and canned) but in 1970 the main emphasis of the total diet survey was on the estimation of mercury intakes. Accordingly, coverage was broadened to include representative amounts of white fish, fatty fish, shellfish, canned salmon, other canned fish and cooked fish, and other fish products. Later, when interest centred on lead intakes, samples of tap-water were added.

During the review of the study, many additional foods were proposed for inclusion on the grounds that they might provide significant quantities of a particular contaminant, either because they were widely consumed (e.g. alcoholic drinks) or because they might be especially prone to contamination (e.g. herbs and spices). Another proposal was that the study should be designed afresh for each contaminant. However, it was decided to keep the study as simple as possible, and there were few additions apart from the fish and water samples mentioned above. Some similar types of food were combined and others deleted, reducing the workload from 78 to 68 separate items. The foods now included, and the way in which they are grouped, are shown in Table 1. The most important changes are that the meat and fish groups have been completely separated, and that tea, coffee, and concentrated and ready-to-drink soft drinks have been brought together as a new group.

Amounts of foods and their preparation

The amounts of foods to be taken were originally based on total household food purchases together with allowances for food used in catering, as derived within the Ministry of Agriculture, Fisheries and Food for use in National Income and Expenditure estimates for 1964. Regional and seasonal differences in consumption were then superimposed upon these on the basis of National Food Survey records (Harries *et al.* 1969). In the new study the amounts have been derived solely from National Food Survey records of household food purchases for 1974 (Ministry of Agriculture, Fisheries and Food, 1976). Although this excludes catering use, it does reflect most major changes in dietary patterns, such as the increased consumption of chicken and quick-frozen foods and decreased bread consumption, and is more amenable to periodic updating. In addition, it was decided to ignore regional differences in food purchases since they are generally small compared with the analytical and other variables in the study. Seasonal differences were also ignored except for the two groups of foods where they are especially important, non-root vegetables and fruits (with sugars). In effect,

Table 1. *Foods used in the preparation of total diet samples, with weights as purchased (g)*

1. Cereals		2. Meats		3. Fish		4. Oils and fats		5. Fruits and sugars*	
White bread	246	Beef or veal	78	White fish	201	Butter	148	Citrus fruits	35–67
Other bread	46	Mutton or lamb	43	Fat fish	44	Margarine	68	Apples	67–84
Flour	46	Pork	33	Shellfish	7	Lard or compound cooking fats	47	Bananas	26–32
Cakes	41	Bacon or ham	53	Canned salmon	22	Other fats or oils	31	Other fresh fruit	16–64
Biscuits	49	Liver	7	Other canned fish	48	Cream	16	Canned or bottled fruit	37–42
Breakfast cereals	25	Other offal	4	Cooked fish or other fish products	178	Condensed milk	36	Fruit juice	11–12
Oatmeal or its products	4	Poultry	54			Cheese	98	Preserves or syrups	25–29
Rice	5	Sausages	37			Ice cream	38	Nuts, other fruit or fruit products	8–21
Puddings	16	Canned or corned meat	22			Baby foods	18	Sugar	134–141
Other cereal products	22	Canned soup	36					Sweets	83–91
		Other meat or meat products	45						
		Eggs	88						

6. Root vegetables		7. Other vegetables*		8. Beverages		9. Milk	10. Water
Potatoes	406	Tomatoes	55–91	Tea	7	Liquid milk *c*800	Tap-water *c*800
Crisps or other potato products	22	Cauliflower	36–64	Coffee	2		
Carrots	26	Cabbage	67–70	Concentrated soft drinks	20		
Onions, leeks or shallots	27	Brussel sprouts	1–65	Ready-to-drink soft drinks	71		
Other fresh root vegetables	19	Peas, fresh or frozen	12–62				
		Peas, canned	34–54				
		Beans fresh or frozen	12–62				
		Beans, canned	43–69				
		Leafy salads	10–29				
		Other vegetables or products	91–112				

*The ranges show the lowest and highest quarterly amounts to be purchased.

therefore, the national average diet was to be collected in different parts of the United Kingdom (as below) and in different seasons of the year, to achieve the most representative examples of that diet.

The amounts of each food to be bought are included in Table 1. Each group except the liquids totals 500 g of food, but the actual quantities in the final samples will be substantially less than this in four groups because of losses of inedible materials such as bones and skins during preparation and further losses during cooking of many of the meats and vegetables. Where a food may be eaten either raw or cooked (as with tomatoes), it is included raw as before (Harries *et al.* 1969) to indicate maximal contamination. Cooking is also omitted for convenience if the quantities are very small, as for rice and offals other than liver. And as the transfer of foodstuffs from one group to another is to be avoided, foods are not fried in fat but simply grilled or boiled. Tea and coffee grounds (if not 'instant') are not directly added to the analytical sample of beverages because many constituents, being insoluble, would not be drunk. Infusions are prepared from the given quantities with as much water as would be used in the home, and strained before inclusion. The volumes are recorded and all the beverages in the group are mixed. A sub-sample is sent for analysis to avoid the inconvenience of posting the full volume, often nearly 1 litre. For any constituent the amount present in the whole sample can then be calculated.

The appropriate amounts of each raw, prepared or cooked food in each group are combined and finely minced or blended to give a homogeneous sample. The ten separate food samples (including the milk and water samples) are finally posted as quickly as possible to the Laboratory of the Government Chemist in London, where they are analysed.

Regional coverage of the survey

In 1966/67, the foods were purchased and prepared in colleges that specialized in home economics or had departments of food science or allied expertise. These colleges were distributed throughout England and Wales (Harries *et al.* 1969). When the study was repeated in 1970/71 the coverage was extended to the whole of the United Kingdom by the inclusion of three colleges in Scotland and one in Northern Ireland, but only 11 of the original 20 colleges were still able to participate. There have been further changes in the colleges since then, and the following colleges participated in 1976/77: Robert Gordons Institute of Technology, Aberdeen; Queen Margaret College, Edinburgh; Queen's College, Glasgow; Loughry College of Agriculture and Food Technology, Cookstown, Northern Ireland; Llandaff College of Education, Cardiff; Northern Counties College, Newcastle; Totley-Thornbridge College of Education, Sheffield; Ilkley College of Housecraft; F. L. Calder College of Education, Liverpool; Radbrook College, Shrewsbury; Worcester College of Education; Leicester College of Education; Brighton Technical College; Brunel Technical College, Bristol; South Devon Technical College, Torquay; Highlands College, St. Helier, Jersey.

London and the industrial Midlands are not represented at present, but the remainder of the United Kingdom is well covered. It is not essential that every region is included, for the level of a constituent in one sample of food from a single college can never be taken as showing the level of contamination in an entire region; nevertheless efforts are being made to interest new colleges in the study to produce a more representative picture of national intakes. It would be essential to include a college near London if unstable constituents such as nitrosamines or vitamins were to be examined, so that delay before analysis could be minimized.

Each college now provides sets of food samples in no more than two quarters of the year instead of all four as required in the first study. Under the direction of the staff member who has been briefed in detail about the purpose of the study and the precautions to be taken, the students buy, weigh, prepare and cook, re-weigh, mix and homogenize the foods required. Where there is a choice of foods, examples are given (e.g. "other meat and meat products" may include rabbit, game, meat pies, sausage rolls, frozen meat products, meat pastes, liver sausage, haggis, polony or Scotch eggs) and the college is asked to select the items most likely to be eaten in their region; similarly the methods of preparation and cooking should also be appropriate to each region.

Table 2. *Relative importance of each food group in the total diet*

Food group	Contribution to household diet as purchased (kg/person/day)	Loss in preparation and cooking (%)	Estimated weight of food eaten (kg/person/day)
Cereals	0·23	0	0·23
Meat	0·19	20	0·15
Fish	0·02	15	0·02
Fats	0·08	0	0·08
Fruits and sugars	0·19	10	0·17
Root vegetables	0·23	20	0·18
Other vegetables	0·13	15	0·11
Milk	0·40	0	0·40
Beverages	0·12	0	0·12
Total....	1·59		1·46
Energy content (kcal/person/day)....	*c.*2450		*c.*2250

Interpretation of the results

To calculate the average dietary intake of any food constituent, the total amount of each group of foods that is eaten must be known, for only then can the analytical results be properly weighted. Although the amounts of foods purchased in Britain are known from the National Food Survey (Ministry of Agriculture, Fisheries and Food, 1976) the total diet analyses will be in terms of food as eaten. The relationships between the two therefore had to be calculated for each food group in the total diet from the detailed records and weighings of every item provided by each college in 1976—the first year in which the new study was operated. The average weightings so derived are shown in Table 2.

The results of using this revised total diet study to determine specific food constituents will be presented in detail in due course.

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T-2 TOXIN MYCOTOXICOSIS IN THE GUINEA-PIG

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Summary—Purified T-2 toxin dissolved in saline–dimethylsulphoxide (DMSO) 10:1 (v/v) was administered by gastric intubation in single doses of 1·85, 2·52, 3·45 and 4·66 mg/kg body weight to male guinea-pigs. The acute oral LD_{50} in the male guinea-pig was estimated to be 3·06 (2·38–3·93) mg/kg. Single doses of 2·5 or 5·0 mg T-2 toxin/kg body weight administered orally in ethanol–DMSO, 1:1 (v/v) resulted in gross lesions including gastric and caecal hyperaemia and haemorrhage, watery-fluid distension of the caecum, oedematous intestinal lymphoid tissue and adrenal gland hyperaemia. Histological alterations included necrosis of lymphoid tissue, bone marrow and testes, and necrosis and ulceration of the gastro-intestinal tract, most severe in the stomach and caecum. Guinea-pigs, treated orally with T-2 toxin dissolved in saline–DMSO, 10:1 (v/v) at the rate of 0·5 mg/kg/day for 21 days and then 0·75 mg/kg/day for 21 days, remained clinically normal and did not have gross or microscopic lesions, but a moderate leucopenia and an absolute lymphopenia were observed. Oral administration of T-2 toxin to guinea-pigs at a rate of 0·9 mg/kg/day for 27 days produced neither clinical disease nor gross and microscopic lesions, but did produce a decrease in erythrocyte numbers, a leucopenia and an absolute lymphopenia. Alterations in erythrocyte morphology and a marked decrease in the lymphocyte content of the bone marrow were also noted.

Introduction

Numerous cases of mycotoxicoses associated with Fusarium species have been recorded in several areas of the world (Hsu, Smalley, Strong & Ribelin, 1972; Ueno, Ishikawa, Nakajima, Sakai, Ishii, Tsunoda, Saito, Enomoto, Ohtsubo & Umeda, 1971). Ingestion of cereal grains contaminated with Fusarium has caused sporadic acute and chronic diseases characterized by nausea, vomiting and anorexia in both man and animals (Ueno *et al.* 1971). A trichothecene mycotoxin known as T-2 toxin has been consistently isolated from several species of Fusarium (Ueno, Ishii, Kanaeda, Tsunoda, Tanaka & Enomoto, 1972; Ueno, Sato, Ishii, Sakai, Tsunoda & Enomoto, 1973) and has been associated with naturally occurring outbreaks of mycotoxic disease in cows (Hsu *et al.* 1972). In rats, T-2 toxin caused necrotic and haemorrhagic lesions in the intestine, kidney and liver and the LD_{50} was 3·8 mg/kg body weight (Kasuir, Smalley & Nichols, 1971). In mice, purified T-2 toxin given ip had a radiomimetic effect, causing destruction of dividing cells in the thymus, bone marrow, small intestine, testes and ovaries (Ueno *et al.* 1973). The LD_{50} for mice after seven consecutive ip doses of T-2 was 5·2 mg/kg body weight (Ueno *et al.* 1972). The acute oral LD_{50} of T-2 toxin was approximately 4 mg/kg body weight in swine and rats and 6·5 mg/kg body weight in rainbow trout (Smalley, 1973).

Clinical signs in cats given T-2 toxin included vomiting, diarrhoea, anorexia, ataxia in the hind legs and an ocular discharge (Sato, Ueno & Enomoto, 1975). Doses of purified toxin given sc at a rate of 0·1 or 0·05 mg/kg body weight at 1–11-day intervals for 4 wk produced a leucopenia terminally. However, a temporary leucocytosis was noted following individual doses. Daily oral dosing with the crude toxin for 17 days at 5–7·5 mg/kg body weight also produced leucopenia. The sc daily doses required to induce leucopenia were 0·1 mg/kg body weight for 4 days or 0·05 mg/kg body weight for 8 days. Lesions in the cats included necrosis of the bone marrow, lymph node and spleen, haemorrhages in the lung and meninges, and vacuolar degeneration of renal tubules (Sato *et al.* 1975).

The clinicopathological features of experimental T-2 toxin poisoning have been evaluated only in the cat. The present studies were designed to evaluate the suitability of the guinea-pig as an experimental animal for studies with the T-2 toxin, to define the pathology of T-2 toxin mycotoxicosis in the guinea-pig and to evaluate any alterations that might occur in the haematopoietic tissue and the peripheral blood of guinea-pigs administered T-2 toxin.

Experimental

Animals. The guinea-pigs used in these studies had originated from the Hartley strain and each weighed approximately 300 g. They were housed in an air-conditioned building with environmental controls. Fresh drinking-water and commercial guinea-pig diet were provided *ad lib.*

Experiment I. Twenty male Hartley guinea-pigs were randomly divided into four groups of five (groups 1, 2, 3 and 4). Each group was housed in a separate cage. Purified T-2 toxin was administered as a single dose of 1·85, 2·52, 3·43 and 4·66 mg/kg body weight to the members of groups, 1, 2, 3 and 4 respectively. The toxin was administered by gastric intubation in saline–dimethylsulphoxide (DMSO, 10:1 (v/v).

After dosing, the guinea-pigs were monitored at 6-hr intervals for 3 days. Those animals found dead were autopsied. Survivors of the 3-day experimental period were killed with ether and autopsied. The median lethal dose was estimated according to the method of Weil (1952).

Experiment II. Thirteen male Hartley guinea-pigs were randomly divided into one group of three animals (group 1) and two groups of five (groups 2 and 3). Purified T-2 toxin in ethanol–DMSO, 1:1 (v/v), was administered to each animal in groups 1 and 2 in a single dose of 2·5 or 5·0 mg/kg body weight, respectively, by gastric intubation. The guinea-pigs were weighed before dosing. Group 3 animals were given the vehicle alone by gastric intubation.

After dosing the guinea-pigs were monitored hourly. Moribund guinea-pigs were killed with ether and autopsied. At autopsy, samples of all major organs were placed in 10% neutral buffered formalin for fixation and were then processed for histopathological evaluation.

Experiment III. Male Hartley guinea-pigs were randomly divided into a test group of five animals and a control group of two animals. The guinea-pigs of group 1 were given daily oral doses of 0·5 mg T-2 toxin/kg body weight for 21 days and then daily doses of 0·75 mg/kg for an additional 21 days. The toxin was administered by gastric intubation in saline–DMSO. 10:1 (v/v). The guinea-pigs of group 2 were given the vehicle alone daily, by gastric intubation.

Blood samples from both groups were collected into powdered EDTA from the pedal vein of each guinea-pig (Vallojo-Freire, 1951) on days 7, 14, 19, 22, 27, 33, 37 and 42 of the trial. Haematological determinations were done on all samples. Guinea-pigs of both groups were killed for autopsy on day 43. Samples of all the major organs were collected in 10% neutral buffered formalin for fixation and were then processed for histopathological evaluation.

Experiment IV. Six female and eight male Hartley guinea-pigs were randomly divided into two groups each consisting of three females and four males. The seven animals in group 1 were given daily oral doses of 0·9 mg T-2 toxin/kg body weight for 27 days. The toxin was administered by gastric intubation in saline–DMSO, 10:1 (v/v). and, the seven animals in Group 2 were given the vehicle alone by gastric intubation.

Blood samples were collected as in Experiment III from each guinea-pig of both groups on the day before dosing and on days 4, 9, 15, 21 and 27 of the experimental period. Bone-marrow aspirates were obtained on day 17 from the left femur while the guinea-pigs were under ether anaesthesia. Guinea-pigs of both groups were killed on day 28 for autopsy. Samples from all major organs were placed in 10% neutral buffered formalin for fixation and were then processed for paraffin sectioning and histopathological evaluation. Bone-marrow smears were prepared from the right femurs of all animals of both groups, by a single gentle brushing of the central portion of the marrow core with a camel-haired artist's brush previously bathed in bovine serum, followed by a similar brushing of clean glass slides.

Results

Experiment I

One guinea-pig given 1·85 mg T-2 toxin/kg was discovered dead 36 hr after dosing. The other animals of this group appeared normal throughout the 3-day observation period. One guinea-pig given 2·52 mg/kg was found dead 24 hr after dosing, and a second guinea-pig was clinically lethargic for the first 24 hr after dosing but recovered and appeared clinically normal for the next 48 hr. The remaining members of group 2 appeared normal for the entire experimental period. Three guinea-pigs from group 3 (3·43 mg/kg body weight) were found dead within 12 hr of dosing. The surviving two guinea-pigs were severely depressed for the first 48 hr after dosing, but were clinically normal at the end of the experimental period. The five guinea-pigs given 4·66 mg/kg (group 4) died between 6 and 12 hr after dosing.

Gross lesions were similar in all the guinea-pigs that died during the 3-day experimental period and consisted of gastric hyperaemia and haemorrhage, watery-fluid distension of the stomach, hyperaemia of the adrenal gland and oedema of the mesenteric lymph node. No gross lesions were found in the guinea-pigs that were killled.

Using the method of Weil (1952), the acute oral LD_{50} of T-2 toxin in guinea-pigs, with the 95% confidence interval, was calculated to be 3·06 (2·38–3·93) mg/kg body weight.

Experiment II

One of the members of group 1 (2·5 mg/kg) was found dead 11 hr after dosing. The two surviving guinea-pigs were moribund at this time and were killed and autopsied. One animal in group 2 (5·0 mg/kg) was found dead 16 hr after dosing. The other four guinea-pigs of this group were found moribund between 5 and 17 hr after dosing and were killed for autopsy between these times. Group 3 guinea-pigs (vehicle controls) remained clinically normal and were killed and autopsied 17 hr after dosing.

Gross lesions in the principal organs of groups 1 and 2 were most prominent in the gastro-intestinal tract and consisted mainly of gastric and caecal hyperaemia and haemorrhage. The caeca were often distended with a watery fluid and the intestinal lymphoid tissue was oedematous. Hyperaemia of the adrenal glands was a consistent finding. Oedema of

Table 1. *Summary of mean complete blood count parameters in T-2 toxin-treated and untreated guinea-pigs in experiments III and IV*

Experiment and group number	Oral treatment	Day	Total red blood cell count ($\times 10^6$/ml)	Total white blood cell count ($\times 10^3$/ml)	Total lymphocyte count/ml	Total plasma protein (g/100 ml)	Packed cell volume (%)	Haemoglobin (g/100 ml)	Total platelet count ($\times 10^3$/ml)	Differential white cell count (%)				
										Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils
III (1)	0·5 mg T-2 toxin/kg body weight for 21 days then 0·75 mg/kg for 21 days, given in 10:1 saline/DMSO	7	5·0 ± 0·4	7·0 ± 2·5	3453 ± 893	4·2 ± 0·1	42 ± 2	13·7 ± 0·2		42 ± 9	51 ± 9	3 ± 1	2 ± 1	1 ± 1
		42	5·4 ± 1·1	4·5 ± 0·6	1316 ± 353	4·8 ± 0·3	39 ± 2	13·7 ± 0·4	530·8 ± 46·5	67 ± 3	29 ± 5	3 ± 2	0	0
III (2)	10:1 saline/DMSO for 42 days	7	5·1 ± 0·6	6·7 ± 1·1	3690 ± 1606	4·2 ± 0·2	43	14·2 ± 0·1		40 ± 13	54 ± 16	5 ± 3	1	0
		42	6·0 ± 0·8	4·6 ± 0·7	2806 ± 111	4·7 ± 0·1	44 ± 5	14·3 ± 0·3	583·8 ± 100·8	34 ± 10	64 ± 8	3 ± 2	0	0
IV (1)	0·9 mg T-2 toxin/kg body weight for 27 days in 10:1 saline/DMSO	0	5·5 ± 0·6	5·8 ± 1·1	3813 ± 729	4·5 ± 0·2	34 ± 1	13·3 ± 0·8	630·7 ± 127·2	32 ± 7	64 ± 9	2 ± 2	2 ± 2	0
		27	4·2 ± 1·0	4·0 ± 1·4	2392 ± 872	4·6 ± 0·3	35 ± 7	11·5 ± 2·5	537·1 ± 140·3	34 ± 14	62 ± 16	3 ± 2	1 ± 1	0
IV (2)	10:1 saline/DMSO for 27 days	0	5·8 ± 0·8	6·4 ± 1·7	4670 ± 1581	4·6 ± 0·3	37 ± 3	13·5 ± 0·8	506·4 ± 112·2	27 ± 7	71 ± 7	2 ± 1	1 ± 1	0
		27	5·4 ± 0·4	6·8 ± 0·9	4818 ± 843	4·8 ± 0·3	45 ± 4	14·5 ± 1·5	613·2 ± 135·2	26 ± 4	71 ± 4	3 ± 1	1 ± 1	0

the mesenteric lymph nodes and haemorrhages in the lung were only occasionally observed.

Lesions were found in group 1 and 2 guinea-pigs in the lymphoid tissue, gastro-intestinal mucosa, bone marrow and testes. Necrosis involved the cortical follicles of the lymph nodes (Figs. 1 & 2) and the lymphoid tissue in other locations, including Peyer's patches, peribronchiolar lymphoid follicles and palpebral lymphoid follicles. Necrosis was less prominent in the thymus and was minimal in the splenic lymphoid tissue (Fig. 3).

Mucosal lesions were present throughout the gastro-intestinal tract, but were most severe in the caecum and stomach. The caecal alterations were mucosal haemorrhage, necrosis of mucosal epithelium and necrosis within the centres of the subepithelial lymphoid nodules (Figs. 4 & 5). In the stomach, the fundic mucosa was most severely involved and the lesions were mucosal haemorrhage, mucosal necrosis and multiple focal ulceration. Focal areas of villar necrosis with pseudomembrane formation as well as cast formation within crypts were found in the small intestine. Similar, but less severe, lesions were found in several portions of both the small and large intestine. Marked mucosal hyperaemia was present in many areas even in the absence of mucosal necrosis.

The bone marrow was remarkably cellular. Necrosis of marrow elements was widespread, as nuclear debris was scattered throughout the marrow. Occasionally, large reticulo-endothelial cells contained quantities of cellular debris (Figs. 6 & 7).

Degeneration and necrosis involved the cells of the seminiferous tubules of the testis. Only primitive spermatogenic cells remained in the seminiferous tubules and many of these were necrotic, as evidenced by nuclear pyknosis and karyorrhexis.

Experiment III

Body-weight gains of the T-2 toxin-treated guinea-pigs (group 1) were much lower than those of the untreated animals of group 2. The total weight gain of the treated animals over the 42-day period was 180 g whereas that of the control animals was nearer 250 g. Other clinical signs of toxicity were not seen.

Haematological determinations for both the T-2 toxin-treated animals in group 1 and the untreated guinea-pigs of group 2 are summarized in Table 1. Total counts for erythrocytes and platelets were similar for guinea-pigs in both control and treated groups throughout the 42-day experimental period. However, total leucocyte counts in the treated guinea-pigs were moderately depressed and this was particularly evident after day 33. Total lymphocyte counts of treated guinea-pigs were also markedly reduced after day 21 compared to the control counts. No gross or microscopic lesions were found in control or treated guinea-pigs.

Experiment IV

Body weights of treated guinea-pigs were reduced compared with the controls. The total weight gain of the treated animals over the 27-day period was 40 g compared with an increase of over 130 g in the control group. No other signs of toxicity were seen as both groups appeared clinically normal.

Haematological determinations for both the T-2 toxin-treated (group 1) and untreated (group 2) guinea-pigs are summarized in Table 1. Total plasma proteins and platelet numbers for both groups were similar over the 27-day experimental period. Erythrocyte numbers were reduced in the T-2 toxin-treated guinea-pigs compared with the controls and the decrease was gradual. There were dramatic differences in the erythrocyte morphology of the two groups, those in the treated animals showing basophilic stippling, fragmentation and increased polychromasia and anisocytosis (Fig. 8) as well as some nucleate forms. These changes became more severe over the experimental period.

Both the total leucocyte and total lymphocyte counts were markedly reduced in the T-2 toxin-treated guinea-pigs and the decline was gradual.

In the bone marrow, the myeloid:erythroid ratio (M:E ratio) was markedly reduced as compared to the controls. A reduction in the number of lymphocytes within the bone-marrow samples was also demonstrated in the treated guinea-pigs of group 1 (Table 2). No gross or microscopic lesions were found in control or treated guinea-pigs.

Discussion

The acute oral LD_{50} of T-2 toxin in guinea-pigs was estimated to be 3·06 (2·38–3·93) mg/kg body weight. This value was lower than values reported for other experimental animals (Kosuri *et al.* 1971; Smalley, 1973; Ueno *et al.* 1972) suggesting greater susceptibility of the guinea-pig.

Lesions observed in the guinea-pig in these studies supported the previous suggestion (Ueno *et al.* 1973) that T-2 toxin has a radiomimetic effect. Tissues most severely affected were the rapidly dividing cells of the gastro-intestinal mucosa, bone marrow, lymph nodes and testicle. Marked lesions in the spleen and haemorrhages in the lung and meninges, as well as renal tubular epithelial degeneration, reported by others (Sato *et al.* 1975) were not found in our guinea-pigs.

Table 2. *Summary of mean bone-marrow parameters in guinea-pigs treated with T-2 toxin and untreated guinea-pigs in experiment IV*

Group	Oral treatment	Day	Myeloid:Erythroid ratio	Lymphoid (% of total nucleated cells)
1	0·9 mg T-2 toxin kg body weight for 27 days, given in 10:1 saline DMSO	17	0·876	35·8
		27	0·938	35·0
2	10:1 saline DMSO for 27 days	17	2·289	45·5
		27	2·099	43·7

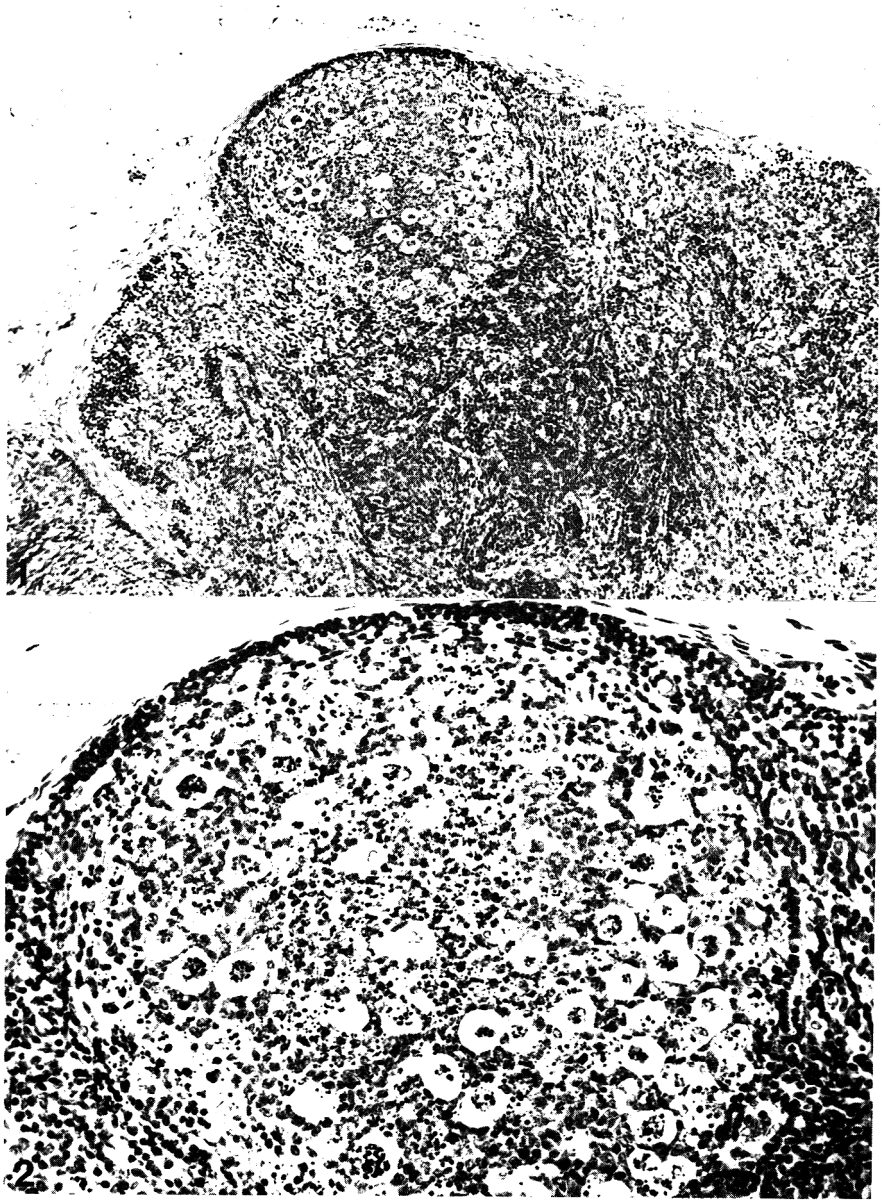

Fig. 1. Cortical follicular necrosis within the mesenteric lymph node. Haematoxylin and eosin (H & E) × 84.

Fig. 2. Higher magnification of lymph node follicle in Fig. 1 demonstrating necrosis characterized by pyknosis, karyorrhexis and karyolysis. H & E × 224.

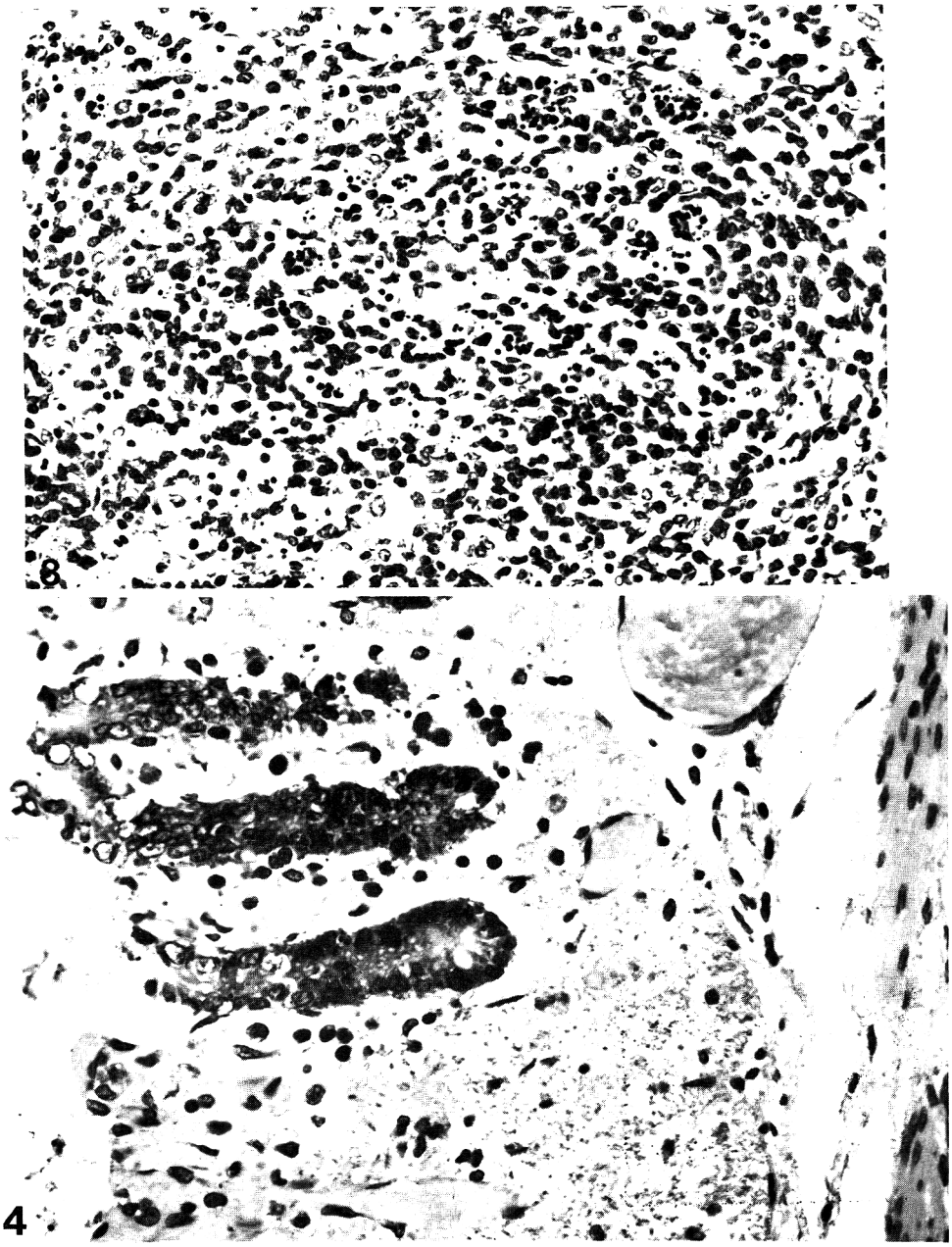

Fig. 3. Moderate lymphoid necrosis in the spleen characterized by pyknosis, karyorrhexis and karyolysis. H & E × 350.

Fig. 4. Pathological changes in caecal mucosa, including coagulative necrosis of the mucosa and congestion. H & E × 350.

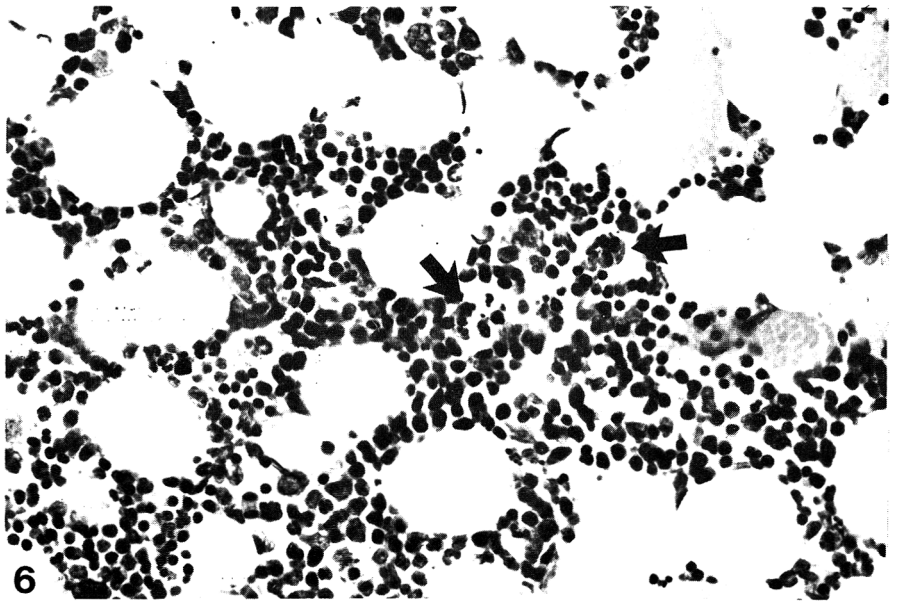

Fig. 5. Necrotizing typhlitis and several degenerating lymphoid follicles. H & E × 56.

Fig. 6. Focal necrosis of marrow precursor cells accompanied by nuclear debris throughout the marrow (arrowed). H & E × 350.

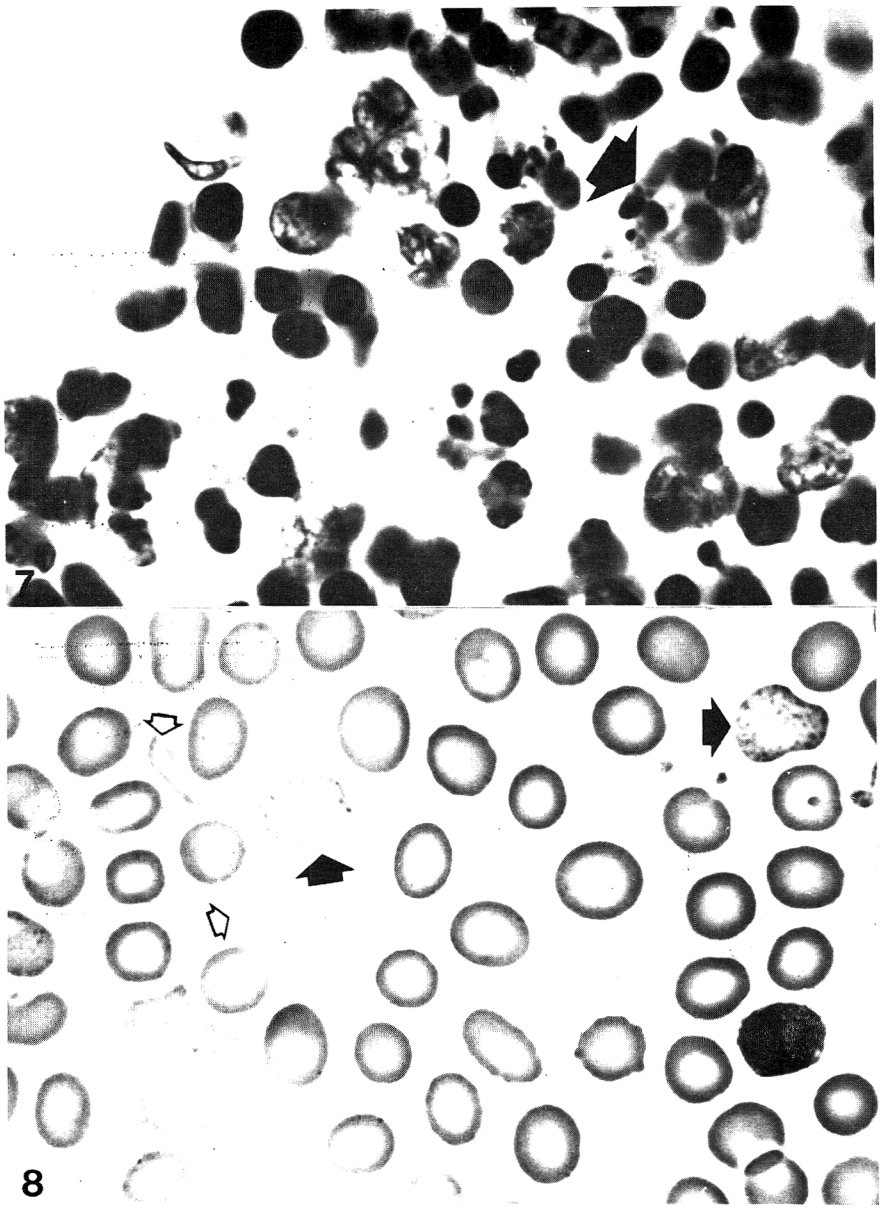

Fig. 7. Macrophages of bone marrow laden with debris (arrowed). H & E × 644.

Fig. 8. Basophilic stippling of polychromatophilic cells, and red-cell fragmentation (arrowed). Wrights stain × 644.

The lower dosage levels administered in Experiment III produced a reduction in body weights, a leucopenia and an absolute lymphopenia. The latter response was reported also in cats receiving T-2 toxin (Sato *et al.* 1975). Because lesions were not seen microscopically in lymphoid tissues in guinea-pigs given these doses of T-2 toxin, the data suggested that the lymphopenia was the result of direct action of the mycotoxin on either lymphocyte production or release.

The slightly higher daily dosage level of 0·9 mg/kg body weight given in Experiment IV also resulted in reduction of body weights, a decrease in leucocyte counts and reduction in total lymphocyte counts. Depression of total lymphocyte counts was further exemplified by a reduction in the lymphocyte percentages in the bone marrow. This dose also produced changes within the erythron as indicated by a gradual decrease in erythrocyte numbers and alterations in erythrocyte morphology. Necrosis of bone marrow and other actively dividing cells (Ueno *et al.* 1973) caused by the administration of T-2 toxin suggests a possible direct destructive action on the erythroid components of the marrow. Changes in morphology of peripheral erythrocytes and the hypercellularity of the bone marrow with a markedly reduced M:E ratio suggests a hyperplastic reaction in the erythroid compartment of the marrow. Such a response would be expected from a sublethal dose of a radiomimetic material.

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INDUCTION OF DUODENAL SEROTONIN PRODUCTION BY DIETARY SODIUM SELENITE AND AFLATOXIN B_1

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Summary—Groups of Mongolian gerbils were fed diets supplemented with 5 ppm sodium selenite, 12·8 ppm aflatoxin B_1 or both. All groups receiving supplemented diets showed increased duodenal serotonin levels relative to the controls, indicating interference with serotonin metabolism by these agents both individually and combined. Aflatoxin B_1 alone had the greatest effect and sodium selenite alone, the least; the mixture had an intermediate effect. The possible mechanisms of this interference are discussed.

Introduction

Interference in tryptophan metabolism by aflatoxin B_1 (AFB_1) has been reported by several earlier researchers. Wogan & Friedman (1965) reported that AFB_1 inhibited the hydrocortisone-dependent induction of tryptophan pyrrolase but did not inhibit induction by the substrate. Using cortisone, Clifford & Rees (1966) observed similar results over a shorter time-course. Lemonnier, Scotto & Thuong-Trieu (1974; 1975) demonstrated a higher percentage of rats showing abnormal tryptophan-load results following intoxication with AFB_1. No previous investigations into AFB_1 effects on serotonin have been reported. The study of AFB_1, of selenium and of serotonin is important because of the increasing evidence that AFB_1 may be an intestinal (colon) carcinogen (Ward, Sontag, Weisburger & Brown, 1975), that selenium may protect against intestinal (colon) cancer (Schrauzer, White & Schneider, 1977; Shamberger, Rukovena, Longfield, Tytko, Deodhar & Willis, 1973) and that changes in serotonin concentrations are associated with intestinal neoplasia (Brown, 1977; Thompson, 1977). The protective action of selenium salts against various toxic reactions initiated by AFB_1 is also important (Aleksandrowicz, Dobrowolski, Lisiewicz, Smyk & Skotnicki, 1975; J. H. Lalor & G. C. Llewellyn, unpublished data, 1978; Newberne & Conner, 1974).

Experimental

Weanling male Mongolian gerbils, *Meriones unguiculatus* Tum, were purchased from Tumblebrook Farms, Inc., West Brookfield, MA. Upon arrival, the animals were inspected for general health and then housed individually in 20 × 50 × 10 cm transparent polystyrene cages with galvanized lids. All the animals were fed on ground rat chow purchased from Ralston Purina, Co., St. Louis, MO, and were given tap-water *ad lib.* during a 2-wk acclimatization period before the start of the dietary treatments. Environmental conditions were almost constant throughout the experiment: temperature, 22·8 ± 2·0°C; humidity, 68·2 ± 3·8%; barometric pressure, 768·8 ± 2·4 mm Hg; 14/10-hr fluorescent light/dark cycle.

Each of four groups of eight animals received one of the following: basal diet (Ralston Purina Co., 0·29% tryptophan), basal diet supplemented with 5 ppm sodium selenite (Na_2SeO_3, Nutritional Biochemicals Corp., Cleveland, OH), basal diet supplemented with 12·8 ppm AFB_1 (Calbiochem Co., La Jolla, CA) or basal diet supplemented with 5 ppm Na_2SeO_3 and 12·8 ppm AFB_1. The dietary concentrations of Na_2SeO_3 were verified by transient signal atomic absorption spectroscopy (Miller, Abel & Williams, 1975). The samples were digested in mixed-acid, diluted and a hydride was generated by $NaBH_4$ reduction. Using a shielded Ar/H_2 flame, the hydride was decomposed for quantitation. Similar analysis of the drinking-water showed no detectable selenium. Qualitative and quantitative methods employed to verify the AFB_1 concentrations used dilutions and visual readings from thin-layer chromatographic plates (Horwitz, Senzel & Reynolds, 1975). No aflatoxin contamination (< 2·0 ppb) was observed in the control diet. During the experiment, water and feed consumptions as well as faecal excretion were measured.

After 12 wk, all the animals were anaesthetized with ether and were exsanguinated from the heart; they were all killed between 1000 and 1300 hr. The proximal 2·5 cm of the duodenum were removed surgically for the serotonin analysis. The sample tissues were homogenized in *n*-butanol and were centrifuged. Serotonin was extracted by addition of a portion of the supernatant to a heptane—0·1 N-HCl mixture (Miller, Cox, Snodgrass & Maikel, 1970). An aliquot of this extract subsequently was reacted with the indicator *o*-phthaldialdehyde (OPT, Regis Chemical Co., Morton Grove, IL) for a period of 1 hr at 40°C. After this period, the conjugation reaction was ended by placing the tubes in ice. Fluorescence determinations were then performed on an Aminco-Bowman spectro-

photofluorometer. The activation and emission wavelengths were set at 360 and 470 nm, respectively. Final concentrations were obtained by extrapolation from the experimental readings on a standard curve determined immediately before the experimental analyses.

Results and Discussion

All three dietary supplements induced significant serotonin increases (Fig. 1). The mechanisms of these increases remain unclear. The increase produced in the group given Na_2SeO_3 may be a result of the metabolism of additional tryptophan absorbed from the gastro-intestinal tract. Another possibility is the increased activity of an enzyme which would accelerate the rate of serotonin biosynthesis. The increase in the serotonin of the AFB_1 group was similar to the increase reported by Thompson (1969). He observed a significant serotonin increase in the upper duodenum of Buffalo rats fed 0·25 g *N,N'*-2,7-fluorenylene-bisacetamide/kg. This increase occurred only in non-malignant carcinogen-exposed tissues and a significant decrease was observed in the malignant tissues examined. The increase in the present AFB_1 group could be explained partially by normal metabolism of higher available levels of tryptophan obtained from the ingested feed. Recently, Jorgensen & Majumdar (1976) have demonstrated that force-feeding tryptophan to rats increased the hepatic microsomal cytochrome *P*-450 content. If greater quantities of tryptophan were absorbed from the gastro-intestinal tract, a similar increase in cytochrome *P*-450 and hydroxylase activity might occur within the cells of the tract. Some of the absorbed tryptophan could then undergo metabolism through this system. If hydroxylation occurred at carbon 5 of the indole ring, this process would resemble the first step in normal serotonin formation catalysed by tryptophan hydroxylase (Thompson, 1977). Continued metabolism would result in serotonin production. This system may also be self-perpetuating. Serotonin has been shown to provoke an increase in the ionic permeability of the neuronal membrane, particularly to Na^+, K^+ and Cl^- (Paupardin-Tritsch & Gerschenfeld, 1973). A similar phenomenon might occur in the plasmalemma of the intestinal epithelial cells. Such a phenomenon may stimulate the transport of many amino acids into the cells since the amino acid transport is associated with the Na^+ flux through the plasmalemma (Heinz, 1967).

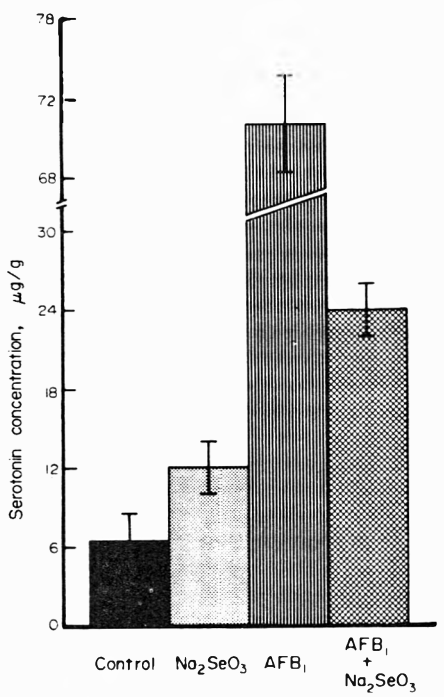

Fig. 1. Mean (±SD) duodenal serotonin levels resulting from treatment with basal diet (■), or basal diet supplemented with 5 ppm Na_2SeO_3 (▨), 12·8 ppm AFB_1 (▩), or both (▩) during a 12-wk experimental period.

Another phenomenon observed in the group given AFB_1 may be similarly related to the neuronal influence of serotonin. It was thought that an increased serotonin concentration might induce increased peristalsis and removal of feed from the intestine. However, a greater percentage of ingested feed was absorbed by the gastro-intestinal tract in the group receiving AFB_1 (J. H. Lalor & G. C. Llewellyn, unpublished data 1978). This contradicts the anticipated result and possibly suggests a decreased rate of peristaltic action, unless mucosal absorption was increased commensurately. If the serotonin is very high, as in the AFB_1 group, it may stimulate all three ionic receptors: for Na^+, for K^+ and for Cl^-. The inhibitory responses due to the K^+ and to the Cl^- may exceed the excitatory responses due to the Na^+ and therefore inhibit neuronal transmission. This may result in decreased peristalsis, with the feed remaining longer in the intestinal tract, and in a subsequent increase in the digestion and absorption of the feed.

The intermediate result shown by the group given both Na_2SeO_3 and AFB_1 may represent a combination of the mechanisms involved in the other two test groups. The serotonin concentration is higher than that in the Na_2SeO_3 group because of the influence of the AFB_1. However, it is lower than that of the group given AFB_1 possibly because of the action of a seleno-enzyme, glutathione peroxidase (GSH-Px). Vilas, Bell & Draper (1976) demonstrated the dependence of the gastro-intestinal activity of this enzyme on the dietary intake of selenium. If this peroxidase functions similarly to other forms, it may catalyse the transformation of serotonin into a product that may not react in the assay utilized. Nelson & Huggins (1975) have demonstrated a transformation of serotonin catalysed by horseradish peroxidase and by a mammalian peroxidase of the spleen. Therefore, the lower serotonin concentration in the group given both AFB_1 and Na_2SeO_3 cannot be stated unequivocably to result from a lower serotonin production relative to the group receiving AFB_1 alone. The possibility of increased catabolism following a similar or greater production of serotonin must be considered. It is also possible that the selenium, when given in combination with AFB_1, gave protection against the stimulatory action of the AFB_1. Sodium selenite previously has been shown to antagonize the activity of known carcinogens, such as *N*-2-fluorenylacetamide (Harr, Exon, Whanger & Weswig, 1972).

In summary, there seems to be adequate evidence that AFB_1 and sodium selenite interact with sero-

tonin metabolism both separately and in combination. The potential of such interactions merits additional investigation in several areas. Among the more important of these are any delayed biochemical and structural alterations induced by aflatoxin and by selenium on the entire gastro-intestinal tract.

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Review Section

REVIEWS OF RECENT PUBLICATIONS

Evaluation of Certain Food Additives. Twenty-first Report of the Joint FAO/WHO Expert Committee on Food Additives. Tech. Rep. Ser. Wld Hlth Org. 1978, **617**. pp. 41. Sw.fr. 5.00 (available in the UK through HMSO).

This publication resulted from the meeting of the Expert Committee held in Geneva in April 1977. Much publicity has already been given to the Committee's recommendations on saccharin but other sweeteners received their share of attention. Cyclamates (the calcium and sodium salts) were awarded a temporary ADI of up to 4 mg/kg, on the basis of a 74-mg/kg no-effect level for cyclohexylamine (CHA) in rats and a conversion rate of 18%. Studies were required by 1980 to determine the no-effect level of CHA-induced embryotoxicity in the mouse and the effect of dose on the degree of cyclamate absorption prior to conversion to CHA, and to define more precisely the level at which CHA induces no testicular effects in the rat, and the percentage conversion of cyclamate to CHA in the human gut. Evaluations of aspartame and xylitol were postponed, pending the validation of studies on the former (*Food Chemical News* 1977, **19**(19), 50) and the assembly of a full toxicological profile, including completed long-term rat and mouse tests, on the latter.

Another substance of particular interest was caramel. Separate specifications were established for caramels produced by the ammonia and the ammonia-sulphite processes, and the ADI for the first type was withdrawn on the ground that lymphocyte depression had been observed at all levels tested. For caramel prepared by the ammonia-sulphite process, the previous ADI of up to 100 mg/kg was temporarily retained, pending adequate carcinogenicity and teratogenicity studies laying stress on bone-marrow effects and immune competence. For both types of caramel it was recommended that the component(s) causing the reported adverse effects should be identified.

Colourings evaluated for the first time were Chocolate Brown HT, Yellow 2G and Red 2G, for which temporary ADIs were allocated of up to 0·25, 0·025 and 0·006 mg/kg respectively. All three were deemed to require reproduction/teratology studies, and metabolic studies were recommended for the first two. For Red 2G, studies on bone marrow to elucidate the toxic effects on erythropoiesis were specified. The previous ADI of up to 15 mg/kg for chlorophyllin copper complex (sodium and potassium salts) was upgraded from temporary to permanent status, but no ADIs could be allocated to a number of other colourings. These included Ponceau SX, for which the Committee recommended that a no-effect level should be defined in the dog for effects observed in previous studies and reproduction/teratology tests should be conducted.

Monographs were prepared on all the above colourings, and on a number of others for which ADIs could not be recommended. These were aluminium (which in its very limited use in confectionery was not considered to present a hazard), Benzyl Violet 4B (which it was recommended should not be used in foods, pending the isolation and identification of the specific carcinogenic component), Black 7984, Brown FK, carthamus, Chocolate Brown FB, chrysoine, cochineal, carmine and carminic acid, Orange I, Orange GGN, Orange RN and silver.

The Committee was able to confirm previous ADIs of up to 5 mg/kg for adipic acid and 30 mg/kg for monosodium L-(+)-tartrate, but an ADI could not be allocated to the DL-tartrate in the absence of adequate long-term studies and in view of indications of renal effects at high doses. For *tert*-butylhydroquinone (TBHQ) the previous requirement for a reproduction study on a mixture of this and other antioxidants was withdrawn, and a group ADI of up to 0·5 mg/kg for TBHQ, butylated hydroxyanisole and butylated hydroxytoluene was recommended. No ADI could be recommended for sucrose acetate isobutyrate pending long-term tests in two species, an adequate 2-year dog study and a reproduction/teratology study.

General principles for the evaluation of food colourings were drawn up at the meeting. The minimum requirements for synthetic colourings were considered to be metabolism studies in several species, preferably including man, short-term feeding tests in a non-rodent mammal, multigeneration reproduction/teratogenicity studies, and long-term studies in two species. The Committee considered that natural colourings isolated in a chemically unmodified form from a recognized foodstuff, and used in that foodstuff at levels normally found, did not require toxicological studies, but all other natural and nature-identical colourings required the same testing as synthetic colourings. Only chemical and microbiological specifications were considered necessary for enzymes obtained from edible animal tissues commonly used as foods, or from edible portions of plants or micro-organisms traditionally accepted as food constituents or normally used in food preparation. However, enzymes from other sources (including non-pathogenic micro-organisms commonly found as food contaminants) were considered to require toxicological testing.

The problem of exposure of infants and children to food contaminants was also discussed. It was concluded that food intended for infants under the age of 12 weeks should (with certain exceptions) not contain any additives, but that the ADIs allocated to most additives could be applied to all children above this age. However, toxicological and metabolic studies were required to include investigations to permit an evaluation of safety for the newborn child and infant.

and the importance of considering the short- and long-term effects of exposure *in utero* and during lactation (including changes in behaviour and immunological mechanisms) was stressed. Further information was requested on the levels of contaminants in mothers' milk, in part to enable the Committee to propose tolerable intakes of lead, mercury, cadmium and polychlorinated biphenyls for children up to the age of 2 years.

Pollution by the Food Processing Industries in the EEC: In the Canning, Beet Sugar, Potato Starch and Grain Starch Sectors. A report prepared for the Directorate-General for Industrial and Technological Affairs and for the Environment and Consumer Protection Service of the Commission of the European Communities by the Institut National de Recherche Chimique Appliquée, France. Graham & Trotman Ltd., London, 1977. pp. xvii + 193. £15.

This report presents a comparative technological survey of the pollution attributable to certain food-processing industries in the nine member countries of the EEC, and if you are concerned with the canning or processing of fruit, meat, potato or grain starch or sugar beet, you should find within its pages a strong inference that you have problems. These relate principally to the discharge of water used in processing, and to the possibilities that the discharge may induce changes in the ecological balance of the receiving environment, contaminate water sources and/or violate some existing or proposed legislation. The volume clearly describes the present scene in terms that, at best, make close surveillance by the staff of each food-processing plant seem imperative.

The text contains much useful data on processing steps, current legislation within each Member State, economic considerations and sources of funds to assist industry in applying anti-pollution measures, and in addition provides a glimpse of the future scene and anticipated requirements. One important conclusion that emerges is that it is not possible to define or quantitate the kinds or degree of pollution resulting from food processing. Nevertheless, the intent to tighten controls on the discharge, amount and composition of effluents is clearly indicated.

This report should certainly be perused by the food-processing industries and by those organizations throughout the EEC with a responsibility for water recovery.

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BOOK REVIEWS

Ecological Effects of Pesticides. Edited by F. H. Perring and K. Mellanby. Linnean Society Symposium Series Number 5. Academic Press, London 1977. pp. xii + 194. £11.

Ever since Rachel Carson's *Silent Spring* increased public awareness of the possible hazards associated with the use of pesticides, demands for less hazardous materials have been the order of the day. This current volume in the Linnean Society Symposium Series represents the deliberations of a symposium held at the Royal Geographical Society in 1976 and provides some insight into the situation in which we find ourselves.

Despite the more wary approach that has now been adopted, we have seen a sharp increase in the UK in the numbers of approved products. From a total of 63 in 1944, the numbers rose to 819 in 1976. The last 10 years have seen the organochlorine insecticides fall from favour, with the contact organophosphates and carbamates moving into the limelight. The uses to which this vast array of chemicals has been put and the benefits they have conferred are described in subsequent sections of the volume. Benefits are, however, difficult to assess, as they have registered themselves alongside other important advances in recent years, such as the production of new and better varieties of crops and fertilizers. It is pointed out that the process may have gone too far in some ways. The production of the unblemished product purely for cosmetic effect is a difficult standard to achieve and requires excessive numbers of pesticide applications. Perhaps the consumer needs educating to a somewhat lower standard. The book is not a face-saver for pesticides, for there certainly has been abuse in the past and this is dealt with in rational terms throughout. Particularly interesting in this respect is a paper on the military use of herbicides in Vietnam.

Other papers in the volume cover the use of pesticides on crops, the ecological effects of herbicides, forest pest and disease management and the uses and future of aquatic herbicides. Unfortunately toxicity is all too frequently considered in acute terms. Precautions against adverse effects require a broader approach, taking into account the possibility of mutagenic, carcinogenic and teratogenic potential.

In some ways, the pesticides have been subjected to a rather over-enthusiastic press, and reporting has not always been correct. J. D. Fryer points out that in connexion with the recent Seveso disaster even *The Times* printed a leading article describing TCDD as a "very effective general purpose weedkiller and defoliant". With false statements like this it is not surprising that the pesticides have found it difficult to gain favour.

Discussion is not restricted to the UK, although the majority of the early papers in the volume draw upon UK figures and examples to put over their points. Three classic human disease problems, those of malaria, trypanosomiasis and onchocerciasis are examined and the ways in which pesticides are helping to overcome them are described. In this context it is of interest to note that the total quantity of pesticide chemicals used against pests of public health importance is only about 3% of the total.

What of the future prospects for both wildlife and man? This is the theme examined in the ultimate and penultimate papers. With the withdrawal of most uses of the more persistent organochlorine insecticides, a serious avoidable pesticide threat to wildlife in the UK is being removed. Nevertheless, several of the papers make the salient point that if one reduces the numbers of an abundant species there will inevitably be repercussions on numbers of other species, because of the nature of ecological interactions. No pesticides, however, have caused serious reductions of populations on a national scale, although with increasing incursions into non-agricultural uses problems may arise.

Although some ways have been found to reduce pesticide use through cultural methods and by adopting biological and integrated control systems, the overall trend is upwards. Development is now becoming a costly process and work on pesticides will become increasingly difficult to justify commercially.

This volume succeeds in highlighting the benefits of pesticide use along with its pitfalls and does so in a balanced fashion very different from the more emotive publications on the subject. Professor Kenneth Mellanby stresses, in the final paper, the need to keep a sense of proportion. Industrialized societies are producing many other more dangerous materials, which are capable of disrupting the ecological matrix. At the same time, the possible hazards of pesticide use are being reduced by the development of chemicals of greater selectivity and the increasing efficiency of methods of application. Perhaps the 'silent spring' of the pesticide has drawn to a close leaving us to a 'summer' of benefits which far outweigh any risks.

Cutaneous Toxicity. Edited by V. A. Drill and P. Lazar. Academic Press Inc., (London) Ltd., 1977. pp. x + 277. £10.30.

After appropriate promptings from the regulatory authorities, industrial toxicology, with its emphasis on inhalation and cutaneous exposure to hazardous materials, has become a 'high-growth area'. In the past, many toxicologists have been concerned mainly with the evaluation of food additives and related materials, and consequently much of their professional expertise has only peripheral relevance to the industrial scene. If the new requirements are to be met, therefore, much more emphasis will have to be placed on toxicity studies involving inhalation and percutaneous absorption. A collection of papers presented at the Third Conference on Cutaneous Toxicity

held in Washington in May 1976 may be of assistance to those caught up in this trend.

An overall picture of cutaneous toxicity is given in this volume, although sensitization and irritancy are the aspects receiving most attention. The review by D. B. Hood of the animal and human tests available for the detection of irritants and sensitizers is comprehensive but is aimed at readers already acquainted with the subject and, for others, would benefit from further description of each method. A more detailed discussion of some common tests is provided by J. F. Griffith and E. V. Buehler, who conclude that man is less sensitive to the effects of irritants than are the rabbit and guinea-pig. While the guinea-pig is useful in the identification of strong allergens, the human repeated-insult patch test is still the method of choice as a preliminary sensitization screen for most materials. The report of P. J. Frosch and A. M. Kligman on their research work with the chamber-scarification test also makes interesting reading. They express the hope that this more sensitive method will eventually replace traditional patch testing for potential irritants.

Percutaneous absorption is covered in four chapters. Of these, the contribution of R. C. Wester and H. I. Maibach, comparing human and animal data, is perhaps the most useful to the general reader. In crude quantitative terms, the monkey and pig are probably the present animal models of choice for measuring percutaneous absorption, the skin of the rat and rabbit being much more permeable than that of man.

For those who promote the use of tissue tests as alternatives to *in vivo* animal studies, the book offers little joy. The paper by Wester and Maibach mentioned above includes a discussion on the *in vitro* methods available for the measurement of percutaneous absorption; on this evidence there are, as yet, no viable alternatives to the live animal, although *in vitro* tests provide a useful preliminary picture. The paper by A. E. Munson and his colleagues similarly indicates that existing *in vitro* techniques for predicting irritancy and sensitization are only in the early stages of development.

Of the remaining papers, an article by M. K. Bruch on the systemic toxicity that can result from the topical application of antimicrobial agents, particularly hexachlorophene, and an interesting review of the toxicity of hair dyes written by C. M. Burnett and J. F. Corbett must be noted. The latter should be of interest to those who consider the case against hair dyes proven.

Books inspired by symposia or conferences are often heterogeneous collections of papers of variable quality. The present work has largely avoided both these drawbacks. With few exceptions, the contributions are relevant, well written and, above all, comprehensible to the non-specialist. Minor irritation stems from a regular smattering of typographical errors and some readers might have been helped by a few more explanatory diagrams in chapters dealing with the physiology of the skin. However, other aspects of presentation are excellent. It is all the more remarkable, therefore, that the cost of the volume has been kept down to what is, at least by present standards, a very reasonable £10. Consequently, in all respects, *Cutaneous Toxicity* can be recommended for those who require some background information in an important area of toxicology.

A Colour Atlas of Neuropathology. By C. S. Treip. Wolfe Medical Publications Ltd., London, 1978. pp. 208. £15.

This book is one of a series of popular medical atlases. Like the previous volumes, it contains numerous well-produced colour photomicrographs, black-and-white photographs demonstrating gross lesions, and a minimal amount of accompanying text.

The book is divided into eleven chapters. The first deals with changes that characterize injury to the neuron and various glial elements, while infections and vascular diseases are covered in chapters 2 and 3. Chapters 4 and 5 deal with degenerative and demyelinating diseases, areas in which important advances are being made. Metabolic and nutritional disorders and traumatic lesions are dealt with in the next two chapters and the distressing conditions of congenital malformation and perinatal disorders occupy the eighth. The last three chapters deal with a simplified classification of tumours of the central and peripheral nervous systems and of the pituitary.

In a presentation of this kind, the quality of reproduction of the photomicrographs is obviously a factor of prime importance and, in general, the standard here is good. The photographs are in most instances well selected and demonstrate the lesion in question clearly. However, the number of illustrations between pages 53 and 56 showing very similar lesions could probably have been reduced without any substantial loss.

The volume is to be recommended to the senior medical students and aspiring pathologists for whom it was compiled. It should also be a useful reference work for persons interested in comparative neuropathology.

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Nonparametric Statistics. A Contemporary Approach. By R. P. Runyon. Addison-Wesley Publishing Company, Reading, MA, 1977. pp. vi + 218. £6.40.

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Information Section

ARTICLES OF GENERAL INTEREST

TOXIC FACTORS IN RAPESEED OIL STILL UNCLARIFIED

Hypotheses continue to be put forward as to the toxicological significance of the mono-unsaturated fatty acid, *cis*-13-docosenoic acid, commonly referred to as erucic acid (EA). This compound, which is present in varying amounts in rapeseed oil, has been held responsible for fatty deposits in heart muscle and associated myocardial lesions in a number of experimental animals (*Cited in F.C.T.* 1975. **13**, 130). Further work, prompted by the growing interest in the use of rapeseed as a valuable oilseed crop, provoked several alternative suggestions (*ibid* 1977, **15**, 348). These have included the possible implication of other long-chain fatty acids, the level of the test oil in the diet rather than its EA content, and the ratio of mono-unsaturated to saturated fatty acids in the rapeseed oil.

Pathological observations

It has now been postulated that linolenic acid may play a role in the aetiology of the cardiac necrosis observed when rats are fed diets containing low-EA rapeseed oils (McCutcheon *et al. Lipids* 1976, **11**, 545). Over a period of 25 weeks, male Wistar rats were fed semi-purified diets containing 20% fat. Ten different oils or oil blends were administered, including rapeseed oils, simulated rapeseed-type oils and modified rapeseed-type oils. The cardiac lesions induced were most severe in the group fed the high-EA oils that also contained linolenic acid. In addition a microvascular alteration (oedematous swelling and loosening of small vessels) was observed. This feature has not previously been described in the literature on rapeseed oil but is suspected to represent an early stage in cardiac pathogenesis. The likelihood that an imbalance in the linoleic:linolenic acid ratio facilitated the lesion formation nevertheless gained only partial support in the study. Although an increase in the linoleic:linolenic acid ratio of the high-EA oils was seen to reduce the incidence–severity rating of the microvascular alteration, it did not affect the incidence–severity rating of myocardial necrosis in the high-EA oil groups. The authors suggested, however, that the presence of more advanced necrotic lesions in the cardiac tissue might well have distorted the picture, in that a far shorter experimental period could have provided more definitive information relating to the correlation of the linoleate:linolenate ratio with early degenerative changes in the myocardium. It was also possible that in view of the propensity of linolenic acid for autoxidation, the destruction of nutrients in the diet or gut as a result of this latter process might have contributed to cardiac lesion formation.

Work undertaken by Astorg & Cluzan (*Annls Nutr. Aliment.* 1976, **30**, 581; *ibid* 1977, **31**, 43) also appears to focus upon the relevance of the linoleic:linolenic acid ratio. Over periods of 3 or 7 days or 16 weeks, male weanling rats were fed diets containing 15% (w/w) of one of the following seven fats: two partially hydrogenated herring oils containing 15 or 30% mixed isomers of the C22:1 acid and supplemented or not with linoleic acid, two mixtures of rapeseed oil and peanut oil possessing the same two levels of EA, and peanut oil (control). The lipids and fatty acids of heart, liver and adipose tissue were analysed at the end of each feeding period and the analyses for total fatty acids, triglycerides and C22:1 isomers and the lipid histochemistry of the heart revealed that the degree of fatty degeneration induced by the oils depended mainly on three factors: the C22:1 content of the oil, the isomeric form of the C22:1 (at a level of 30% in the oil, EA in rapeseed oil had a greater effect than its isomers in herring oil), and the presence of linoleic acid. When added to the herring oil containing 30% C22:1, linoleic acid apparently inhibited the fatty degeneration and the incorporation of EA in the heart tissue.

Results in general accord with other studies were obtained by Hung *et al.* (*Lipids* 1977, **12**, 215). They were able to show that the content of EA in the dietary oil of rats correlated fairly well with the degree of cardiac lipidosis but not as well with the incidence of myocardial lesions. Any attempt to explain the rise in concentration of cardiac triglycerides without an accompanying increase in phospholipids observed when rats were fed high-EA rapeseed oils remained unsuccessful. Both these lipids are synthesized *de novo* from precursor 1,2-diglycerides and it may well be that other metabolic alterations associated with impaired fatty-acid oxidation or an increased uptake of fatty acid by cardiac tissue are responsible for the phenomenon.

The androgens have also attracted attention for their possible involvement in the formation of myocardial lesions (Hulan *et al. Can. J. Physiol. Pharmac.* 1977, **55**, 265). In studies to examine the effects of castration on the incidence of myocardial lesions in rats, two factors were examined: sex (entire males, castrated males, entire females and castrated-ovariohysterectomized females) and diet (5% corn oil, 20% corn oil and 20% *Brassica napus* var. Zephyr rapeseed oil). Following a period of 16 weeks administration, results indicated that castration had no effect on cardiac fatty acid composition. However, whilst the incidence of myocardial lesions in entire and castrated females and in castrated males was similar, significantly more entire males developed lesions. An involvement of androgens in the formation of myocardial lesions was suggested, as castration significantly lowered the incidence in males but not in females.

The authors also drew attention to the point that cardiovascular damage may be brought on by various dietary stress situations. This feature is not uncommon in growing rodents and they suggested that the results observed in their studies might indicate that the mere presence of an excess amount of oil in the diet of the young growing rat might also create a stress situation, thereby influencing the incidence of myocardial necrosis and fibrosis.

That the sex or strain of the experimental animal may play a part in the cardiopathogenic effects of EA has been previously commented upon (*Cited in F.C.T.* 1977, **15**, 349). So as to examine further the influence of the strain of rat on the development of myocardial lesions, Wistar, Sherman, Chester Beatty (Hooded) and Sprague-Dawley (derived from two sources and designated Sprague-Dawley A and Sprague-Dawley C) rats were administered diets containing 5% corn oil, 20% corn oil or 20% *B. napus* var. Zephyr rapeseed oil (Hulan *et al. Can. J. Physiol. Pharmac.* 1977, **55**, 258). No marked differences were observed in the fatty acid composition of the total cardiac lipids among strains. However, for all strains except the Hooded, rats fed the diet containing 20% Zephyr rapeseed oil had a significantly higher incidence of myocardial lesions than rats fed the 20% corn oil diet. Similarly, significantly more Sprague-Dawley C than Sprague-Dawley A rats had myocardial lesions, regardless of diet. This was an interesting feature, since Sprague-Dawley C rats consumed less of each diet and exhibited a slower rate of growth than did Sprague-Dawley A rats, thus implying that strains of rat that grow at a slower rate and consume a smaller amount of feed (and therefore have a lower absolute fat intake) do not necessarily have a lower incidence of myocardial lesions than other strains, which grow faster and consume more feed. Of greater importance was the absence of myocardial lesions in the Hooded rats fed on a diet containing 20% Zephyr rapeseed oil. The Hooded rat is known to absorb and transport a major portion of the fatty acids via the portal circulation. In contrast, in albino rats the main portion of fatty acids appears in the form of triglycerides in the thoracic-duct lymph. The investigators therefore considered that when Hooded rats were fed a diet containing Zephyr rapeseed oil, fatty acids reached the heart by way of the liver in the free form, bound to albumin, whereas in albino strains these fatty acids reached the heart in the form of triglycerides which had been re-esterified in the intestinal mucosa cells.

It is of interest to note that in the chicken, the major route of fat transport is also by way of the portal system to the liver. Earlier studies by Lall & Slinger (*Poult. Sci.* 1973, **52**, 1729) showed that diet was not responsible for any histopathological abnormalities of the hearts of hens fed diets containing 10 or 20% high- or low-EA rapeseed oil over a period of 12 weeks. It is possible that the action of the liver in both the chicken and Hooded rat in some way alters the absorbed fat prior to its reaching the heart, thus accounting for the absence of myocardial lesions when diets containing long-chain monoenes were fed. Further work with the chicken has been undertaken by Kramer & Hulan (*Can. J. Anim. Sci.* 1977, **57**, 305). Male White Leghorn chicks were fed either a basal diet or diets supplemented with soya-bean oil or four different rapeseed oils at 20% (w/w) of the diet from 1 day of age to 3 days and 1, 2 and 4 weeks. The four rapeseed oils contained different levels of EA (0·9–22·3%) and eicosenoic acid (1·5–12·3%). From this the authors were able to provide a species comparison of the incorporation of eicosenoic acid and EA into cardiac lipids of chicks fed high-EA rapeseed oil with that in rats and pigs fed diets containing the same high-EA rapeseed oil (Kramer *et al. Nutr. Metabol.* 1975, **19**, 279). Rats accumulate very high levels of these acids during the first week of feeding, although these levels then decline on continued feeding. In contrast, the pig incorporates much lower levels of these monoenes than the rat and these levels remain unchanged after the first week. Chicks deposit more eicosenoic acid and less EA than rats, but more of both than pigs. Despite the fact that fairly large amounts of eicosenoic acid and EA accumulate in the cardiac lipids of chicks fed diets containing rapeseed oil, there is as yet no evidence attributing histopathological changes in the myocardium to rapeseed oil.

Previous investigations in the pig have provided conflicting results. In a recent study (Friend *et al. Can. J. Anim. Sci.* 1976, **56**, 361), 60 boars were administered either a corn/soya-bean/starch-type control diet to which oil was not added or the control diet with 20% corn oil, 20% low-EA rapeseed oil or 20% high-EA rapeseed oil replacing the starch. Cardiac lesions were characterized by focal areas of myocardial necrosis accompanied by an infiltration of mononuclear cells and eosinophils, or by focal interstitial infiltrations of mononuclear cells and eosinophils. In older lesions there was a replacement of myocardium by fibrous connective tissue resulting in scar formation. However, an analysis of the lesion data revealed no significant interaction between the diets and the presence of cardiac lesions. The necrotic myocardial lesions observed in the study were similar in character to those previously described by Friend *et al.* (*ibid* 1975, **55**, 571) and Aherne *et al.* (*ibid* 1975, **55**, 77). The latter investigators suggested that migration of the larval parasite *Ascaris suum* might have been involved in the aetiology of the lesions. Friend *et al.* (1976, *loc.cit.*) have now postulated that they are caused by eosinophil chemotaxins generated by the necrotic myocardium.

Biochemical studies

It is evident that a better insight into the biochemical mechanisms involved in the cardiopathogenic effects is required before the issue is clarified. Although it has been shown that the inclusion of high-EA rapeseed oil in the diet of rats has resulted in dramatic changes in the fatty acid composition of total cardiac lipids, changes have also been reported in the classes of lipid in the rat heart. Blomstrand & Svensson (*Acta med. scand.* 1975, **198**, 51) investigated the lipid classes and fatty acid pattern of rat-heart homogenate and mitochondria after feeding a diet containing 9·8% (w/w) EA for 10 days and 1·4 and 2·6% EA for 28 days. The rats fed 9·8% EA exhibited a significant increase in the triglyceride content of the heart mitochondria, a tendency which was much less pronounced in those rats treated with 1·4

or 2·6% EA. The total phospholipids showed a decrease following administration of 9·8% EA and a slight increase with 1·4 and 2·6% EA. There was a tendency towards an increased concentration of phosphatidylcholine and a decreased concentration of phosphatidylethanolamine in both the mitochondria and heart homogenate after the feeding of 9·8% EA as rapeseed oil over the 10-day period. No significant difference was observed in the relative distribution of different phospholipids between mitochondria and homogenate. In all experiments the triglycerides of the heart mitochondria showed a high EA content. The fatty acids of the cholesterol esters of the heart mitochondria were also influenced by dietary rapeseed oil but to a lesser extent than the triglycerides.

Although the fatty acids of phosphatidylcholine, phosphatidylethanolamine and cardiolipin were all influenced by the EA content of the dietary rapeseed oil, the EA appeared to have a specific affinity for cardiolipin. Following the administration of 9·8% EA as rapeseed oil for 10 days, isolated cardiolipin was found to contain 12·0% EA. Of equivalent interest, however, was the decrease observed in the linoleic acid content of the cardiolipin from 82 to 47%. As the phospholipids are membrane constituents, some knowledge of their distribution and metabolism is understandably a necessary prerequisite for a better indication of the phenomena involved in heart lipidosis and other disease of the heart muscle.

The incorporation of EA by the cardiolipin molecule is of particular interest, since cardiolipin is synthesized by the mitochondria and may be necessary for the integrity of the mitochondrial inner membrane. It is known that the molecular structure of fatty acids in lipid molecules has a profound influence upon the packing of these molecules in a bilayer. In general, the longer the fatty acid the more tightly packed are the molecules in a monolayer; the greater the unsaturation the more expanded the film. EA and other long-chain mono-unsaturated fatty acids have physical characteristics similar to those of the saturated fatty acids. Thus an incorporation of EA into cardiolipin together with a decrease in the linoleic acid might influence the physical properties of this phospholipid characteristic of the mitochondrial inner membrane. The mechanism by which specific distribution of the EA in membrane phospholipids is brought about remains to be established (Blomstrand & Svensson, *loc.cit.*).

Results that contrasted to a certain extent with those of the above study were recorded in studies on newborn rat heart cells in culture (Rogers, *Lipids* 1977, **12**, 375). EA labelled with ^{14}C was incorporated into foetal calf serum and incubated with cultures of beating myocardial cells from neonatal rats. Of the ^{14}C activity taken up, 26% was present in extractable lipid, two thirds of this being in the neutral lipid and one third in the phospholipid. Within the phospholipid fraction 66% of the ^{14}C activity was in phosphatidylcholine, 14% in phosphatidylethanolamine, 6% in sphingomyelin and only 1% or less in cardiolipin. Nevertheless, there was basic agreement with Blomstrand & Svensson (*loc.cit.*) in the author's suggestion that the altered fatty acid composition associated with the incorporation of EA or its metabolites into phospholipids such as phosphatidylcholine, phosphatidylethanolamine and sphingomyelin might well be responsible for the effects on the integrity and function of heart cell membranes.

Although a clarification of EA metabolism by myocardial cells is of great importance towards providing a solution to the problem, some knowledge of the incorporation and metabolic conversion of EA in other tissues is also desirable. Previous work has shown that EA is transformed in various organs, but primarily the liver, into other mono-unsaturated fatty acids such as oleic acid and nervonic acid. However, these transformations have only been studied over relatively long experimental periods (at least 2 hours) in animals on high-lipid diets (10–15%, w/w). Under these conditions it was not always easy to interpret the results observed. Studies have therefore been undertaken (Ong *et al. Lipids* 1977, **12**, 563) over shorter periods of 2–30 minutes and with rats administered a balanced diet of normal lipid content. A comparison was made of the fate of [14-^{14}C]EA and [9,10-^{3}H]oleic acid in various organs following the simultaneous injection of these two fatty acids. The results indicated that when injected in a single dose, in animals whose diet was free of it, EA was taken up more slowly than oleic acid, although the difference was not very significant and did not appear to persist, since 16 minutes after the injection there was less ^{14}C than ^{3}H radioactivity in the free fatty acids of the blood. This phenomenon does not explain the very rapid accumulation of EA in heart triglycerides following the single injection nor can it explain the high percentage of ^{14}C radioactivity observed in the kidney and spleen, a finding which has not previously been reported. The studies also revealed that transformation of EA into other mono-unsaturated acids, mainly oleic acid, was very rapid and took place in all the organs studied except the heart. It appears, therefore, that the organism uses EA mainly after metabolic conversion to oleic acid in the liver, which largely compensates for the difference in complete β-oxidation between EA and oleic acid. However, with regular ingestion of rapeseed oil this process may not be adequate to eliminate EA totally from the various tissues.

Although data from exposed human populations have not been particularly forthcoming, some information is now available concerning the effects of high levels of fats rich in EA on the non-human primate species *Macaca fascicularis* (cynomolgus monkey) over a period of 4 months (Ackman & Loew, *Fette Seifen AnstrMittel* 1977, **79**, 1). The control group was fed a diet containing 25% lard and corn oil in a 3:1 mixture and the other groups received either 25% rapeseed oil or 25% partially hydrogenated herring oil. The docosenoic acids formed approximately 25% of the rapeseed oil (as EA) and of the partially hydrogenated herring oil (as a mixture of cetoleic acid and cetelaidic acid). In the two experimental groups skeletal and cardiac muscle showed lipidosis. This was especially evident in the apex of the heart. The histopathology of the primate hearts in all groups revealed a few mild foci of inflammation which could not be associated with diet, whereas identical diets fed to male weanling rats induced severe necrotic lesions. The results did not indicate any major difference in basic fatty acid metabolism between the rat and the

cynomolgus monkey although biochemical studies of a more refined nature are needed to determine whether basic biochemical differences do exist. Despite the absence of serious myocardial necrotic lesions in the oil-fed primates in this study, further information from non-human primate species is required to aid in the assessment of myocardial data generated from animal studies in relation to the likelihood of cardiopathogenic effects in man.

In the meantime, whilst the toxicologists strive to pin-point the mechanisms underlying the pathological effects in animals, plant breeders continue to direct their attentions to the development of new rapeseed breeding lines exemplified by 'low erucic', 'high linoleic' and 'low glucosinolate' strains (Jönsson. *Hereditas* 1977, **87**, 205).

[S. P. Johnson—BIBRA]

MERCURIAL DANGERS FOR NEONATES

Mercury poisoning has become a familiar disaster area in recent years. Reports of foetal poisoning, particularly from Japan (Matsumoto *et al. J. Neuropath. exp. Neurol.* 1965, **24**, 563) indicate that man may be particularly susceptible to mercury toxicity in the early stages of his development. Concern about the possibility of damage to the foetus from levels of mercury that do not harm adults has brought about a large number of investigations, mainly in experimental animals.

The transfer of chemicals across the placenta is inevitably controlled by the mechanism of their transfer across cell walls, but is not entirely dependent on molecular size, the lipid-solubility and degree of ionization of the compound being important factors (Tuchmann-Duplessis, *Proc. Eur. Soc. Study of Drug Toxicity* 1970, **11**, 36). The metabolism of both the mother and the placenta will also contribute to the degree of placental transfer. There are significant differences between the metabolism of the placenta of the rat and that of man (Tuchmann-Duplessis, *loc.cit.*) just as there are structural differences between the placentae of the two species. However such differences have not been systematically compared in the context of the placental transfer of mercury (Suzuki, in *Advances in Modern Toxicology. Vol. 2. Toxicology of Trace Elements*, p. 1: Hemisphere Publishing Corp., Washington, 1977). These differences must be borne in mind, since most experimental work has involved trace studies, using the radioisotopes ^{197}Hg and ^{203}Hg, with rats as the subjects. Some investigations in man and the monkey have been reported however.

One Japanese group (Nishimura *et al. Biologia Neonat.* 1974, **24**, 197) tried to discover the normal levels of mercury in human embryos and foetuses in Japan. Total mercury was measured and the ratio of methylmercury to total mercury was assumed to be 0·8. Mercury was detected in all of 67 embryos from 5 to 7·5 weeks old and in all the foetal organs of 28 foetuses between 4 and 10 months old but there was considerable individual variability. Levels of mercury in foetal livers and kidneys were not significantly different from those of infants but were significantly lower than adult values. However, no significant difference was found between the mercury levels in the brain at the three stages. The only significant change with the age of the foetus was a decrease in the average mercury level of third-trimester livers. This could be related to the onset of detoxication by the foetal liver.

Fujita & Takabatake (*Bull. env. contam. & Toxicol. (U.S.)* 1977, **18**, 205) studied the relationships between mercury levels in the blood and hair of both mother and foetus and in the breast milk. The only significant correlations amongst any of these pairs were between neonatal hair and neonatal blood and between neonatal hair and maternal blood. Mean levels were 3·6 ppb (b = 10^9) in breast milk, 3·3 ppm in maternal hair, 4·3 ppm in neonatal hair and 25 ppb in maternal and neonatal blood. These levels were much higher than that found in the study cited below but were apparently much lower than levels previously found in Japanese women from an agricultural area (Wakatsuki *et al.* in *Environmental Pollution and Health Hazards*, Kodansha, Tokyo, 1973). Probably the most surprising finding of this study was that foetal blood-mercury levels were not related to maternal blood levels.

A similar approach was taken by Pitkin *et al.* (*Proc. Soc. exp. Biol. Med.* 1976, **151**, 565) in tests on women from a largely rural inland population in Iowa, USA. The total mercury in maternal blood ranged from 0 to 8 ppb and the range in cord blood was from 0 to 6 ppb, with averages of 1·01 and 1·24 ppb respectively, the two blood levels being significantly related. The maternal level was related to age. In 38 placentae the mean mercury concentration was 2·28 ppb and that in milk samples was 0·93 ppb. Although the mercury levels were relatively low in this group of women, the trends found were similar to those in other studies.

Reynolds & Pitkin (*ibid* 1975, **148**, 523) administered methylmercury chloride by catheter into a maternal or (in one case) foetal vein of rhesus monkeys and determined blood-mercury levels over the ensuing 5–7 hours. Methylmercury reached the foetus within 10 minutes but the ratio of maternal to foetal blood mercury did not fall below about 10:1, in cells and plasma, over a 5-hour period. Minimal amounts of the labelled mercury reached the amniotic fluid. After administration of mercury directly into the foetal blood the ratio of foetal to maternal blood mercury was never lower than 25:1 for red blood cells and 36:1 for plasma. The fact that these ratios were considerably greater than the maternal to foetal ratios found after mercury injection into the maternal circulation suggests that placental transfer takes place more readily from the mother to the foetus than *vice versa*. More mercury reached the amniotic fluid after foetal administration than after maternal administration, possibly reflecting the greater importance of foe-

tal mechanisms in amniotic-fluid formation. The placenta and chorioamnion contained high levels of mercury after maternal administration, and accumulation in these tissues probably retards the movement of mercury into the foetal circulation and amniotic fluid respectively. Levels in the foetal brain were far below those in the kidney, liver and gastro-intestinal tract even after 5–7 hours, suggesting the operation of a blood–brain barrier. Levels were higher in the cerebellum than in other parts of the brain, possibly because of an association of mercury with grey matter.

A comparison of the rates of uptake of inorganic and organic mercury in the rat was made by Mansour *et al.* (*Envir. Res.* 1973, **6**, 479). The progeny of pregnant rats that had been injected with labelled doses of either mercuric nitrate or methylmercury were killed at 3 weeks of age. The relative amounts of the tracer in the young rats, their mothers and the placentae were determined. Maternal clearance of the organic isotope was about twice as fast as clearance of the inorganic isotope. In the placenta the concentration of inorganic mercury was about 6–17 times higher than in the neonatal rat, whereas the levels of methylmercury in the placenta and newborn animals were roughly equal. Although a greater proportion of organic than inorganic mercury was transferred across the placenta, both types of label were equally prevalent in the milk.

The maternal kidney had the highest tissue level of either form of mercury. Of the infant tissues, the hair contained the highest concentration of methylmercury. With the exception of the maternal kidney, all tissues showed much lower concentrations of inorganic than of organic mercury. These authors conclude that the high levels of mercury in the hair of the newborn provide an indication of the mercury exposure level and suggest that the placenta could be used to monitor mercury levels in the newborn infant. Because of the transfer of mercury through the milk it would seem to be best for women with a history of mercury exposure to avoid nursing.

In a later study by the same group (Mansour *et al. Am. J. Obstet. Gynec.* 1974, **119**, 557), it was found that methylmercury was transferred from dam to foetus more freely than mercuric nitrate regardless of the day of gestation, the difference being equally apparent at 1 and 120 hr after the injection. The fact that methylmercury is more lipid-soluble than mercuric nitrate may partially explain this. The difference in the placental transfer of two types of organic mercury was studied by Yamaguchi & Nunotani (*Envir. Physiol. Biochem.* 1974, **4**, 7). Female rats were each given a total of approximately 10 mg Hg as either methylmercury chloride or phenylmercuric acetate in a series of thrice-weekly sc injections over a 4-week period prior to mating. The increase in the mercury content of foetuses was much greater in those exposed to methylmercury chloride than in those from dams given the phenylmercuric acetate.

Using the hamster, Gale & Hanlon (*Envir. Res.* 1976, **12**, 26) investigated the effects of *in utero* exposure to three different types of mercury compound. Solutions of mercuric nitrate, mercuric acetate and phenylmercuric acetate were injected iv in doses of 5 ml/kg body weight on day 8 of pregnancy. The animals were killed after 24 or 96 hours. Although the level of phenylmercuric acetate in the embryo was higher than those of the other two mercury compounds at day 9, levels in the placenta and the 12th day embryo were higher for both the mercuric nitrate and the mercuric acetate than for the phenylmercuric acetate. The concentration of mercury in the placenta was far in excess of that in the embryo, particularly on day 12 of pregnancy. The yolk sac placenta may therefore protect the embryo from high concentrations of mercury.

In many of the animal studies undertaken, interest has centred on the foetal organs in which mercury accumulates. Yang *et al.* (*Proc. Soc. exp. Biol. Med.* 1972, **141**, 1004) gave rats a labelled dose of methylmercury by gastric intubation on day 16 of pregnancy and killed them after 1–5 days. They then determined the distribution of mercury in maternal and foetal brains. The mercury levels were fairly even in the different parts of the maternal brain, except in the pituitary which contained the highest level and the medulla with the lowest concentration. The overall percentage of the dose present in the brain remained the same over the 5 days, whereas the mercury level in the foetal brain increased more than threefold from day 17 to day 21, by which time it was about three times the level in the maternal brain. The foetal cerebellum contained the highest levels. The findings of Null *et al.* (*Life Sci.* 1973, **12**, 65) were broadly in agreement with this study. Methylmercury hydroxide was administered sc to pregnant and non-pregnant rats at levels of 0–40 mg Hg/kg. Seven days after administration, mercury concentrations in the brains of foetuses were twice those in the brains of the pregnant rats. The brains of the non-pregnant rats accumulated slightly more mercury than those of the pregnant females. This could result from the effects of hormonal changes on the supply of metal-binding proteins or from the foetus acting as a sink, removing mercury from the circulation. A higher proportion of mercury in foetal than maternal brains of rats was also demonstrated by King *et al.* (*Teratology*, 1976, **13**, 275), who administered methylmercury chloride by gastric intubation on day 10, 13 or 19 of pregnancy at a dose of 10 μg Hg/kg. The dams were killed 24, 48 or 72 hours later. After 24 hr, the foetal brain concentration was 3·4 times higher than the maternal brain concentration of mercury. Although maternal blood levels decreased with time after administration, foetal brain levels were once again found to rise. This may result from the fact, discussed above, that the transfer of mercury from the maternal to the foetal circulation is more rapid than that in the opposite direction. However, concentrations of mercury in the foetal kidney were considerably lower than those in the maternal kidney. A similar picture was unfolded by Wannag (*Acta pharmac. tox.* 1976, **38**, 289) who gave pregnant rats methylmercury chloride in a single iv dose of 1 mg/kg at various stages of pregnancy, and corrected the resulting organ levels of mercury for the blood level of the organ. The ratio of foetal to maternal brain levels of mercury was somewhat lower than those determined by other workers. When the mother was injected 14 days before term, the near-term foetal brain contained 1·4 times as much mercury as the maternal brain, the foetal liver contained

2–2·6 times as much mercury as the maternal liver and the foetal kidney contained 13–23 times less mercury than the maternal kidney.

The independent contribution of the placenta to the foetal burden of inorganic mercury was determined by Kelman (*Toxic. appl. Pharmac.* 1977, **41**, 659). To separate foetal uptake from placental transfer, mercury transport across the guinea-pig placenta was measured *in situ* by a perfusion technique in dams injected iv with labelled mercuric chloride about 5 days before the end of pregnancy. There was no significant relationship between the rate of radiomercury clearance from the maternal plasma and the rate of flow of the perfusing solution, most of the variability in clearance being directly attributable to changes in the maternal blood flow to the placenta. The clearance of inorganic mercury was low but the small amount that did cross the placenta did not depend on the presence of the foetus. Clearance of organic mercury, previously measured under identical conditions, was more than twelve times the clearance of inorganic mercury; this difference would account for the observed differences in foetal absorption of organic and inorganic mercury.

Garrett *et al.* (*ibid* 1972, **22**, 649) determined the placental/foetal distribution of radioactive mercury 30 minutes after intracardial injection of 2 μCi $^{203}HgCl_2$ to rats on day 16 of pregnancy. The chorioallantois and yolk sac were shown to accumulate mercury from the maternal blood but to restrict the passage of the metal to the foetus; the chorioallantois and visceral yolk sac, respectively, contained concentrations of mercury 73 and 36 times as high as that in the foetus. High concentrations of the mercury were found in the intracellular fraction containing the stroma and this suggests a strong affinity of the placental cell surface for mercury. The authors conclude that the placenta provides a strong barrier to mercury. However, they were using inorganic mercury which has been shown to penetrate the placenta much less freely than the organic compounds (Mansour *et al.* 1973, *loc.cit.*).

Several routes of administration were used in the foregoing studies. The effects of exposure by two different routes were compared by Greenwood *et al.* (*Experientia* 1972, **28**, 1455). Pregnant rats were killed either immediately after a 2·5-minute inhalation of mercury vapour or 2·5 minutes after receiving an equivalent dose of mercuric chloride by iv injection. The amount of mercury in the maternal blood was 25 times greater in injected animals than in vapour-exposed animals, probably because of the relatively rapid diffusion of metallic mercury from blood to tissues demonstrated in earlier studies. In spite of this difference in blood levels, the total amount of mercury taken up by the placenta and foetus combined was roughly equal in the two groups, but nearly half of this total had reached the foetus in the vapour-exposed animals whereas only 1% had reached the foetus in the group injected with inorganic mercury. This suggests that there is considerable danger of damage to the foetus in cases of occupational exposure to mercury vapour in activities such as preparing thermometers and calibrating pipettes.

Yang *et al.* (*Proc. Soc. exp. Biol. Med.* 1973, **142**, 723) studied the mammary transfer of mercury from mothers to their progeny. On day 16 of lactation, rats were intubated with a dose of ^{203}Hg-labelled methylmercury chloride and nursing was continued. The pups were killed 1–5 days later and finally the mothers were killed. In pups the concentration of mercury in the brain increased from day 1 to day 5 after treatment, the final levels in the different parts of the brain being more than twice those found in the corresponding parts of the maternal brain on day 5.

A number of studies have been carried out in species other than the rat. Childs (*Archs envir. Hlth* 1973, **27**, 50) supplemented the diets of mice with dried tuna to achieve levels of 0·005 to 1 ppm mercury for 10 days before mating and then throughout gestation. The concentration of mercury in the foetuses was proportional to the maternal dietary level and to the mercury content of the dams. Although the foetus may contain up to twice the maternal concentration of mercury at dietary levels up to 0·005 ppm, the maternal load was greater at a level of 0·05 ppm and above.

Uptake of methylmercury by the mouse foetus was studied by Olson & Massaro (*Toxic. appl. Pharmac.* 1977, **39**, 263). Single doses equivalent to 5 mg Hg/kg were administered sc to pregnant mice on one of days 7–13 of gestation. Dams were killed at intervals up to 29 days after treatment and offspring up to 21 days after birth. Foetal accumulation increased with foetal age at time of administration. With treatment between day 10 and 13 of pregnancy, higher foetal than maternal blood-mercury levels were reached 7 or 5 days after administration, which fits in with the pattern of a more rapid transfer from the mother to the foetus than *vice versa*. The peak foetal mercury concentration (4·8 mg/kg) followed injection on day 13 and was reached 3 days after administration. Placental mercury concentrations were equal to or higher than mercury concentrations in maternal blood. An increase in the net rate of maternal liver elimination was associated with the increased foetal sequestration in the later gestational stages. Maternal blood elimination rates increased slightly throughout the same period. Offspring were cross-fostered to determine the effects of mercury uptake via the milk. The offspring exposed to mercury via the placenta had tissue mercury concentrations 15 to 30 times greater than those receiving mercury via the milk.

In a later study by the same workers (*idem. Teratology* 1977, **16**, 187) methylmercury was administered to mice at a dose of 5 mg Hg kg on day 12 of gestation. A high incidence of cleft palate (72–40%) was found in foetuses examined on days 15–17 and foetal protein and DNA concentrations were reduced by as much as 28 and 32%, respectively, within 3 days of treatment. The low foetal protein level was apparently the result of a reduced rate of protein synthesis found during the first 24 hours after mercury administration and attributed principally to an impairment of placental-foetal amino acid transport. Free amino acid concentrations in the foetus were reduced 12 hours after the mercury injection, a 23% reduction in the uptake of the essential amino acid, cycloleucine, being particularly significant.

Fowler & Woods (*Lab. Invest.* 1977, **36**, 122) found a dose-related decrease in the density of foetal liver mitochondria after exposure of rats to doses of 5 or

10 ppm methylmercury hydroxide in the drinking-water for 4 weeks before mating and through to day 19 of gestation. They also found a decrease in mitochondrial protein synthesis and lowered activities of some mitochondrial enzymes, in broad agreement with the second study of Olson & Massaro mentioned above.

With the possibility of very different placental functions in man and the animals used in these studies, it is particularly difficult to assess the relevance of the experimental data to man. However, it does seem clear that the placenta provides some barrier to the transfer of mercury but that methylmercury crosses this barrier more rapidly than other mercury compounds. It also appears that the placenta allows transport of mercury from mother to foetus more readily than in the opposite direction. The very much higher levels in the foetal brain than in the maternal brain may go some way to explaining the cases of poisoning encountered in human foetuses. However, similarly high ratios were not demonstrated for foetal and maternal brain levels in the study of a normal Japanese population (Nishimura *et al. loc.cit.*).

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TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS

PRESERVATIVES

3347. Nitrosamines in food surveyed

Gough, T. A., Webb, K. S. & Coleman, R. F. (1978). Estimate of the volatile nitrosamine content of UK food. *Nature, Lond.* **272**, 161.

Sensitive methods have been developed for determining volatile nitrosamines in food products (Goodhead & Gough, *Fd Cosmet. Toxicol.* 1975, **13**, 307). Combined gas chromatography–mass spectrometry is a reliable method with a typical detection limit of 1 μg/kg food, and the more recent use of thermal energy analysis has lowered the detection limit further (Fine *et al. Analyt. Chem.* 1975, **47**, 1188). Both of these methods have been used by the authors cited above to provide data for an assessment of the intake of volatile nitrosamines in foods commonly encountered in the UK. Foods examined included bacon, canned luncheon and other cured meats, fresh meat and meat products, fish and fish products, cheese, yogurt, desserts, canned fruit and jams, frozen and fresh vegetables, soups, beverages and baby foods, as well as complete meals, both domestically prepared and commercially packaged. The samples were studied for their content of *N*-nitrosodimethylamine (NDMA), *N*-nitrosodiethylamine (NDEA), *N*-nitrosodi-*n*-propylamine (NDPA), *N*-nitrosodi-*n*-butylamine (NDBA), *N*-nitrosopiperidine (NPIP) and *N*-nitrosopyrrolidine (NPYR).

All samples of fried bacon contained NPYR (1–20 μg/kg and occasionally up to 200 μg/kg). NPIP (0·08–0·25 μg/kg) and NDMA (up to 5 μg/kg). In other cured meats, NPYR did not exceed 1 μg/kg, but some contained several nitrosamines (0·1–1 μg/kg). Of 36 uncured meats and meat extracts, one contained NDMA (2 μg/kg) and six contained a mixture of dialkylnitrosamines (up to 0·2 μg/kg). Uncooked and fried fish contained NDMA, but only one sample in 70 contained any other nitrosamine (NPYR, at 0·01 μg/kg). Five out of 24 samples of salted, pickled, smoked and canned fish contained NDMA. In cheeses only NDMA occurred, usually in low concentrations, and 16 of 76 samples contained no nitrosamine: NDMA appeared to be no more likely to occur after toasting or addition of nitrate to the cheese. Of 22 other dairy products, three contained NDMA (up to 0·1 μg/kg) but no heterocyclic nitrosamines. Six canned desserts for babies contained no detectable nitrosamines (i.e. not more than 0·01 μg/kg). Five of 12 samples of canned fruit contained NDMA (less than 0·1 μg/kg) and one contained NPYR (0·09 μg/kg). Vegetables were nitrosamine-free. Of 30 soups, three contained NDMA (less than 0·09 μg/kg), two contained NDBA (0·2–0·5 μg/kg) and one contained NPIP and NPYR both at 0·06 μg/kg. Of 13 prepacked meals, five contained 0·01–0·3 μg *N*-nitrosodialkylamines/kg, while of ten canned baby foods, nine contained no volatile nitrosamines, the exception being one that included bacon. Nitrosamines were present in a very small proportion of laboratory-cooked pastry-based foods, stews, casseroles and fried meals. *N*-Nitrosoethylmethylamine (0·2 μg/kg) occurred only in four meals containing mushrooms, and was not accompanied by other nitrosamines.

Of the total of 493 samples analysed, the major source of volatile nitrosamines was cured meat. This was followed by fish and then cheeses. On the basis of this study, the nitrosamine intake per person from a normal UK diet has been estimated as about 1 μg/wk for nitrosodialkylamines and about 3 μg wk for volatile heterocyclic nitrosamines.

3348. Nitrosamines and stomach cancer

Ruddell, W. S. J., Bone, E. S., Hill, M. J. & Walters, C. L. (1978). Pathogenesis of gastric cancer in pernicious anaemia. *Lancet* **I**, 521.

A high stomach pH has been found to be associated with high concentrations of total bacteria, of bacteria with nitrate-reductase activity and of nitrite, the last presumably derived by bacterial action (*Cited in F.C.T.* 1977, **15**, 665). Many enteric bacteria can also catalyse nitrosamine formation from nitrites at neutral pH (*ibid* 1971, **9**, 739). These findings may explain the fact that gastric cancer is five times more common among patients with pernicious anaemia (PA), in which achlorhydria (absence of stomach acid) is a prominent feature, than in the general population, a possibility that has been explored by the authors cited above.

Thirteen patients with PA were found to have a mean gastric nitrite concentration of 120 μmol/litre, nearly fifty times greater than that of age-matched controls (2·7 μmol/litre). Stomach pH was neutral in PA subjects, compared with a range of 1·2–2·8 in the controls. Marked increases were also found in the PA group in the counts of total bacteria and of bacteria showing nitrate-reductase activity. Direct analysis for nitrosamine content proved impossible in the small amounts of viscid gastric juice extracted from these patients. However, when a bacterial culture from PA subjects was added to neutralized gastric juice from controls and nitrite was admixed to achieve a concentration of about 150 μmol/litre, small quantities of several carcinogenic nitrosamines could be detected after incubation for 6 hr at 37 C.

In four experiments using three different pooled samples of gastric juice and bacterial cultures derived from four PA patients, combined gas chromatography and thermal energy analysis demonstrated the presence of *N*-nitrosodimethylamine and *N*-nitrosodiethylamine in all the samples, at concentrations of 1·4–16·2 and 1·0–2·0 μmol/litre, respectively. Lower

concentrations of *N*-nitrosodi-*n*-propylamine. *N*-nitrosopyrrolidine and *N*-nitrosomorpholine were also found in isolated cases. No nitrosamines were detectable in control incubations containing no added nitrite, confirming their absence from the pooled gastric juice and the bacterial cultures. As gastric amine concentrations are higher in PA patients than in normal subjects, the amount of nitrosatable amines in the media for these *in vitro* studies was probably lower than would be present *in vivo*. It might, therefore, be predicted that amounts of nitrosamines formed in the stomachs of PA sufferers could be somewhat greater than the small amounts demonstrated *in vitro*. No quantitative conclusions can be drawn from this study, however, although the findings do suggest a possible mechanism for the observed association between gastric cancer and achlorhydria or, more specifically, PA.

3349. More negative results for irradiated wheat

Reddi, O. S., Reddy, P. P., Ebenezer, D. N. & Naidu, N. V. (1977). Lack of genetic and cytogenetic effects in mice fed on irradiated wheat. *Int. J. Radiat. Biol.* **31**, 589.

There is reluctance on the part of legislative bodies to promote irradiation as a method of food preservation, despite the fact that irradiated animal feeds, milk products, wheat and potatoes have failed to demonstrate mutagenic or other effects (*Cited in F.C.T.* 1978, **16**, 71). Although the numbers of both children and monkeys participating in studies of the effects of irradiated foods in malnutrition have been limited, evidence derived from both human and animal studies continues to accumulate.

In a study of irradiated wheat, male mice were fed a diet containing 60% wheat flour, which had been irradiated with 20 or 200 krad for 180 days and stored for 30 days before use, and were then mated for a dominant lethal assay and for the detection of mutations at specific loci. A group of females was also fed the test diet for 180 days prior to mating with control males. Further groups of males and females were fed the irradiated diets for up to 1 yr, during which the animals were killed at 3-monthly intervals for chromosome studies on the testes and seminiferous tubules or on the ovaries.

In the dominant lethal assay in males, litter sizes and sex ratios and the body weights of the offspring showed no significant differences between the test and control groups, and there was no indication of an increase in pre- or post-implantation losses or total losses as a result of treatment. Neither was there any significant deviation from control values in the average counts of corpora lutea or in implantation losses in females fed the irradiated diets prior to mating. No effects on the survival of either spermatogonia and spermatocytes or oocytes were detected in mice fed the irradiated-wheat diet for 90–360 days. Finally, the search for mutants at specific loci in the offspring of treated males mated with females from a multiple recessive seven loci tester stock was negative. Thus, none of the studies in this group gave positive evidence that the irradiated diet had any genetic or cytogenetic effects.

AGRICULTURAL CHEMICALS

3350. Dichlorvos not a methylating agent in practice

Wooder, M. F., Wright, A. S. & King, L. J. (1977). *In vivo* alkylation studies with dichlorvos at practical use concentrations. *Chemico-Biol. Interactions* **19**, 25.

Dichlorvos has been shown to possess weak alkylating activity *in vitro*, including an ability to alkylate mammalian DNA (*Cited in F.C.T.* 1974, **12**, 769). The possibility that it might alkylate DNA in mammalian tissues *in vivo* at concentrations approaching those encountered during its use as an insecticide has therefore been investigated.

Rats were exposed to an atmosphere containing [Me-^{14}C]dichlorvos in a concentration of 0·064 μg/litre for 12 hr, each rat inhaling a total dose of 6 μg dichlorvos. DNA, RNA and protein were isolated from homogenates of the main organs from the exposed animals and their radioactivity was determined. Macromolecular fractions from all organs showed traces of activity. Among the DNA fractions examined, that from spleen had the highest specific activity and that from liver the lowest. For the RNA fractions, activity was highest in the fraction from the heart lung homogenate and lowest in that from the brain. However, no labelled 7-methylguanine moieties could be detected in either DNA or RNA from the exposed rats. Limits of detection of methylation were one methyl group per 6×10^{11} and 2×10^{9} nucleotide units for DNA and RNA, respectively. Thus, there was no evidence that, over a period constituting a significant fraction of the half-life of 7-methylguanine moieties in DNA, nucleic acids were methylated by concentrations of dichlorvos approaching those encountered under practical conditions of use. This failure to methylate mammalian nucleic acids *in vivo* is ascribed to a combination of the low methylating potential of dichlorvos towards nucleic acids and the efficiency of dichlorvos metabolism in mammals.

3351. Gut reaction to diquat

Crabtree, H. C., Lock, E.A. & Rose, M. S. (1977). Effects of diquat on the gastrointestinal tract of rats. *Toxic. appl. Pharmac.* **41**, 585.

Small doses of the bipyridylium herbicide diquat have provoked diarrhoea, dysphagia and ulceration

of the tongue and pharynx, and after ingestion of a massive dose in an ultimately successful suicide bid, necrosis of the mocosa of the oesophagus and haemorrhage from the stomach wall was observed (*Cited in F.C.T.* 1972, **10,** 876). Nevertheless the immediate cause of diquat-induced death has not been established. Diquat lacks the specific effects on the lung associated with paraquat, to which it is closely related structurally and chemically, and the histological changes recorded have not been considered to be sufficiently severe to account for death.

In an attempt to clarify the situation, rats were dosed by oral intubation or sc with 900 or 90 μmol diquat/kg, respectively, these doses being the approximate LD_{50}s, and were observed for 8 wk. After the oral dose the animals appeared normal, apart from having a lower water intake than the controls. After 24 hr they were subdued, showed signs of piloerection and excreted green mucoid faeces. Death occurred within 9 days, half the deaths occurring within 3 days. After the sc dose, the animals were slightly subdued and showed piloerection within 4–6 hr. Water intake was reduced significantly over the first 24 hr, all animals losing weight over this period, but no deaths occurred within 5 days of dosing.

Orally administered diquat produced a rapid accumulation of water in the gastro-intestinal tract; this was maximal after 1–2 days, subsequently decreased in survivors until day 8, and was most marked in rats that failed to gain weight after dosing and died within 3 days. Diquat given sc did not increase the water content of the tract significantly until day 3, but after this both the water content and dry weight of the tissue increased. After 14 days some animals showed severe abdominal distension, although others appeared normal. Dehydration of blood, muscle and liver was observed 24 hr after oral administration but not after sc injection. The animals that were distended 14 days after the sc injection showed a significantly increased hydration of kidney, skin and gastro-intestinal tract compared with both the non-distended treated animals and the controls, and all died eventually, some as late as 8 wk after dosing. Haemoconcentration, observed 0·5 hr after an oral dose of diquat, persisted for 6 days and in a subsidiary study was shown to be dose-related at 24 hr.

Early deaths from orally administered diquat are evidently associated with rapid fluid loss into the gastro-intestinal tract, but death after sc injection appears to be an unrelated phenomenon.

THE CHEMICAL ENVIRONMENT

3352. Dimethylformamide: a hazard for beer drinkers?

Chivers C. P. (1978). Disulfiram effect from inhalation of dimethylformamide. *Lancet* **I,** 331.

Some years ago, a report from Germany (Reinl & Urba, *Int. Arch. Gewerbepath. Gewerbehyg.* 1965, **22,** 33) suggested that, in some people, dimethylformamide (DMF) exposure may lead to an adverse reaction, similar to that elicited by prior treatment with 'Antabuse' (disulfiram), when alcohol is consumed. This possibility was underlined by the recent experience of a 34-yr-old maintenance fitter, who spent some 4 hr repairing a blocked pipe under a reaction vessel, during which time he was exposed to DMF, initially at less than the threshold limit of 10 ppm but later at 30 ppm. In addition to breathing the vapour, he may have soaked part of his overalls in liquid DMF, although this could not be confirmed. Soon after the exposure he drank half a pint of beer, which turned his face blotchy red and caused a feeling of tightness in the chest. Recovery from this episode took about 2 hr, as did recovery from a similar reaction which followed the consumption of 2 pints later in the day. On the next day, consumption of 2 pints of beer, followed 2 hr later by a further 6 pints, brought a recurrence of the symptoms. The following morning, the patient's lung function was found to be normal. He reduced his normal beer consumption for a week, during which his symptoms were negligible, and on resuming his habitual 6–8 pints per night he experienced no ill effects. His serum γ-glutamyl-transpeptidase value was abnormally high, but serum glutamic-oxalacetic transaminase and bilirubin were within normal limits. The man experienced another episode of facial flushing when he drank beer after a second exposure to DMF. This effect, analogous to disulfiram sensitization to ethanol, appeared in none of the patient's fellow workers.

3353. Toluene distribution

Pyykkö K., Tähti, H. & Vapaatalo, H. (1977). Toluene concentrations in various tissues of rats after inhalation and oral administration. *Arch. Tox.* **38,** 169.

The retention and fate of volatile solvents is of particular importance during the industrial exposure of workers, and we have referred to the respiratory uptake and elimination of a number of solvents, including toluene, in man (*Cited in F.C.T.* 1975, **13,** 591).

In the study now reviewed, the uptake and elimination of [4-^{3}H]toluene were determined in Sprague-Dawley rats exposed to the solvent by gastric intubation or by inhalation. After administration by intubation, radioactivity in the blood reached a peak at 2 hr and at the same time activity in the stomach tissue began to decline. The highest relative activity in the blood was about 10%, the relative activity being the percentage calculated on the administered dose and the weight of the organ or tissue in relation to total body weight. Peak activity in heart, lung, liver, thyroid and adrenal tissue also occurred about 2 hr after dosing, while in spleen, kidney, brain, bone-marrow, muscle and brown adipose tissue it was delayed to 3 hr. Activity in white adipose tissue increased slowly for up to 5 hr.

After inhalation of 20 ppm [^{3}H]toluene for 10 min, radioactivity was rapidly distributed through all tissues apart from white adipose tissue, to reach a peak 15–30 min after exposure. In white adipose tissue the peak concentration occurred after 1 hr. The ratio of tissue to blood radioactivity was similar to that following oral administration. During the first 9 hr after exposure the rate of elimination of activity from all tissues was more rapid after inhalation than after intubation, but between 12 and 24 hr the rate was similar for both exposure routes. Elimination from white adipose tissue was much slower than from other tissues, with activity falling to 10% of the initial value only after 12 hr and to 1% after 24 hr compared with 4 hr and 12 hr respectively for other tissues. Little elimination from bone marrow was apparent during the first 24 hr after the inhalation.

The findings indicate that evaluating exposure to toluene by monitoring concentrations in expired air, blood and urine may over-simplify the situation, since accumulation in tissues that are not readily sampled may present a hazard.

NATURAL PRODUCTS

3354. Dehydroabietic acid shows a relatively clean sheet

Villeneuve, D. C., Yagminas, A. P., Marino, I. A. & Becking, G. C. (1977). Toxicity studies on dehydroabietic acid. *Bull. env. contam. & Toxicol.* (*U.S.*) **18**, 42.

Dehydroabietic acid (DHA) is a resin acid produced by coniferous trees. In paper mills, resin acids are present in the pulp and paper wastes and have proved toxic to fish at a level of 0·44 ppm. DHA has been identified as a toxic component in pulp-mill effluent.

DHA of 78·3% purity was given to Sprague–Dawley rats either by gastric intubation, dissolved in corn oil, to determine its acute toxicity or by inclusion in the diet in a subacute toxicity study. In two experiments, the LD_{50} values for DHA were 4000 and 3690 mg/kg in males and 1710 and 3690 mg/kg in females. Lethal doses caused neuromuscular incoordination, hind-leg paralysis and diarrhoea. When fed to rats at a dietary level of 50, 500 or 5000 ppm for 14 and 28 days, DHA did not affect weight gain, food consumption, water intake or haematological data. At the 500- and 5000-ppm levels, liver and spleen weights were lower at day 14 but not at day 28. Liver-protein levels were not affected, but aniline-hydroxylase activity showed an increase at day 28 in rats on 5000 ppm DHA. At all feed levels, serum protein was lower after 14 days but normal after 28 days. In the 5000-ppm group, alkaline-phosphatase activity was unchanged at day 14 but was higher than the control value after 28 days. These findings suggest that DHA is no more than moderately toxic to rats, whatever it may do to fish.

3355. Fading role of shikimic acid in bracken carcinogenicity

Hirono, I., Fushimi, K. & Matsubara, N. (1977). Carcinogenicity test of shikimic acid in rats. *Toxicology Lett.* **1**, 9.

The carcinogenicity of bracken (*Pteridium aquilinum*) has sometimes been attributed to its content of shikimic acid (*Cited in F.C.T.* 1975, **13**, 405), but there are several other contenders for the role of chief carcinogen (*ibid* 1975, **13**, 481; *ibid* 1976, **14**, 514 & 651). The brief report cited above throws further doubt on the importance of shikimic acid in this connexion.

Rats fed a diet containing 0·1% shikimic acid for 142 days and then a normal diet were killed and autopsied either when they became moribund or after 480 days and tissues were removed for histopathological examination. Of the six males and six females treated, one male and two females died of pneumonia. The other nine rats survived the observation period but, as in the control group, none showed any tumours or even any histological changes suggesting that intestinal or bladder tumours would develop later. These rats were estimated to have consumed more than twice as much shikimic acid as others which, in another study, were fed for 120 days on a bracken-containing diet (Hirono *et al. J. natn. Cancer Inst.* 1970, **45**, 179), yet all the bracken-fed animals surviving beyond 7 months developed intestinal tumours. These findings thus support other indications that responsibility for the observed carcinogenicity of bracken, at least in the rat, lies with some compound or compounds other than shikimic acid.

3356. Tea and tumours: a complex situation

Bogovski, P. & Day, N. (1977). Accelerating action of tea on mouse skin carcinogenesis. *Cancer Lett.* **3**, 9.

The consumption of tea has been considered as a possible contributory factor in the aetiology of human oesophageal cancer, either as a result of burning by the hot liquid or of chemical irritation; the mutagenic effect of caffeine itself is not firmly enough established to offer a likely explanation (*Cited in F.C.T.* 1970, **8**, 86).

In a study of possible co-carcinogenic effects of tea infusion, skin treatment of albino mice with 1% benzo[*a*]pyrene was followed by thrice-weekly painting of the same area with black-tea infusion, oak-sawdust extract, or a solution of tannic or gallic acid for a total of 80 applications. The tannin content of each solution was 1%. The mice were observed for 567–580 days. The number of deaths was similar in

all the treated groups. There was also a similar incidence (30–36%) of skin tumours, but the malignancy varied between groups. In the animals painted with tea infusion there were three squamous-cell carcinomas, two of which appeared earlier than any tumours in animals treated with benzo[*a*]pyrene alone. One malignant tumour appeared in each of the groups treated with oak or tannic acid, but none occurred in that treated with gallic acid. The latency of skin tumours in mice painted with tea or oak-dust infusions was significantly shortened in comparison with the tumours induced by benzo[*a*]pyrene alone. These findings suggest that the action of the infusions is complex, and that it may depend upon the relative proportions of various constituents.

COSMETICS, TOILETRIES AND HOUSEHOLD PRODUCTS

3357. The mutagenicity of 2,4-toluenediamine

Pienta, R. J., Shah, M. J., Lebherz, W. B., III & Andrews, A. W. (1977). Correlation of bacterial mutagenicity and hamster cell transformation with tumorigenicity induced by 2,4-toluenediamine. *Cancer Lett.* **3**, 45.

2,4-Toluenediamine (*m*-toluenediamine: MTD) has produced hepatomas when fed to rats in a semi-synthetic diet (*Cited in F.C.T.* 1969, **7**, 697) and carcinogenicity was also indicated in a recent National Cancer Institute feeding study (*Food Chemical News* 1977, **19**(32), 19). However, when low doses have been applied to mouse skin there has been no definite evidence of carcinogenicity (*Cited in F.C.T.* 1975, **13**, 353: Giles *et al. J. Toxicol. envir. Hlth* 1976, **1**, 433). In several rodent species, the predominant metabolites of MTD were phenolic in nature, the principal product being 5-hydroxyMTD, although small amounts of the 4-acetyl derivative were also produced (*Cited in F.C.T.* 1977, **15**, 261). MTD hydrochloride was mutagenic in *Salmonella typhimurium* strain TA1538 after metabolic activation (Ames *et al. Proc. natn. Acad. Sci. U.S.A.* 1975, **72**, 2423) and further evidence of this property is now presented.

When MTD was incubated with *S. typhimurium* strain TA1538, TA98 or TA100, no increase in mutations was obtained in the absence of metabolic activation. However, in the presence of a rat microsomal-enzyme preparation to effect such activation, the first two strains (both indicators of frameshift mutation) responded with a significant increase in reversions. Secondary hamster embryo cells treated with MTD at a concentration of 0·5 μg/ml showed 2/511 neoplastic transformations (assessed by extensive three-dimensional growth and random disorientation of peripheral cells), although higher concentrations were cytotoxic and negative results were obtained at 0·05 μg/ml. The carcinogens 3-methylcholanthrene and benzidine produced a similar number of transformations at 0·5 μg/ml, while the non-carcinogen phenanthrene was inactive even at 20 μg/ml. It was concluded that the observed mutagenicity of MTD was due to a metabolite, which in the second experiment was produced by the hamster cells themselves.

3358. Hazards of the beauty parlour

Menck, H. R., Pike, M. C., Henderson, B. E. & Jing, J. S. (1977). Lung cancer risk among beauticians and other female workers: Brief communication. *J. natn. Cancer Inst.* **59**, 1423.

The exposure of beauticians to a wide variety of chemical compounds, viewed in conjunction with allegations that some hair dyes are mutagenic and therefore conceivably carcinogenic, has stimulated comparisons of cancer rates among female beauticians with those in the female population at large. Findings have suggested that the risk of lung cancer may be substantially increased among female beauticians (*Cited in F.C.T.* 1977, **15**, 654).

This conclusion is echoed in a study of 22,792 women included in a cancer surveillance programme. The two statistical measures of risk used were the proportional incidence ratio, for which those expected to develop cancer by age group were calculated proportionally with the use of the age-specific site distribution of all cancer among people of the same sex with reported occupations, and the standard incidence ratio, for which those expected to develop cancer by age group were calculated from the sex-specific populations at risk by occupation. Of the 135 beauticians in the survey, 20 had lung cancer. Within the whole group, the most frequent cancers reported were those of the breast (32% of total cancers), genital organs (22%) and lungs (15%). The increased risk was only significant in the case of lung cancer, which showed a proportional incidence ratio of 1·76. In the population at risk, the standard incidence ratio among beauticians was 2·37. In two other occupational groups of women, factory assemblers and waitresses, a significantly increased risk of lung cancer appeared. In another study by the same authors, 199 women with lung cancer and 187 age-matched controls were compared. In each group were six former beauticians who had practised for longer than 10 years, giving a relative risk of lung cancer of 0·94. The two analyses offer scant basis for any assertion that lung cancer is more likely to affect beauticians than other women, but suffer from the lack (shared with other studies) of any reliable data regarding the smoking habits of the women concerned.

Fd Cosmet. Toxicol. Vol. 16, p. 633. Pergamon Press 1978. Printed in Great Britain

LETTER TO THE EDITOR

LIVER CHANGES ASSOCIATED WITH FEEDING BACTERIAL SINGLE-CELL PROTEIN TO BIRDS

Sir,—Well-developed changes in the livers of chicks fed diets containing methanol-fermented bacterial single-cell protein (SCP) have recently been reported (Farstad, *Acta Agric. scand.* 1977, **27**, 129; Whittemore *et al. Br. Poult. Sci.* 1978, **19**, 283). We have observed enlarged livers with necrotic areas varying from single foci to almost complete coverage of the liver in broiler chicks receiving a methane-fermented mixed-culture bacterial SCP at dietary concentrations from 5·0 to 30%.

The results from 15 studies, in which a total of about 2000 birds were fed rations containing SCP, suggest that the incidence of liver lesions was dose dependent. However, even at the highest SCP inclusion (30%), hepatic damage was seen in only half the treated birds. At the concentrations of SCP likely to be used in commercial practice (up to 7·5%), the birds had a growth rate comparable with that of birds fed control rations and appeared normal, although some had a degree of liver damage discernible at autopsy.

Histological examination of the affected livers has identified lesions of both parenchymal cells and portal tracts. The parenchymal cell lesion was associated with cellular necrosis, dilated sinusoids and infiltration by lymphoid series cells. In some affected livers eosinophil infiltration was widespread, and occasionally granulomatous changes occurred with the presence of multinucleated giant cells. The portal lesion showed bile-duct proliferation with fibrosis, with infiltration by round cells and sometimes by eosinophils. Occasionally there was focal cholangitis with inflammation of the stroma of the major bile ducts and necrosis of ductule epithelium. Mitotic figures have been seen in ductule epithelium. The development of this lesion varied from mild proliferative changes in a few portal areas to extensive proliferation involving all portal areas and forming a general network throughout the parenchyma.

Lesions could be detected in bird livers after only 6 days on diets containing SCP. Any damaged liver tended to have lesions at the same stage of development, with no evidence of progression from focal necrosis through confluent necrosis to widespread granuloma formation. This suggested a critical time factor for the onset of these pathological changes.

As a result of these findings we feel that further work is required to evaluate the biological implications of feeding bacterial SCP to birds.

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Fd Cosmet. Toxicol. Vol. 16, p. 634. Pergamon Press 1978. Printed in Great Britain

CORRIGENDUM

Volume 16 (1978)

p. 259, question 9: *For* Is it a lactone fused to another ring, or a five- or six-membered α,β-unsaturated lactone? *read* Is it a 4-membered lactone, or a 5- or 6-membered α,β-unsaturated lactone, or is it a lactone fused to another ring?

FORTHCOMING PAPERS

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

Studies on the fate of quinoline yellow in the rat. By B. Wahlström, G. Blennow and C. Krantz.

Mutagenicity studies of R-amino salt, a metabolite of amaranth (FD & C Red No. 2), in mouse lymphoma cells heterozygous at the thymidine kinase locus and in the rat dominant lethal test. By K. A. Palmer, C. W. Sheu and S. Green.

Two-year oral toxicity and multigeneration studies in rats on two chemically modified maize starches. By R. Truhaut, B. Coquet, X. Fouillet, D. Galland, D. Guyot, D. Long and J. L. Rouaud.

The metabolism of beef tallow sucrose esters in rat and man. By J. W. Daniel, C. J. Marshall, H. F. Jones and D. J. Snodin.

Long-term toxicity study of quillaia extract in mice. By J. C. Phillips, K. R. Butterworth, I. F. Gaunt, J. G. Evans and P. Grasso.

Contamination of beer with trace quantities of *N*-nitrosodimethylamine. By B. Spiegelhalder, G. Eisenbrand and R. Preussmann.

Long-term toxicity and carcinogenicity studies of the bread improver potassium bromate. 1. Studies in rats. By N. Fisher, J. B. Hutchinson, R. Berry, J. Hardy, A. V. Ginocchio and V. Waite.

Long-term toxicity and carcinogenicity studies of the bread improver potassium bromate. 2. Studies in mice. By A. V. Ginocchio, V. Waite, J. Hardy, N. Fisher, J. B. Hutchinson and R. Berry.

Physiopathology of haemorrhagic syndrome related to ochratoxin A intoxication in rats. By P. Galtier, B. Boneau, J. L. Charpenteau, M. Alvinerie and J. More.

Production of [^{3}H]patulin of high specificity activity. By T. J. Gillespie and R. L. Price.

The toxicity of α-tomatine to *Tetrahymena pyriformis*. By J. G. Surak and A. V. Schifanella.

Absorption of BLANKOPHOR BHC from a detergent solution applied to the skin of pigs. By K. Patzschke, L. A. Wegner and H. Weber.

Supplement Number 2 1978 to the journal *Food and Cosmetics Toxicology*

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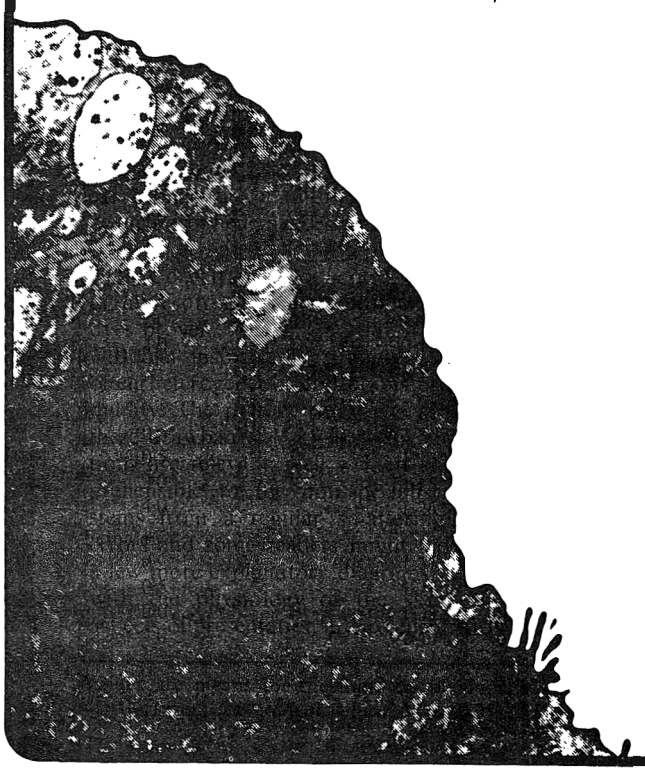

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[*Contents continued*]

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Contents

Volume 16 Number 1

Volume 16 Number 2

Volume 16 Number 3

Volume 16 Number 4

Volume 16 Number 5

Volume 16 Number 6

Volume 16 Supplement

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